OPTIMIZED SOLID-STATE AND SUBMERGED FERMENTATION OF PEA PROTEIN-ENRICHED FLOUR TO COMPARE THE EFFECTS ON PROTEIN QUALITY AND FUNCTIONAL PROPERTIES

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ABSTRACT

The goal of this research project was to benchmark the optimal solid-state (SSF) and submerged (SmF) fermentation parameters for five generally regarded as safe (GRAS) microorganisms on pea protein-enriched flour (PPEF) to achieve limited protein hydrolysis (10% DH) and evaluate the effects of microbial SSF and SmF on the protein quality and functional properties of PPEF. PPEF was inoculated with Aspergillus oryzae NRRL 5590, Rhizopus oryzae NRRL 395, Rhizopus oligosporus NRRL 2710, Lactobacillus plantarum NRRL B4496, and Bacillus subtilis ATCC 6051 and evaluated at two temperatures (30°C and room temperature) over a 120-h fermentation time course. A degree of hydrolysis of 10% DH was chosen as the limit for protein hydrolysis to improve the protein quality and functional properties of PPEF. Under SSF at 10% DH, protein content for all samples significantly increased and lipid content decreased significantly, while ash content did not change. Surface properties of all SSF samples showed an increase in zeta potential while surface hydrophobicity decreased. Under SSF, the functional properties of PPEF were affected significantly but did not improve with the exception of water hydration capacity (WHC) and oil-holding capacity (OHC): Solubility decreased in all fermented samples at both pH 4 and pH 7; Emulsifying activity (EA) decreased for all fermented samples at pH 4, but at pH 7 PPEF fermented with A. oryzae and R. oligosporus increased; Emulsion stability (ES) increased for all fermented samples at pH 7, but at pH 4 only PPEF fermented with A. oryzae and R. oligosporus increased; Foaming capacity (FC) and foam stability (FS) decreased for all fermented samples at both pH 4 and pH 7 whereas WHC and OHC increased for all fermented PPEF samples. Under SmF at 10% DH, protein, ash, and lipid contents all increased significantly. Under SmF, the surface charge (zeta potential) of PPEF increased for all samples while the surface hydrophobicity of PPEF decreased for all samples, as seen for SSF. The functional properties of PPEF subjected to SmF was significantly affected but also did not improve any parameters other than WHC and OHC: Solubility decreased for all SmF fermented PPEF samples at both pH 4 and pH 7, along with the following functional effects: EA decreased at both pH 4 or pH 7, ES increased at pH 4 but decreased at pH 7, FC decreased at both pH 4 and pH 7, and FS increased at pH 4 but decreased at pH 7. Protein digestibility of PPEF increased following fermentation with either
platform (i.e., SSF, SmF), but due to limiting amino acids, their protein digestibility-corrected amino acid score (PDCAAS) values were below 1.0. Solid-state and submerged fermentations of PPEF with microorganisms have significant potential to improve the functional properties and protein digestibility of PPEF, but at 10% DH, only WHC and OHC were improved. Different degrees of hydrolysis of PPEF should be explored to further examine the possibilities of improvement in functionalities as novel food ingredients.
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LIST OF ABBREVIATIONS AND SYMBOLS

a.u. – Arbitrary unit
ANOVA – Analysis of variance
ANS – 8-anillino-1-naphthalenesulfonic acid
d.b. – Dry weight basis
DH – Degree of hydrolysis
EA – Emulsifying activity
ES – Emulsion stability
EAA – Essential amino acid
FAO – Food and agriculture organization
FC – Foaming capacity
FS – Foam stability
IVPD – *In vitro* protein digestibility
IV-PDCAAS – *In vitro* protein digestibility-corrected amino acid score
OHC – Oil holding capacity
PDA – Potato dextrose agar
pI – Isoelectric point
PPEF – Pea protein-enriched flour
SSF – Solid-state fermentation
SmF – Submerged fermentation
ZP – Zeta potential
1. INTRODUCTION

1.1. Overview

The goal of this project is to benchmark and optimize submerged (SmF) and solid-state (SSF) fermentation platforms/techniques to modify and improve the protein functionality, quality and digestibility of pulse crops like field pea (*Pisum sativum* L.) in order to apply and utilize it for human consumption as a protein supplement and ingredient for novel foods. Microorganisms suitable and commonly used for the fermentation of plant products will be employed to ferment dry fractionated pea protein-enriched flour (PPEF), and will include two bacterial and three fungal GRAS microorganisms: *Lactobacillus plantarum*, *Bacillus subtilis*, *Rhizopus oryzae*, *Rhizopus oligosporus*, and *Aspergillus oryzae*. Optimal fermentation conditions (i.e., pH, temperature, time, moisture content) for each strain will be determined and benchmarked for both SmF and SSF platforms. Degrees of protein hydrolysis (DH) will be used to benchmark optimal fermentation parameters for each of the five microorganisms for both fermentation platforms (SmF vs SSF) and will employ a target DH of 10%. Optimal fermentation conditions will vary depending on the microorganism employed as well as the fermentation platform used.

1.2. Objectives

- Determine optimal fermentation conditions (i.e., temperature, pH, time, moisture content) for each of the five GRAS microorganism to achieve 10% degree of hydrolysis for PPEF under solid-state fermentation (SSF).
- Using the benchmarked conditions for SSF, evaluate the hydrolyzed PPEF for the improvement of its functional properties.
- Using the benchmarked conditions for SSF, evaluate the hydrolyzed PPEF for the improvement of protein quality and digestibility.
- Determine optimal fermentation conditions (i.e., temperature, pH, time, moisture content) for each of the five GRAS microorganism to achieve 10% degree of hydrolysis for PPEF under submerged fermentation (SmF).
- Using the benchmarked conditions for SmF, evaluate the hydrolyzed PPEF for the improvement of its functional properties.
- Using the benchmarked conditions for SmF, evaluate the hydrolyzed PPEF for the improvement of protein quality and digestibility.
- Determine the optimal fermentation platform (SSF vs SmF) for PPEF.
- Determine the best microorganism for PPEF fermentation.

1.3. Hypotheses

- Fermentation (SSF and SmF) of PPEF to 10% DH will improve protein content
- Fermentation (SSF and SmF) of PPEF to 10% DH will improve its functional properties
- Fermentation (SSF and SmF) of PPEF to 10% DH will improve its protein quality and digestibility properties
- Under SSF, the three fungal strains (*R. oryzae*, *R. oligosporres*, and *A. oryzae*) will be more effective in improving the functional properties and protein quality of PPEF compared to the two bacterial strains (*L. plantarum* and *B. subtilis*)
- Under SmF, the two bacterial strains (*L. plantarum* and *B. subtilis*) will be more effective in improving the functional properties and protein quality of PPEF compared to the three fungal strains (*R. oryzae*, *R. oligosporres*, and *A. oryzae*)
2. LITERATURE REVIEW

2.1. Introduction

The global protein ingredient market is growing at a compound annual growth rate (CAGR) of 10.1% since 2019, where it was valued at USD 38 billion, and is expected to continue until 2028 (Ismail et al., 2020). This surge in demand for protein ingredients over the last few years includes significant growth in the plant-based protein market. The increase in usage of plant proteins in food products is being driven by the increasing vegan, vegetarian and flexitarian populations as the global population increases, as well as consumers becoming more educated and aware of the negative environmental factors that impact animal protein consumption is having on the planet. Plant proteins are produced at a lower cost, allowing its use in manufacturing a wide range of natural products that are affordable, which offsetting market share from animal protein products such as dairy, egg, and meat (Ismail et al., 2020).

This global challenge to address food security and preserve the natural environment and resources due to climate change, population growth, and changing diets is fueling the need for novel alternative protein ingredients that are environmentally sustainable. Protein allergenicity is another issue that must be addressed; hence, it is a contributing factor for research and development of novel plant protein ingredients that are non-allergenic, since dairy, egg and soy protein products contain major allergens. Furthermore, plant proteins generally have good functional properties, thus novel plant protein ingredients that can replace synthetic ingredients such as synthetic emulsifiers are of interest (Ismail et al., 2020).

Proteins from pulse crops (i.e., peas, chickpeas, lentils and fava beans) have drawn tremendous interest from researchers, producers, and consumers due to their promise as novel food ingredients, as there is only limited knowledge of plant proteins outside of soy proteins. Pulses are the dry edible seeds of legume crops that are highly nutritious with a rich source of protein, carbohydrates, fiber, and micronutrients (Wang et al., 2020).

2.2. Peas (Pisum sativum L.)

Dry peas are predominantly used as a food ingredient for human consumption either as whole vegetable or as dry flour. Dry peas are inexpensive, readily available and rich in nutrients, primarily providing a good source of protein, complex carbohydrates, vitamins, minerals and other
beneficial phytochemicals, while containing very little fat or cholesterol and also being allergen-free (Dahl et al., 2012).

Canada is the largest producer and exporter of dried peas in the world. Peas are one of Canada’s largest pulse crops seeded across Alberta, Saskatchewan and Manitoba. It is a good rotational crop that can increase cereal crop and oilseed crop yields, and can break disease cycles (Tulbek et al., 2017). Peas, which are rich in protein, fiber, carbohydrate, vitamins, and minerals, can be milled and separated into functional ingredients. Peas have a low-fat content, which eliminates the need for an oil extraction stage prior to protein extraction. Depending on the process method, pea protein can be prepared as either whole pea flour (starch-rich flour), protein-enriched flour, and protein isolate. Dry milled dehulled peas yields whole pea flour. Pea protein-enriched flour is primarily prepared by dry fractionation via air classification, while pea protein isolates are produced by wet fractionation.

The protein fraction of peas is the most valuable extractable ingredient. Pea protein is a good alternative to the well-established and versatile soy protein products that traditionally have dominated the plant-based protein market (Sandberg, 2011). These pea-derived functional ingredients offer various applications in food systems, such as an egg replacement in baking, high-protein snack products, texturizer in soups, and emulsifiers in meat and sauce products. In addition, peas are known to reduce blood glucose levels, improve gastrointestinal health, and enhance satiety (Tulbek et al., 2017).

2.3. Pea Protein Extraction

2.3.1. Wet Fractionation

Wet fractionation is a proven and common method to yield pea protein isolate and is utilized by the food processing industry to enhance the nutritional quality and texture of food products. It starts with soaking milled pea flour in alkaline water to dissolve and extract the proteins while suspending the starch granules, and then using a hydrocyclone whereby the protein solution is separated from the starch granules (Pelgrom et al., 2013). In the protein solution, the pH is adjusted to the isoelectric point of the proteins to precipitate them out, followed by readjusting the pH to neutral. A drying step is then required to obtain the final pea protein isolate (Boye et al., 2010). Although this yields up to 90% protein purity, the disadvantage of this method is that the pH
adjustments and the drying step causes partial loss of the native functionality of the proteins. In addition, this method is quite costly and inefficient in terms of energy and water use (Schutyser & van der Goot, 2011).

2.3.2. Dry Fractionation

A more viable alternative method of separating whole peas and other legumes is dry fractionation by fine milling followed by air classification (Bergthaller et al., 2001). The primary advantages of dry fractionation are lower energy and water use and a higher native protein functionality retention rate (Schutyser & van der Goot, 2011). The fine milling process using impact and jet milling separates the larger starch granules from the smaller protein particles by detaching them physically (Tyler & Panchuk, 1982). In order to optimally separate starch granules from the protein bodies, peas are milled under various conditions. Classifier wheel speeds are varied to obtain pea flours with varying particle size distributions, starch levels and protein content. Subsequently, the optimal milling conditions are verified by scanning electron microscope imaging and particle size analysis (Pelgrom et al., 2013).

Air classification is applied to the fine-milled pea flour to separate the fine protein fraction from the coarse starch fraction to obtain pea protein-enriched flour. Pea flour is taken up in the air classifier chamber via air flow, with lighter and smaller particles being carried higher than the large particles due to lower weights. These small particles go through a wheel with slits at the top of the air classifier while the large particles are unable to pass through and collect at the bottom (Pelgrom et al., 2013).

2.4. Pea Protein

Peas are a valuable alternative source of protein for both human consumption and animal feed. The protein content of peas can range from 13.7 to 30.7% depending on environmental conditions and genetic factors (Dahl et al., 2012). An average protein content of 22.3% was reported by Tzitzikas et al. (2006) based on a study from fifty-nine pea varieties. Pea protein primarily exist as globulins (65-80%). Globulins are composed of storage proteins that include legumin (11S) and vicilin (7S), the major proteins in pea seeds. Legumin (11S) has a hexameric quaternary structure composed of six subunits, where each subunit has one basic and one acidic section linked by a disulfide bond (Meng & Cloutier, 2014). Vicilin (7S) comprises approximately 35% of the total
protein in peas, and is a trimer composed of three subunits (Liang & Tang, 2013). Legumin has better emulsifying ability than vicilin, while vicilin has better emulsifying stability due to its low molecular weight (Koyoro & Powers, 1987). The nutritional value of pea protein is based on their amino acid compositions (Boye et al., 2010). Pea protein was shown to be high in glutamine, aspartic acid, arginine and lysine, and lower in methionine, tryptophan and cysteine (Tömösközi et al., 2001).

Despite the high nutritive value and non-allergenic characteristics, applications of pea protein in the food industry have been limited by its weaker functional properties. Additionally, the presence of anti-nutritional factors such as protease inhibitors reduces the digestibility of pea protein (Boye et al., 2010). Hydrolysis of pea protein by way of fermentation can not only improve protein digestibility by generating shorter peptides with bioactivities but can also improve protein quality by protein enrichment and modification of its functional properties (Espinoza et al., 2011).

2.5. Pea Protein Quality and Digestibility

2.5.1. Protein Quality

Food protein quality is an indicator of how well a protein meets the requirements of essential amino acids (EAAs) and the physiological and nutritional needs for humans (Kurpad, 2013). The quality of a protein source can be determined by the measurements of the overall digestibility and bioavailability of its amino acids (Sarwar Gilani et al., 2012). The amino acid composition of food proteins and how efficiently they can be digested to allow amino acid absorption determines their capacity to provide nitrogen and EAAs for human growth and functions. Therefore, protein quality influences dietary requirements; if the protein quality is high, the required dietary protein intake is low and vice versa (Kurpad, 2013).

2.5.2. Amino Acid Score

The concept of assessing protein quality based on a protein’s constituent amino acids was introduced in the late 1940’s (Kurpad, 2013). An amino acid score (with 1.00 meeting the required amount for dietary purposes) is an indicator of the available essential amino acids derived from food proteins. A popular modern method used to determine the amino acid score of a food protein involves the hydrolysis of the protein with acid or alkaline, followed by a separation process of
the released amino acids which are quantified using high-performance liquid chromatography (HPLC).

Amino acid data are calculated as milligrams of particular amino acids per gram of protein. Occasionally, these data are reported as milligrams amino acid per gram of nitrogen; in such cases, the data can be converted to protein equivalents via multiplying by specific protein factors that range from 5.7 (17.5% nitrogen) to 6.4 (15.6% nitrogen) for major protein sources in the diet (Kurpad, 2013).

Essential amino acid proportions that are in excess of the required amounts are still assigned a fractional score of 1.00 (100%), despite the mathematical calculation giving a higher value. The EAAs with the lowest value (the limiting amino acid) determines the protein’s amino acid score; usually sulfur-containing amino acids (methionine and cysteine), along with lysine, threonine, and tryptophan are most likely to limit the protein quality (Kurpad, 2013).

2.5.3. Correction for Protein Digestibility

The capacity of a food protein to deliver nitrogen and essential amino acids for proper human growth and function may be limiting, despite having a good amino acid composition, if the protein cannot be fully digested. Various factors can influence the extent of digestion, absorption, and utilization of food proteins: the inherent differences in their source, protein configuration and amino acid binding properties, dietary fiber, tannins and polyphenols, anti-nutritional factors such as trypsin inhibitors and lectins, and processing methods that can alter the nature of the protein (Sarwar Gilani et al., 2012). Therefore, amino acid scores are considered to integrate protein digestibility and amino acid availability (Kurpad, 2013). For proteins to meet essential amino acid requirements, the extent of their digestibility must be considered to achieve higher amino acid scores (Boye et al., 2012).

2.5.4. Protein Digestibility and PDCAAS

Traditionally, food protein digestibility and quality are evaluated using a variety of methods related to animal responses (i.e., rats) after feeding them the protein, such as protein efficiency ratio (PER), net protein ratio (NPR), or net protein utilization (NPU) (Schaafsma, 2012). However, these numbers do not incorporate the contribution of essential amino acids. In 1989, the FAO and WHO recommended the use of the protein digestibility corrected amino acid score (PDCAAS)
method, which considers both the essential amino acid content of the test protein as well as its digestibility (Tavano et al., 2016).

PDCAAS is now used widely for protein quality evaluation. It is a chemical score derived from the ratio between the first limiting amino acid in a sample protein and the corresponding amino acid in a reference amino acid pattern and corrected for true fecal nitrogen digestibility (Schaafsma, 2012). During digestion, some essential amino acids can be lost in the colon due to the activity of native intestinal flora in animal digestive tracts (Schaafsma, 2012). The use of rat assays for true fecal nitrogen digestibility is less informative compared to the use of ileal-fistulated pigs. Ileal digestibility is more appropriate for quantifying digestion of food proteins, but it is less practical and more difficult to replicate than rat assays. Overall, in vivo assays are generally more costly and time consuming, with a minimum requirement of 9 days (McDonough et al., 1990). In addition, increasing ethical objections to animal use (Schaafsma, 2012) have also helped the development and employment of alternative in vitro methodologies (Tavano et al., 2016).

The in vitro protein digestibility methods measure the potential susceptibility of protein peptide bonds to proteolysis by digestive enzymes. These assays use enzyme mixtures or different enzyme sequences for proteolysis of protein samples in an effort to mimic the effects of the human digestion process or processes of in vivo protein digestion (Tavano et al., 2016).

The amino acid score is equivalent to the ratio of the content of the first limiting amino acid (methionine and cysteine) in the fermented pea protein and the corresponding content of this amino acid in the WHO/FAO/UNU reference pattern; whereas PDCAAS is equal to the product of the measured in vitro protein digestibility and the above-mentioned amino acid score. (Schaafsma, 2012, Boye et al., 2012, Leser, 2013).

2.6. Pea Protein Functional Properties

Although field peas are a widely consumed legume seed, pea protein ingredients are not yet fully utilized to their potential in food applications due to limited information on the functional properties of pea proteins (Adebiyi & Aluko, 2011). In order to successfully apply pea protein as a food ingredient, the protein should ideally demonstrate certain desirable characteristics and functional properties. Therefore, the relationship of pea protein quality with processing parameters (i.e., fermentation) that affect the protein functionality is worthy of investigation (Ragab et al., 2004).
The functional properties of proteins are intrinsic physicochemical characteristics that affect their behaviour in food systems during processing, manufacturing, storage and preparation (Kinsella, 1979). Functional properties represent complex interactions between the conformation, structure, composition, and physicochemical properties of proteins under the influence of other food components and the environment (Mirmoghtadaie et al., 2016). The main functional properties of proteins are the ability to form and/or stabilize foams, emulsions and networks (gels and films). Mirmoghtadaie et al. (2016) listed the different mechanisms that underlay these properties: (1) hydration that can affect absorption of water and oil, wettability, solubility and thickening characteristics of protein; (2) protein surface activity, such as hydrophobicity, hydrophilicity, net charge, or charge distribution which can affect the formation of protein-lipid films, foaming, and emulsifying activities; (3) alteration in protein structure, such as shape, size, amino acid composition and sequence that can alter rheological characteristics, including viscosity, elasticity, adhesiveness, aggregation and gelation.

2.6.1. Solubility

Solubility is an important property of proteins, as it helps with uniform distribution of proteins within food products (Adebiyi & Aluko, 2011). It is the amount of protein in a sample that dissolves into solution and is a physicochemical property that is useful for understanding the utilization of proteins in foams, emulsions, and gels; Protein solubility significantly affects texture, colour and sensory properties of products, including emulsifying, foaming and gel forming properties (Shand et al., 2007). It is the main characteristic of proteins in liquid food and beverage applications. Protein solubility is influenced by ionic strength, solvent type, pH, temperature, and processing conditions. Amino acid composition and sequence and molecular weight also affects solubility (Zayas, 1997). The solubility of pea protein is lowest at pH values between 4 and 6 and highest between pH 8 and 9 (Boye et al., 2010). Good solubility can markedly expand potential applications of proteins.

2.6.2. Surface Hydrophobicity

Food proteins are involved in many functional processes due to their three-dimensional structures. Hydrophobic, steric, and electrical parameters are important variables that affect the structure and thus the functionality of proteins. Hydrophobicity describes the tendency of non-
polar solutes to adhere to one another in an aqueous environment (Alizadeh-Pasdar & Li-Chan, 2000). Although protein solubility may affect other functional properties, better correlations to the various protein functionalities were obtained when hydrophobic parameters were used in conjunction with solubility (Hayakawa & Nakai, 2006).

### 2.6.3. Emulsification

A fundamentally important property in food processing is emulsification. Food emulsions are usually two-phase systems of either oil-in-water (O/W) or water-in-oil (W/O) where a discontinuous phase is dispersed as small droplets into a continuous phase; O/W emulsions such as salad dressing or plant-based milks tend to have a creamier texture, while W/O emulsions such as mayonnaise have a greasy texture (Zayas, 1997). These systems with two immiscible liquids must be formed and stabilized using emulsifiers.

Proteins can be emulsifiers due to their hydrophilic and hydrophobic properties by forming a layer of film around dispersed oil droplets in an aqueous medium, which prevents coalescence, creaming, sedimentation, or flocculation and lowers interfacial tension. To evaluate the emulsifying properties of protein flours, three indices are considered, including emulsifying activity, emulsifying capacity, and emulsifying stability. Emulsifying activity is the ability of the protein to participate in emulsion formation, emulsifying capacity is the amount of oil that is emulsified under specific conditions by 1 gram of protein, and emulsifying stability is the capacity of emulsion droplets to remain dispersed without separation (Boye et al., 2010).

In an O/W emulsion, amphiphilic protein molecules can migrate to the oil-water interface and change their configuration to align the hydrophilic and hydrophobic groups to the water and oil, respectively and inhibits oil droplet flocculation and coalescence (Zayas, 1997). Partial denaturation of protein can enhance emulsifying activity by exposing otherwise buried hydrophobic groups and balancing the ratio of hydrophilic and hydrophobic groups for a stable emulsion formation.

Plant-based proteins such as those from soybeans, rapeseed, and sunflower seed are a good source as cheaper and healthier emulsifying agents due to their comparable functional properties and amphiphilic nature as they readily diffuse into oil-water interfaces (Imbart et al., 2015). Due to its similar characteristics to soy protein, pea protein has the potential to be a novel emulsifier in food manufacturing.
2.6.4. Foaming

In food processing, foam is defined as the entrapment of air in a liquid or solid to form small bubbles that aid in the texture of food products; the air pockets result in uniform rheological properties (lightness and smoothness) as well as even dispersion of flavour compounds (Kinsella, 1981). These systems are inherently unstable and require the use of a surfactant to interact at the air-water or air-solid interface to stabilize the foams (Makri et al., 2005). Usually synthetic or natural proteins are employed as foaming agents and stabilizing surfactants; the ability to form and stabilize a foam are independent of one another (a good foaming agent may not necessarily have to be a good foam stabilizer) (Sathe et al., 1982).

Foams are formed when proteins unfold to create an interfacial skin that holds air bubbles in suspension and prevents their collapse. Protein dispersions at specific concentrations are homogenized at high speed to induce foam formation, at which point foam expansion and foam capacity are measured and expressed as the volume percentage increase due to whipping. Foam stability is measured as the change in the volume of foam over a specified time (Zayas, 1997). The ability of proteins to form stable foams is essential in the production of foods such as beverages, cakes, mousses, and whipped toppings (Chandra-Hioe et al., 2016); the taste and texture of these foods are dependent on stable foam formation (Green et al., 2013). The foam product’s textual properties and appearance are affected by the size distribution of air bubbles.

Foam formation is a function of the ability of proteins to rapidly diffuse and adsorb to the air-water or air-solid interface, while the foam stability is based on its ability to reduce interfacial tension after it has been adsorbed (Martin et al., 2002). The interfacial tension is pivotal in the stability of foams; high interfacial tension causes the air bubbles to coalesce, destabilize, and burst. Therefore, low molecular weight peptides that can favorably interact at the air-water interface and reduce interfacial tension are ideal.

Common foaming agents include egg whites, gelatins, casein, gluten, soy proteins and whey proteins (Barac et al., 2014). However, the allergenicity of these proteins have limited their use and thus a good non-allergenic alternative is needed in the market. Pea protein is a viable replacement as it has low allergenicity and is cheap and widely available. Pea protein is made up of 11S and 7S globular proteins that cannot unfold at the interface, thus explaining its weak functional properties such as foam capacity compared to other proteins (Kaur & Singh, 2007).
However, partial protein denaturation by fermentation may be able to aid in its alignment at the interface and thus improve its foaming properties.

2.6.5. Water Hydration and Oil Holding Capacity

Water hydration capacity (WHC) and oil holding capacity (OHC) of proteins are two important functional properties in food application in processing and manufacturing, as well as having influence on a product’s storage and shelf-life (Kumitch et al., 2020).

The WHC is defined as the maximum amount of water absorbed per gram of protein. It affects the texture, colour, and sensory properties of products. Functional properties, storage stability, and eating quality are also affected, therefore it is an important property for food formulation and application (Zayas, 1997). Proteins that have high water hydration capacity may render food products dry and brittle during storage while proteins with low water hydration capacity cannot hold water effectively (Boye et al., 2010). The WHC affects other functional properties of proteins that are dependent on the protein-water interaction and hydrogen bonds, including solubility and emulsifying properties (Zayas, 1997).

In protein-water interactions, a protein’s structure holds water as absorbed water and retained water. Absorbed water binds to the exterior of the protein structure by surrounding it at charged sites via hydrogen bonds. The first layer is known as vicinal water and is the product of protein-water interactions, whereas the second layer is a consequence of water-water interactions via hydrogen bonds is known as multi-layer water and covers the remaining exposed sites on the protein complex. Retained water is bulk water trapped within the protein matrix (Zayas, 1997). The many intrinsic properties of the protein, such as amino acid composition, structure, size, isoelectric point, concentration, polarity all affect its absorbed water amount. Protein concentration influences its WHC by reducing the available protein sites for protein-water interactions, i.e., a higher protein concentration favours more protein-protein interactions than protein-water interactions (Zayas, 1997). The globular structure of pea protein has less water-binding sites available due to its folded nature as compared to fibrous proteins with significantly more exposed polar sites; therefore, enzymatic hydrolysis by fermentation to partially unfold them to expose hydrophilic sites may improve the WHC of pea protein.

The oil holding capacity of proteins is the weight of oil absorbed per weight of protein powder (Boye et al., 2010). It is important in food formulations, especially emulsions. Binding of oil with
proteins also influences the textural properties and flavor absorption. The protein source, processing conditions, particle size and temperature all significantly affect oil absorption. The OHC of pea protein was found to be 1.2 g/g (Fernandez-Quintela et al., 1997).

2.7. Fermentation

2.7.1. Solid-state Fermentation

Solid-state fermentation (SSF) is a biomolecule manufacturing process applied to many cereals and legumes where growth of microorganisms, on moist solid substrates without free-flowing water, is induced. The growing microorganism subsequently produce bioactive compounds that metabolize and hydrolyze the substrate and modify the functional properties of, for example, protein flour, by forming shorter chain peptides with lower molecular mass (Xiao et al., 2015). Fermentation also increases nutritional quality by reducing antinutritional factors such as phytate, oxalates, and complex polysaccharides, while increasing mineral bioavailability. Microorganisms such as filamentous fungi are better suited for this technique as they do not require a high moisture content in order to grow (Novelli et al., 2016).

2.7.2. Submerged Fermentation

In contrast to SSF, submerged fermentation (SmF) uses free-flowing liquid substrates submerged in water, where the bioactive compounds are secreted. Microorganisms such as bacteria are more suitable for this fermentation technique due to their ability to grow unicellularly and thus distribute uniformly within the fermentation vessel (Subramaniyam and Vimala, 2012).

2.7.3. Solubility of Fermented Proteins

Pulse crops such as peas contain globulins and albumins, with globular proteins making up the majority. The solubility of pea protein is low because of its globular nature with few exposed hydrophilic binding sites. Fermentation of pulses have been demonstrated to improve the solubility; Prinyawiwatkul et al. (1997) reported an increase in the solubility of heat-denatured cowpea flour after fungal fermentation with Rhizopus oligosporus. Fungal proteases cleaving peptides into smaller sizes along with increased hydrophilic binding sites exposed as a result of fermentation were attributed to the increase in solubility.
2.7.4. Emulsification of Fermented Proteins

It has been reported that partial denaturation of plant-based proteins by fermentation can improve its emulsifying properties. The above study by Xiao et al. (2015) fermented chickpea flour with Cordyceps militaris and improved its emulsifying properties due to the activity of fungal enzymes’ proteolytic activity exposing hydrophobic groups which promoted emulsification as the hydrophobic-hydrophilic group ratio became ideal for such function. The authors also claimed that smaller hydrolyzed peptides as a result of fermentation also made it easier for them to migrate to the oil-water interface to stabilize the emulsion.

In another study, velvet bean (Mucuna cochinensis) protein isolate was subject to natural fermentation for up to 72 hours and demonstrated a significant increase in its emulsifying capacity. The authors also attributed the improvement to partial hydrolysis of protein resulting in the exposed hydrophobic groups interacting with the oil-water interface (Udensi & Okoronkwo, 2006).

2.7.5. Foaming of Fermented Proteins

The use of plant-based proteins such as pea protein as novel ingredients are only possible if its functional properties are enhanced by some type of processing, but not to a point where the protein quality is adversely affected. Partial hydrolysis of the protein by fermentation is a viable option to not only improve its functionality, but also to improve their quality. There have been studies that sought to better understand the effect of fermentation on the foaming properties of plant-based proteins. In the above-mentioned study by Udensi & Okoronkwo (2006), the foaming properties of velvet bean (M. cochinensis) protein isolate significantly increased after fermentation, with the newly exposed hydrophobic groups balancing the hydrophobic-hydrophilic group ratio to make the protein amphipathic, thereby reducing the interfacial tension.

2.7.6. Water Hydration and Oil Holding Capacity of Fermented Proteins

Microbial fermentation causes the hydrolysis of proteins by secreted proteases which then alters the protein structure. This action affects the protein’s intrinsic factors that dictate its functionalities such as WHC and OHC by exposing previously inaccessible hydrophilic and/or hydrophobic binding sites. Previous studies on the effect of fermentation on various proteins’ functionality reported increased WHC and/or OHC by Obatolu et al. (2007), Yu et al. (2007), and Xiao et al. (2015).
A study by Xiao et al. (2015) fermented chickpea flour with *C. militaris* and reported an increase in its WHC and OHC and attributed this improvement to the proteolytic activity by the fermenting microorganism exposing hydrophilic and hydrophobic binding sites. The same reason was given by Obatolu et al. (2007) after observing increased WHC of yam bean flour fermented with lactic acid bacteria. Yu et al. (2007) reported the increase in OHC of roasted peanut flour after fermentation with *R. oligosporus* was due to increased exposure of hydrophobic interior proteins.

2.8. Microorganisms

In this thesis project, five GRAS (generally regarded as safe) microorganisms that are traditionally used in fermentation of plant products will be employed as they vary in terms of metabolite production which will offer a range of treatment options for this project. A brief description of these organisms is provided below.

2.8.1. *Aspergillus oryzae*

*Aspergillus oryzae* is an aerobic filamentous fungus that has a long history of safe use in food fermentation, and the lack of aflatoxin (a toxic compound) production has earned it GRAS status. It produces many extracellular enzymes that degrade polypeptides, carbohydrates, and nucleic acids. Hence, it has been used traditionally in East Asia as starter culture for preparation of koji in the production of fermented foods and alcoholic beverages such as soy sauce, miso, sake, and vinegar. Koji refers to the solid-state fermentation involving *A. oryzae* and the fermented substrate that is rice, soybean, and wheat. *A. oryzae* can be isolated from soils and plants such as rice. It has an optimal growth temperature of 32-36°C and an optimal growth pH of 5-6, and generally can grow on media with a water activity above 0.8 (Gomi, 1999).

*Aspergillus oryzae* produces the required enzymes for transforming raw materials into more readily digestible components but also affects the colour, aroma, flavour and texture of the fermented products. Due to its high-level of enzyme production and high degree of safety, *A. oryzae* has seen use for organic compounds such as glutamic acid as well as commercial enzymes such as α-amylase, glucoamylase, cellulase, and proteases; it has been employed in starch processing, baking, producing detergents, and brewing. *A. oryzae* protease is used in bread-making to release amino acids and peptides for yeast growth and gas production. It can express high levels
of heterologous enzymes, including a recombinant lipase with commercial application in detergents (Gomi, 1999). Recombinant proteins are primarily produced using SmF because it is easier to cultivate. However, it has been shown that using SSF yields a greater quantity of enzymes compared to SmF, thus garnering increased interest (Oda et al., 2006).

2.8.2. *Rhizopus oryzae*

*Rhizopus oryzae* is a complex of closely related, heterothallic species of filamentous fungi most commonly found in soil and rotting vegetation. *R. oryzae* strains are isolated as active components in the production of traditional Asian foods and alcoholic beverages primarily in China, Japan, and Indonesia. They have long been used for enzyme production such as glucoamylases and lipases, organic acid synthesis, as well as various applications in food fermentation (Mertens et al., 2006). The pathogenicity of *R. oryzae* to plants can be attributed to the presence of large number of carbohydrate-digesting enzymes such as cellulases and hemicellulases. Thompson and Eribo (1984) found that *R. oryzae* secretes many other enzymes including protease, urease, ribonuclease, pectate lyase and polygalacturonase. In addition to enzyme production, *R. oryzae* has been found to be capable of producing several organic acids, alcohols and esters, which makes it an effective microorganism for applications in the food and pharmaceutical industries. It is an effective biocatalyst for different agro-industrial products and can produce protein- and fat-rich mycelia during SSF that results in significant enrichment of several essential amino acids such as valine, leucine, threonine, isoleucine, and arginine (Ibarruri & Hernandez, 2017).

2.8.3. *Rhizopus oligosporus*

*Rhizopus oligosporus* is a fungus that is widely used as a starter culture for soybean fermentation to make tempeh. It produces white mycelia that binds the soybeans together to create an edible, cake-like textured product. *R oligosporus* grows effectively at 30-40°C and possess strong lipolytic and proteolytic activity; it can also produce metabolites that can inhibit the growth of other fungi and Gram-positive bacteria (Nout, 1989). *R. oligosporus* is the domesticated variant form of *Rhizopus microsporus* which produces some potentially toxic metabolites such as rhizoxin and rhizinins A and B; however, the domesticated variant *R. oligosporus* has mutations in its genome that led to the loss of genetic material responsible for toxin production, thus allowing it to obtain GRAS status (Jennessen et al., 2005).
Although it is most-commonly utilized for the fermentation of soybeans during production of tempeh, a food staple in Southeast Asia and increasingly becoming a popular meat substitute worldwide, this fungal strain can utilize a wide-range of substrates to support its growth. It is able to produce phytase which catalyzes the hydrolysis of phytic acid, releasing inorganic phosphorus, and is often used to increase the nutritional value of animal feed (Surya et al., 2013).

2.8.4. Lactobacillus plantarum

*Lactobacillus plantarum* is a widely prevalent rod-shaped, Gram-positive lactic acid bacterium that is common in human and animal gut mucosa, it is also part of the microbiota of many foods and feeds, including dairy, meat, fish, fermented fruits and vegetables such as must, sauerkraut, pickles, sourdoughs, and silage as it is considered a safe probiotic. *L. plantarum* is used commonly in controlled fermentations due to its desirable effects and properties in many fermented foods and enhances their quality and/or associated health benefits (Corsetti & Valmorri, 2011). The physiological adaptability of *L. plantarum* is very wide, as it can utilize a large range of carbohydrates (producing lactic acid as an end metabolite), thus enabling it to grow in different ecological niches (Mayo & Flórez, 2020). They can grow at temperatures between 15-45 degrees Celsius and at pH values as low as 3 and are acid and bile salt tolerant (Matejčeková et al., 2016).

*Lactobacillus plantarum* is found in a wide range of food products, making it an ideal candidate for probiotics development. A 2008 study demonstrated that *L. plantarum* was effective in reducing the allergenicity of soy flour with a significant reduction of IgE immunoreactivity (Frias et al., 2008). The potential health effects of *L. plantarum* has been the subject of many studies due to it being so abundant in nature and humans and is so easy to grow. It has been shown that *L. plantarum* has significant antioxidant activities and helps to maintain intestinal permeability (Bested et al., 2013).

2.8.5. Bacillus subtilis

*Bacillus subtilis* is a rod-shaped, Gram-positive spore-forming bacteria naturally found in soil, roots of plants, and aquatic environments (Martinez, 2013). It is a ubiquitous microorganism that has GRAS status and can also be found in the gastrointestinal tract of animals. They are widely used in biotechnology due to their ability to efficiently secrete enzymes such as amylase, protease, pullulanase, chitinase, xylanase, and lipase (Morikawa et al., 2006); *B. subtilis* is utilized in the
production of up to 60% of commercially available enzymes (Martinez, 2013). In addition, B. subtilis plays a major role in the food industry as producer of flavour enhancers, sweeteners, and animal feed additives, as well as being utilized to produce fermented foods. B. subtilis is utilized in traditional Asian and African fermented soybean foods such as Japanese natto, Indian kinema, Thai thua-nao and West African dawadawa (Kiers et al., 2000b). Previously, Li et al. (2014) used camellia meal as substrate to study the optimal fermentation conditions for this GRAS strain and reported an optimum initial pH of 6.8 and fermentation temperature at 37°C. Metabolites of B. subtilus are also commonly used in the production of household detergents, antibiotics, and vitamins, and the development of vaccines among other uses in the biotechnology sector (Martinez, 2013).
3. MATERIALS AND METHODS

3.1. Materials

Dry fractionated and air-classified pea protein-enriched flour (PPEF) was obtained from Parrheim Foods (Saskatoon, SK, Canada). All chemicals used were of reagent grade and obtained from Sigma-Aldrich Co. (Oakville, ON, Canada). A Millipore Milli-Q water purification system (Millipore Corp., Etobicoke, ON, Canada) produced the water used in this study. Canola oil was sourced commercially (Great Value, Wal-Mart Canada Corp., Mississauga, ON, Canada). The microorganisms (Aspergillus oryzae NRRL 5590, Rhizopus oryzae NRRL 395, Rhizopus oligosporus NRRL 2710, Lactobacillus plantarum NRRL B4496, Bacillus subtilis ATCC 6051) were obtained from the Agriculture Research Service Culture Collection, USDA (Peoria, IL, USA) and American Type Culture Collection (Manassas, VA, USA).

3.2. Methods

3.2.1. Fermentation

Microorganism and Inoculum Preparation

The five microorganisms used in this study are generally recognized as safe (GRAS) on the basis that they are common and routinely utilized for fermentation of plant-based food products. The fungal strains (A. oryzae NRRL 5590, R. oligosporus NRRL 2710 and R. oryzae NRRL 395) were cultured on Potato Dextrose Agar (PDA). In an aseptic environment, colonies from previously cultivated plates were transferred and placed into 8 mL peptone water. The samples were vortexed for 30 seconds to ensure uniform suspension of spores. A 100 µL aliquot from each suspension was pipetted and spread onto PDA plates, and subsequently incubated and incubated at 30°C for 7 days under aerobic conditions. Fungal spores from each PDA plate were suspended in 10 mL deionized water and the spore concentrations were adjusted to 10⁶ conidiospores/mL, as determined by direct microscopy counting (Leica, Model S6E, Wetzlar, Germany) via a hemocytometer (Bright-Line, Horsham, PA, USA).
The bacterial strains *L. plantarum* NRRL B4496 and *B. subtilis* ATCC 6051 were cultured in MRS broth for approximately 24 hours at 30°C. Growth was monitored until an absorbance that corresponded to a bacterial concentration of \(10^6\) cells/mL was attained. Enumeration of *L. plantarum* and *B. subtilis* were carried out by plating onto MRS medium. Bacterial cultures were then centrifuged, and the cell-free supernatant removed before the bacterial pellet was re-suspended in saline solution.

**Solid-state Fermentation**

PPEF samples were inoculated with 1 mL aliquots of the inoculum per gram of substrate at 50% initial moisture. The moisture contents were calculated using the below equation:

\[
M_n = \left( \frac{W_w - W_d}{W_w} \right) \times 100 \quad \text{(Eq. 1)}
\]

where \(M_n\) represents the moisture content (%) of material n, \(W_w\) is the wet weight of the sample, and \(W_d\) is the dry weight of the sample. Each sample was thoroughly mixed and homogenized prior to being spread evenly onto aluminum baking pans. The samples were covered with aluminum foil with small holes for aeration and incubated at 30°C in an Isotemp incubator (Fisher Scientific, Model 650D, Waltham, MA, USA). PPEF samples were fermented for 120 hours. Samples were taken at the time of initial inoculation (0% DH), and every 24 hours thereafter. Fermentations were done in triplicate.

**Submerged Fermentation**

Inoculated PPEF was suspended/submerged in 400 mL of deionized water in Erlenmeyer flasks loosely covered with a plastic screw-cap. Subsequently, the inoculated PPEF suspensions were incubated in an orbital shaker operated at 100 rpm for periods of up to 120 hours at 30°C. Triplicate samples were taken every 24 hours for analyses.

Fermented PPEF samples were removed from each batch at the defined intervals and the microorganisms deactivated at 80°C in a water bath. All samples were freeze-dried (Labconco, Freezone 12, Kansas City, USA) prior to further analysis.
3.2.2. Determination of pH

All pH measurements were conducted at room temperature using a Fisher-Brand pH meter (Fisher Scientific, Toronto, Canada).

3.2.3. Determination of Degree of Hydrolysis

Fermented PPEF samples (1 g) were individually added to 20 mL of pH 8.2 (w/v) sodium phosphate buffer (0.1 M) in 50-mL centrifuge tubes and capped. The tubes were placed in a hot water bath (95°C for 2 min) to kill microorganisms and inactivate enzymes. Subsequently, the samples were cooled to room temperature (RT) before being centrifuged (8,228 × g for 30 min) using a 5804R centrifuge (Eppendorf, Hamburg, Germany). Using the method described by Adler-Nissen (1976), the supernatant was removed for analysis. The Adler-Nissen method is based on measuring the light absorption change during the reaction between protein and picrylsulfonic acid, yielding N-trinitrophenyl-protein derivatives. The samples were analyzed in plastic cuvettes using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, Madison, WI, USA) at 340 nm. Measurements were conducted in duplicate and reported as the mean ± standard deviation. A glycine solution (1.5 mM) was used to create a standard curve.

Hydrolysis equivalence at complete hydrolysis to amino acids ($h_{tot}$) was measured as a part of the %DH-calculation, according to methods described by Adler-Nissen (1979) and Jung et al. (2005), by adding 24 mg of PPEF to a screw-cap Pyrex tube with 15 mL of 6.0 N HCl, after which the tubes were purged with O2-free nitrogen gas and incubated in a forced-air oven at 110°C for 20 h. After 20 h, the tubes were removed and adjusted to pH 7.0 with 2 M NaOH. The samples were then filtered using Whatman Grade 3 filter paper. Following filtration, a 250-µL sample aliquot was added to 2.00 mL of 1% SDS solution in sodium phosphate buffer. A pH 7 solution was used as a blank solution. Samples were measured in plastic cuvettes using a Genesys 10S UV-VIS spectrophotometer at 340 nm. All measurements were performed in duplicate.

%DH were calculated using the following formulas (Equations 2 and 3) (Adler-Nissen, 1979):

$$h = (h_t - h_c) \times DF$$ and $$h_{tot} = (h_t - h_c) \times DF$$

(Eq. 2)

$$\%DH = \frac{h}{h_{tot}} \times 100$$

(Eq. 3)
where $h$ is the yield of hydrolysis of $\alpha$-NH$_2$-glycine equivalents, $h_t$ is the concentration of $\alpha$-NH$_2$-glycine equivalents (mM), $h_c$ is the concentration of $\alpha$-NH$_2$-glycine equivalent taken at time 0 (before a fermenting organism was added; mM), and DF is the dilution factor.

Fermented PPEF samples were recorded daily to determine the time needed to achieve an arbitrarily determined %DH of 10%.

### 3.2.4. Proximate Composition

The proximate composition values of fermented PPEF were determined using methods from the Association of Official Analytical Chemists (AOAC). Crude protein was determined according to method 984.13A, crude ash was determined using method 923.03, and crude lipid from method 920.87. Proximate composition values were reported on a dry weight basis (d.b.), with measurements made in triplicate and reported as mean ± standard deviation.

### 3.2.5. Surface Properties

#### Surface Charge

Freeze-dried fermented and unfermented PPEF powders were suspended in deionized water at a concentration of 0.05% (w/w) and pH was adjusted to pH 4 or pH 7. The surface charge (i.e., zeta potential) of each sample was determined according to Karaca et al. (2011); electrophoretic mobility measurements were performed using a Zetasizer Nano instrument (Malvern Instruments, Westborough, MA, USA). Measurements were done in triplicate and reported as mean ± standard deviation. Using Henry’s equation (Equation 4, below) and the measured electrophoretic mobility ($U_E$), the zeta potential ($\zeta$; mV) was determined.

$$U_E = 2\varepsilon \times \zeta \times f(\kappa \alpha) / 3\eta$$

(Eq. 4)

where $\eta$ is the dispersion viscosity, $\varepsilon$ is the permittivity, and $f(\kappa \alpha)$ is the function related to the ratio of particle radius ($\alpha$) and the Debye length ($\kappa$).
**Surface Hydrophobicity**

Freeze-dried fermented and unfermented PPEF powders were suspended in deionized water at a concentration of 0.025% (w/w) and pH was adjusted to pH 4 or pH 7. Surface hydrophobicity of fermented samples were determined using the method described by Kato and Nakai (1980) using 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescent probe. Fluorescence intensity was measured using a FluoroMax-4 spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with a slit width of 1 mm, and excitation and emission wavelengths of 390 nm and 470 nm, respectively. Measurements were done in triplicate and reported as mean ± standard deviation.

**3.2.6. Functional Properties**

**Solubility**

Protein solubility was determined according to Stone et al. (2015), with minor modifications. In brief, 1% (w/w) suspensions of freeze-dried fermented PPEF were prepared in deionized water, and pH was adjusted to pH 4 or pH 7. Afterwards, the suspensions were centrifuged at 3,000 × g for 10 min. Following centrifugation, 5.0 mL of a supernatant was transferred into a digestion flask for nitrogen content determination using Kjeldahl Microdigestor (Model 6030000; Labconco, Kansas City, MO, USA) and a distillation unit (Rapid Still I; Labconco, Kansas City, MO, USA). Measured protein contents in the supernatants were divided by the total protein contents in the initial samples to calculate protein solubilities in percentage relative to the initial samples.

**Emulsification**

EA and ES of fermented and unfermented PPEF samples were determined according to Yasumatsu et al. (1972) and Naczk et al. (1985). In brief, a 1-g portion of a freeze-dried sample was suspended in 14.2 mL of deionized water and pH was adjusted to pH 4 or pH 7. The solution was homogenized using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) equipped with a 20-mm saw tooth probe at the speed “4” (~10,000 rpm) for 30 s. Afterwards, 7.1 mL of canola oil was added to the solution and homogenized for 30 s; then another 7.1-mL portion of canola oil was added to the solution and homogenized for 90 s. After homogenization, the emulsion was centrifuged at 1,300 × g for 5 min, and the emulsification height (cloudy), as well as non-emulsified (clear) phase, were measured. Then EA was determined using Equation 5:
\[ EA = (H_e / H_t) \times 100\% \quad (\text{Eq. 5}) \]

where \( H_e \) is the height of an emulsified phase; \( H_t \) is the total height of a centrifuged suspension.

ES was measured using the emulsions prepared as described above. A prepared emulsion was heated in a water bath at 85°C for 30 min, following heating in a second water bath at 23°C for 15 min. Then the emulsion was centrifuged at \( 1,300 \times g \) for 5 min, and ES was determined using Equation 6:

\[ ES = (EA_h / EA_0) \times 100\% \quad (\text{Eq. 6}) \]

where \( EA_h \) is the emulsifying activity after heating; and \( EA_0 \) is the emulsifying activity before heating. Measurements were done in triplicate and reported as mean ± standard deviation.

**Foaming**

FC and FS were determined using the methods described by Lim et al. (2010). Suspensions of freeze-dried powders were suspended in deionized water (1\% w/w), and pH was adjusted to pH 4 or pH 7. A 15-mL suspension aliquot was transferred to a 400-mL beaker for homogenization using the Omni Macro Homogenizer, equipped with a 20-mm saw tooth probe, at the speed “4” (~10,000 rpm) for 5 min. Afterwards, each homogenized sample was transferred into a 100-mL graduated cylinder. Foam volumes were measured immediately (at time “zero”) and after a 30-min period following a sample transfer into the cylinder. FC and FS were determined as in equations 7 and 8, below:

\[ FC = (V_{F0} / V_{\text{Sample}}) \times 100\% \quad (\text{Eq. 7}) \]

\[ FS = (V_{F30} / V_{F0}) \times 100\% \quad (\text{Eq. 8}) \]

where \( V_{F0} \) is the foam volume at the time 0, \( V_{\text{Sample}} \) is the initial volume of a sample (15 mL), and \( V_{F30} \) is the foam volume after a 30-min period. Measurements were done in triplicates and reported as means ± standard deviations.
**Water Hydration and Oil Holding Capacity**

WHC and OHC were determined using the methods described by Stone et al. (2015). For measuring WHC, 0.5 g of a freeze-dried sample was mixed with 5 mL of distilled water in a pre-weighed 10-mL graduated centrifuge tube. The samples were vortexed for 10 s every 5 min for 30 min. Afterwards, the samples were centrifuged at 11,000 \( \times g \) for 15 min. The supernatant was discarded, and the tubes were re-weighed. For measuring OHC, a freeze-dried sample (0.5 g) was mixed with 5 mL of canola oil; this was followed by the same steps as those for WHC determination described above. WHC and OHC were calculated by dividing the mass of bound water or oil by the mass of the dry PPEF (0.5 g). Measurements were done in triplicate and reported as mean ± standard deviation.

**3.2.7. Protein Quality**

**Amino Acid Composition**

The amino acid profiles of all fermented PPEF samples were determined using a Pico-Tag MT amino acid analysis system (Waters Corporation, Milford, MA, USA) and a high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) instrument at the University of Manitoba (Winnipeg, MB).

**Digestibility**

Fermented PPEF (10% DH) digestibility was determined *in vitro* (IVPD) according to the pH drop method described by Kumitch et al. (2019). A multi-enzyme solution to simulate the human digestive tract was prepared by mixing 31 mg of chymotrypsin, 16 mg of trypsin and 13 mg of protease into 10 mL of deionized water. The pH of the solution was adjusted to 8.0 and stored at 37°C. Approximately 60 mg of fermented PPEF was suspended in 10 mL of deionized water and stirred for 1 hour at 37°C, and subsequently the pH adjusted to 8.0. The multi-enzyme solution (1 mL) was mixed into the protein solution, and the pH was monitored and recorded every minute for 10 min. Equation 9 was used to determine *in vivo* protein digestibility, as described below:

\[
IVPD = 65.66 + 18.10 \times \Delta pH_{10\text{min}} \quad (\text{Eq. 9})
\]
where $\Delta p_{H10\text{min}}$ is the change in pH after 10 minutes.

**Protein Digestibility-Corrected Amino Acid Score**

The PDCAAS of fermented pea protein was determined according to the method described by Kumitch *et al.* (2019), where it is the product of the amino acid score and *in vitro* protein digestibility. Measurements were done in triplicate and reported as mean ± standard deviation.

**3.2.8. Statistical Analyses**

All measurements were made in triplicate (either triplicate batches or triplicate of the composite batch (n=3) and reported as the mean ± standard deviation (except for the amino acid profile, which was measured for a single batch. IBM SPSS Statistics Software Version 1.0.0.1508 was used to complete all statistical analyses. A one-way and two-way analysis of variance (ANOVA) was used to test for significant differences within the main effects of fermenting microorganism and pH, along with their associated interactions.
4. RESULTS AND DISCUSSION

4.1. Solid-state Fermentation of Pea Protein

4.1.1. Optimized SSF Conditions for Fermenting Microorganisms

Pea protein-enriched flour (PPEF) may be hydrolyzed as a result of microbial fermentation. The microorganism secretes hydrolytic enzymes such as amylases, lipases, and proteases that aid in its biomass growth and protein hydrolysis. The ideal method of modifying legume protein is through limited protein hydrolysis, as controlling partial protein hydrolysis to a targeted DH to produce smaller sized peptides and exposing previously buried polar and non-polar amino acid side chains, can result in more amphiphilic, hydrophilic, and hydrophobic groups of peptides that can improve functionalities such as solubility, emulsification and foaming capacities (Barac et al., 2004). Therefore, a partial protein hydrolysis of 10% DH was chosen to be the benchmark for the optimization studies described below regarding temperature, initial moisture content, and time.

Solid-state fermentation of PPEF was conducted under two different temperatures and five different initial moisture contents for each microorganism in order to optimize the fermentation conditions to achieve a partial degree of protein hydrolysis (10% DH). All five microorganisms demonstrated a preference for 50% initial moisture for optimal growth and partial protein hydrolysis. The fungal strains were evaluated under 24°C and 30°C.
Figure 4.1 Degree of hydrolysis (%) of SSF PPEF over a 120-hour fermentation time inoculated with (a) *A. oryzae* NRRL 5590, (b) *R. oryzae* NRRL 395, (c) *R. oligosporus* NRRL 2710, (d) *L. plantarum* NRRL B4496 and (e) *B. subtilis* ATCC 6051.

Data represents the mean values from triplicate PPEF samples ± standard deviation (n=3).

*Abbreviations:* SSF (solid-state fermentation); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); PPEF (pea protein-enriched flour).

Fungal strains *R. oryzae* and *R. oligosporus* achieved 10% DH faster at room temperature (24°C) compared to 30°C; while *A. oryzae* fermentation achieved 10% DH faster at 30°C. The bacterial strains were evaluated under 30°C and 37°C. PPEF fermented with *L. plantarum* achieved 10% DH sooner at 30°C, whereas PPEF fermented with *B. subtilis* was optimal at 37°C. Figure 4.1 above shows the degree of hydrolysis of PPEF as a function of fermentation time for each microorganism. PPEF fermented with *A. oryzae* achieved 10% DH after 48 hours, while PPEF fermented with *R. oryzae, R. oligosporus, L. plantarum,* and *B. subtilis* all achieved 10% DH at 72 hours. The optimized conditions for SSF for each microorganism was utilized to achieve partially hydrolyzed (10% DH) PPEF and subsequently for digestibility and functional properties assays.
4.1.2. SSF Effect on Proximate Composition of Pea Protein

Table 4.1 summarizes the changes to the proximate composition (protein, ash, and lipid content) of PPEF as a result of microbial fermentation under SSF. The samples were prepared from fermenting PPEF in triplicate batches with five different microorganisms to a degree of protein hydrolysis (DH) of 10% under SSF. The fermented PPEF samples were freeze-dried and ground into fine powder prior to being analyzed for proximate composition. Unfermented control PPEF samples had a proximate composition of 46.3% protein, 4.4% ash and 1.6% lipid. Analysis of variance (ANOVA) showed that PPEF fermented with different microorganisms had significantly different effects on protein and lipid contents compared to control samples (p<0.001), but not for ash content (Table 4.2).
### Table 4.1 Proximate composition of SSF PPEF at 10% DH vs unfermented PPEF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (%), d.b.</th>
<th>Ash (%), d.b.</th>
<th>Lipid (%), d.b.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unfermented (0% DH)</strong></td>
<td>46.31 ± 0.59</td>
<td>4.41 ± 0.21</td>
<td>1.62 ± 0.17</td>
</tr>
<tr>
<td><strong>A. oryzae (10% DH)</strong></td>
<td>51.26 ± 0.67</td>
<td>4.59 ± 0.04</td>
<td>0.77 ± 0.14</td>
</tr>
<tr>
<td><strong>R. oryzae (10% DH)</strong></td>
<td>52.09 ± 0.72</td>
<td>4.71 ± 0.16</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td><strong>R. oligosporus (10% DH)</strong></td>
<td>48.87 ± 1.09</td>
<td>4.46 ± 0.28</td>
<td>0.47 ± 0.27</td>
</tr>
<tr>
<td><strong>L. plantarum (10% DH)</strong></td>
<td>53.18 ± 0.16</td>
<td>4.25 ± 0.36</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td><strong>B. subtilis (10% DH)</strong></td>
<td>49.54 ± 1.48</td>
<td>4.11 ± 0.12</td>
<td>0.69 ± 0.21</td>
</tr>
</tbody>
</table>

Data represents the mean values from triplicate batches of composite PPEF samples ± one standard (n=3).

Abbreviations: SSF (solid-state fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); d.b. (dry weight basis).

### Table 4.2 Analysis of variance of the composition, surface properties, and functional properties of SSF PPEF.

<table>
<thead>
<tr>
<th></th>
<th>Composition</th>
<th>Surface properties</th>
<th>Functional properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main effects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microorganism</td>
<td>Protein</td>
<td>Ash</td>
<td>Lipid</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Interactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microorganism x pH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Composition and Surface properties were conducted in triplicate on the composite sample blend from three batches of fermentation (n=3).

Functional properties data represents the mean of triplicate measurements on PPEF ± one standard deviation (n=3).

Microorganisms used (Aspergillus oryzae, Rhizopus oryzae, Rhizopus oligosporus, Lactobacillus plantarum, Bacillus subtilis); pH (4.0 and 7.0).

Abbreviations: EA (emulsifying capacity); ES (emulsion stability); FC (foaming capacity); FS (foam stability); WHC (water hydration capacity); OHC (oil-holding capacity); SSF (solid-state fermentation); PPEF (pea protein-enriched flour); NS (not significant)
Protein contents increased for all fermented PPEF samples at 10% DH; the protein content of PPEF fermented with *R. oryzae* increased protein content by 5.8% to 52.1%, PPEF fermented with *A. oryzae* increased by 5.0% to 51.3%, and with *R. oligosporus* it increased by 2.6% to 48.9%. Fermenting PPEF with these three fungal microorganisms increased the proximate protein content to varying degrees, because they are filamentous fungi that grow hyphae and biomass primarily composed of proteins. These fungi are capable of secreting extracellular enzymes such as protease to break down proteins into shorter peptides, amylase and/or lipase to break down starch and lipids and use the resulting simple sugars or fatty acids as fuel to grow its biomass (Sugai-Guérios *et al.*, 2015). The secreted exogenous microbial proteins also influenced the different final protein content of PPEF. In a study by Pedersen and Nielsen (2000), the growth of biomass protein and α-amylase enzyme by *A. oryzae* fermentation was profiled and were shown to use starch efficiently for biomass production. However, in the current study, the lipid content of *A. oryzae* fermented PPEF was significantly reduced. Therefore, it may be assumed that carbohydrate was the preferred source of energy for biomass growth but due to the limited availability of starch in the PPEF, once the carbohydrates were depleted, lipase was produced to use the lipids as energy source for biomass growth. A study by Souza Filho *et al.* (2018) comparing the edible protein production by filamentous fungi using starch plant wastewater showed that the biomass production by *R. oryzae* was lower compared to *A. oryzae*, but it had the highest protein content. Accordingly, the increase in protein content of fermented PPEF fermented with *R. oryzae* was higher at 5.8% compared to 5% for *A. oryzae* and 2.6% for *R. oligosporus*. Protein content of PPEF (at 10% DH) fermented by *L. plantarum* increased the most by 6.9% to 53.2%, while PPEF fermented with *B. subtilis* increased protein content by 3.2% to 49.5%. These increases may also be attributed to the increased biomass protein of the microorganisms, as both are known to produce the necessary enzymes such as amylases and lipases to utilize either carbohydrates or lipids as energy source; The ecological adaptability of *L. plantarum* was greater than the other microorganisms used in this study, as reflected by the fact that the protein content in fermented PPEF was greatest at the 10% DH point.

Lipid content of fermented PPEF at 10% DH decreased for all samples (Table 4.1). This decreasing trend suggests all five microorganisms utilized lipids as fuel for growth and membrane proliferation in addition to using carbohydrates, as all five microorganisms can produce the necessary lipolytic enzymes *i.e.*, lipases. The metabolic pathway chosen by the microorganisms were dictated by the availability of nutrients, and in this case, it is hypothesized that the hydrolysis
of protein-lipid complexes freed up lipids, making them readily available for microbial utilization.

A similar result of reduced total lipids was observed by Oliveria et al. (2011), where they evaluated fermented rice bran lipid contents in a solid-state fermentation system with *R. oryzae* to find a 9.2% reduction in its lipid contents. In this study, the lipid contents of PPEF decreased from 1.6% to 0.5-0.8% depending on the level of production of lipase by the fermenting microorganism (Table 4.1). Lipase is an enzyme which catalyzes the hydrolysis of lipids into glycerol and free fatty acids. Numerous species of fungi, yeast and bacteria produce lipases. Fermentation of PPEF with *A. oryzae* resulted in the smallest change in lipid content, with a decrease of 0.8% to a final value of 0.77%. Ohnishi et al. (1994) studied the production of lipase by *A. oryzae* and concluded that only a small amount of lipase is produced in solid culture (0.05-0.8 U/wet-g of solid medium). In contrast, lipase production in submerged cultures was higher at 0.46 U/ml); this is in line with the above-mentioned affinity for carbohydrates by *A. oryzae*, although it is capable of utilizing lipids if needed. PPEF fermented with *R. oryzae* and *R. oligosporus* resulted in the largest decreases in their lipid contents, each by 1.1% to 0.52% and 0.47%, respectively; these microorganisms are traditionally used in tempeh, and they can produce lipases among other enzymes during fermentation. Sudarmadji and Markakis (1978) studied the changes to lipid contents during tempeh fermentation (soybean meal fermented with *R. oligosporus*) where they observed a rapid increase in free fatty acid content during the first 30 hours of fermentation before reaching a plateau, indicating a significant lipase activity by *R. oligosporus*. PPEF fermented with *L. plantarum* and *B. subtilis* each decreased their respective lipid contents by 0.9% to 0.73% and 0.69%. The observed differences among strains are due to their different lipase activities and preference toward a source of carbon utilization.

4.1.3. **SSF Effect on Surface Properties of Pea Protein**

Table 4.3 shows the surface properties of fermented PPEF. The surface charge (zeta potential) of fermented PPEF increased for all samples and the variances between fermenting microorganisms were significant (p<0.01, Table 4.1). The net increase in surface charge can be attributed to the proteolytic activity of the microorganisms; protease hydrolyzing the PPEF into smaller peptides would cause the liberation of hydrophilic proteins and charged amino acids such as lysine, glutamine, and histidine.
Table 4.3 Surface properties of SSF PPEF at 10% DH vs unfermented PPEF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta Potential (mV)</th>
<th>Surface Hydrophobicity (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>-14.86 ± 0.74</td>
<td>16.24 ± 0.45</td>
</tr>
<tr>
<td>A. oryzae (10% DH)</td>
<td>-17.21 ± 0.76</td>
<td>12.37 ± 0.84</td>
</tr>
<tr>
<td>R. oryzae (10% DH)</td>
<td>-15.82 ± 0.65</td>
<td>11.09 ± 0.38</td>
</tr>
<tr>
<td>R. oligosporus (10% DH)</td>
<td>-17.83 ± 0.37</td>
<td>11.78 ± 0.33</td>
</tr>
<tr>
<td>L. plantarum (10% DH)</td>
<td>-18.07 ± 0.41</td>
<td>9.26 ± 0.71</td>
</tr>
<tr>
<td>B. subtilis (10% DH)</td>
<td>-16.27 ± 0.94</td>
<td>7.93 ± 0.47</td>
</tr>
</tbody>
</table>

Data represents the mean values from triplicate batches of composite PPEF samples ± one standard (n=3).

Abbreviations: SSF (solid-state fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); mV (millivolts); a.u. (arbitrary units).

PPEF fermented with L. plantarum resulted in the largest increase in surface charge by 3.2 mV from 14.86 mV to 18.07 mV. R. oligosporus fermentation increased surface charge by 2.9 mV to 17.83 mV, A. oryzae fermentation increased the net charge by 2.3 mV to 17.21 mV, B. subtilis fermentation increased surface charge by 1.4 mV to 16.27 mV, while R. oryzae fermentation resulted in the smallest change in surface charge by only 0.9 mV, to 15.82 mV. The observed variances are due to the amphiphilic nature of the hydrolyzed pea proteins which can vary in the degree of charged amino acids that are available or freed-up as a result of fermentation. Overall, the zeta potential of the fermented PPEF samples ranged between -18.1 mV and -15.8 mV, which is still quite low for the stability of the system and did not generate sufficiently strong repulsive forces to prevent protein-protein interactions which ultimately caused protein aggregation that contributed to the reduced solubility and other functional properties that will be discussed below.

Wu et al. (2015) described that a stable protein-water system requires a zeta potential greater than 30 mV.

Surface hydrophobicity results of fermented PPEF are also shown in Table 4.3, and it decreased for all fermented samples with the variance between the fermenting microorganisms being significant (p<0.001, Table 4.2). PPEF fermented with B. subtilis resulted in the greatest decrease in hydrophobicity, by 8.3 a.u. from 16.24 a.u. to 7.93 a.u., L. plantarum decreased hydrophobicity by 7.0 a.u., to 9.26 a.u., R. oryzae fermentation reduced hydrophobicity by 5.2 a.u. from 16.24 a.u. to 11.09 a.u., R. oligosporus by 4.5 a.u. to 11.78 a.u., while fermentation with A.
$_{oryzae}$ resulted in a decrease in hydrophobicity by 3.9 a.u. to 12.37 a.u. The variances also have more to do with the amphiphilic nature of the hydrolyzed PPEF than the microorganisms themselves; the differences are mainly due to the different ratios of hydrophilic to hydrophobic protein groups that are present in each sample. The general trend of decreased hydrophobicity usually indicates that a higher ratio of hydrophilic protein moieties was liberated as a result of protein hydrolysis, which is also supported by the fact surface charge for all fermented samples increased as discussed above. Accordingly, the increased surface charge prevented hydrophobic interactions by electrostatic repulsive forces. However, a decreased surface hydrophobicity would be expected to also show higher solubility, but this was not the case as solubility decreased for all fermented samples. This indicates that hydrophobic protein moieties were present in a sufficient amount to reduce solubility. Therefore, it is hypothesized that during the solubility assay, hydrophobic protein moieties were in abundance as a result of the proteolytic activity of the fermenting microorganisms. Over an extended period, the hydrophobic interactions could not be prevented due to the weak repulsive forces, causing the hydrophobic protein surfaces to aggregate and bury the hydrophobic sites back into the interior of the aggregated proteins which resulted in a decrease in surface hydrophobicity.

4.1.4. SSF Effect on Functional Properties of Pea Protein

Solubility

Solubility results of fermented PPEF under SSF at pH 7.0 and pH 4.0 are summarized in Table 4.4. Protein solubility is defined by the nitrogen solubility index as the amount of nitrogen present in a protein that can be dissolved in an aqueous solution compared to the total nitrogen contained in the protein (Adler-Nissen, 1976). At pH values away from the pI of the protein, electrostatic repulsive forces are greater which allows protein-water interactions to be favoured over protein-protein interactions, and typically results in higher solubility of the protein (Wu et al., 1998). The solubility of a protein also plays a major role in how its other functional properties, such as emulsifying and foaming characteristics, behave (Kinsella, 1979).
Table 4.4 Solubility (%) of SSF PPEF at 10% DH vs unfermented PPEF at pH 4.0 and pH 7.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solubility (%) at pH 4.0</th>
<th>Solubility (%) at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>25.4 ± 0.8</td>
<td>59.8 ± 0.6</td>
</tr>
<tr>
<td>A. oryzae (10% DH)</td>
<td>21.3 ± 0.7</td>
<td>54.4 ± 0.3</td>
</tr>
<tr>
<td>R. oryzae (10% DH)</td>
<td>19.7 ± 0.6</td>
<td>52.5 ± 0.4</td>
</tr>
<tr>
<td>R. oligosporus (10% DH)</td>
<td>17.8 ± 0.5</td>
<td>49.2 ± 0.4</td>
</tr>
<tr>
<td>L. plantarum (10% DH)</td>
<td>16.9 ± 2.1</td>
<td>52.3 ± 1.2</td>
</tr>
<tr>
<td>B. subtilis (10% DH)</td>
<td>18.8 ± 0.9</td>
<td>55.8 ± 0.2</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

Abbreviations: SSF (solid-state fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis).

A two-way analysis of variance determined the effects of the fermenting microorganisms and pH were both significant (p<0.001), but their associated interaction was not significant (Table 4.2). At pH 4.0 the solubility of PPEF was significantly lower (25.4%) compared to its solubility at pH 7.0 (59.8%), as shown in Table 4.4. This was expected, as the isoelectric precipitation point (pI) of pea protein is very close at 4.6 compared to pH of 4.0, where weak electrostatic repulsive forces promoted protein-protein interactions that cause flocculation and coagulation which decreases solubility. The solubility decreased at 10% DH in all fermented samples (Table 4.4). Fermented PPEF samples had 4.0% – 10.6% lower protein solubilities compared to unfermented control samples (0% DH). PPEF fermented with R. oligosporus reduced protein solubility by 10.6% to 49.2%, R. oryzae fermentation lowered solubility by 7.3% to 52.7%, while the reduction was 5.4% for A. oryzae fermented PPEF at 54.4% (Table 4.4). Similar findings were reported by Kumitch et al. (2020), where PPEF fermented (SSF at 10% DH) with A. oryzae and A. niger demonstrated lower solubilities by 4.1% and 4.2%, respectively.

A decrease in solubility indicates the liberation of hydrophobic protein moieties due to the fermenting microorganisms’ proteolytic activities, allowing for protein-protein interactions and causing protein aggregation. The increase in biomass protein with a presumed lower solubility than that of PPEF also contributed significantly to the decrease in solubility of fermented PPEF.

Filamentous fungi, such as A. oryzae, R. oryzae, and R. oligosporus, are widely used GRAS microorganisms that are able to grow in different environments by hyphal extension and branching,
allowing the growing biomass to secrete large amounts of enzymes that hydrolyze proteins in the medium (Parachin et al., 2011). Among many studies of fungal cell wall and mycelial morphology, Müller et al. (2002) determined chitin synthesis is an important factor in the process that regulates hyphal growth and morphology, specifically two chitin synthase gene products: ChsB and CsmA. Chitin synthase is the essential enzyme used in the chitin synthesis pathway, using UDP-N-acetylglucosamine to produce the chitin polymer. Chitin is an abundant aminopolysaccharide polymer which is the building material that gives strength to the cell walls of fungi, but most importantly it binds with proteins via covalent bonds. The structure of chitin is like that of cellulose except the hydroxyl group is replaced with an amine-linked acetyl group. The acetyl groups contribute to the formation of stable hydrogen bonds along its inter sheets, which inhibits its association with water molecules. Therefore, due to this hydrophobic property of the acetyl group, chitin is insoluble in water. Thus, an increase in chitin, which is bound to the growing fungal biomass protein, could have contributed to the insolubility of fermented PPEF.

In the case of bacterial fermentation with *L. plantarum* and *B. subtilis*, the solubilities were reduced by 7.5% and 4.0% to 52.3% and 55.8%, respectively. Their proteolytic activities exposing hydrophobic protein moieties would be the primary contributor to the observed lowered solubility. However, the production of hydrophobic bacteriocins may have compounded the reduced solubility of fermented PPEF (Arnaouteli et al., 2016). Lactic acid bacteria can produce various inhibitory compounds with activity against other microorganisms, such as bacteriocins, phenyllactic acid, peptides, and fatty acids with possible application in food biopreservation. Bacteriocins are ribosomally-synthesized proteins categorized in three classes, of which Class II are described as small, hydrophobic, and heat-stable (Perez et al., 2014). *Lactobacillus plantarum* can produce many Class II bacteriocins including plantaricin C19, 423, EF, JK, S, and NC8, and pediocin AcH. pH and temperature are important factors for plantaricin production, with maximum yield obtained at neutral pH (Perez et al., 2014). Some strains of *L. plantarum* can produce surface proteins that are mucus-binding, aggregation-promoting, and function in intracellular adhesion (Corsetti & Valmorri, 2011). Thus, there are multiple factors that contribute to the increase in protein hydrophobicity which led to the reduction of solubility of PPEF fermented with *L. plantarum* and *B. subtilis*. 
Emulsification

The emulsifying properties of fermented PPEF are one of the functional properties studied to determine its potential as a functional ingredient. The emulsifying activity (EA) and emulsion stability (ES) of fermented PPEF as a function of fermenting microorganism and pH are given below in Table 4.5. The emulsifying activity is defined by the exposure of hydrophobic protein moieties and its ability to be flexible in terms of conformation and prevent flocculation and coalescence (Ahmed et al., 2011); EA is measured by the height of the emulsion. A two-way ANOVA (Table 4.2) shows that the effect of the fermenting microorganisms and pH was significant for EA of PPEF (p<0.001) but their interaction was not. Emulsion stability is the ability of the emulsion to hold its structure and resist changes over time (Ahmed et al., 2011). The effects of fermenting microorganisms (p<0.01) and pH (p<0.001) and their interactions (p<0.05) on the ES of fermented PPEF were all significant (Table 4.2).

The EA of PPEF decreased for all samples at pH 4 by an average of 24.2% compared to its EA at pH 7 (Table 4.5). EA was greater at pH 7 compared to pH 4 regardless of the fermenting microorganism, since fermented PPEF at pH 4 was very close to the isoelectric precipitation point of pea protein at 4.6. The low solubility and the coagulation of precipitated proteins at pH 4 prevented emulsion formation, thus lowering the emulsifying activity significantly (Ahmed et al., 2011).

At pH 7, the EA of PPEF fermented with A. oryzae increased by 3.4% to 41.8%. A 2018 study by Sadh et al. found similar results of increased emulsifying properties of peanut oil cake after SSF with A. oryzae. Kumitch et al. (2020) also demonstrated a slight increase in EA of PPEF at pH 7 when fermented with A. oryzae. In this thesis research, the EA of PPEF fermented with R. oligosporus increased by 2.4% to 40.8%, while the EA of PPEF fermented with B. subtilis, R. oryzae, and L. plantarum all decreased by 4.2%, 1.8%, and 1.2%, to 34.2%, 36.6%, and 37.2%, respectively.
Table 4.5 Emulsifying activity (%) and emulsion stability (%) of SSF PPEF at 10% DH vs unfermented PPEF at pH 4.0 and pH 7.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EA (%) at pH 4.0</th>
<th>EA (%) at pH 7.0</th>
<th>ES (%) at pH 4.0</th>
<th>ES (%) at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>15.8 ± 0.5</td>
<td>38.4 ± 0.9</td>
<td>12.1 ± 1.1</td>
<td>86.9 ± 1.3</td>
</tr>
<tr>
<td>A. oryzae (10% DH)</td>
<td>17.2 ± 0.2</td>
<td>41.8 ± 0.7</td>
<td>15.1 ± 0.5</td>
<td>93.2 ± 0.4</td>
</tr>
<tr>
<td>R. oryzae (10% DH)</td>
<td>12.5 ± 0.7</td>
<td>36.6 ± 0.4</td>
<td>10.3 ± 0.4</td>
<td>88.6 ± 0.8</td>
</tr>
<tr>
<td>R. oligosporus (10% DH)</td>
<td>12.9 ± 0.3</td>
<td>40.8 ± 0.2</td>
<td>14.2 ± 0.6</td>
<td>90.2 ± 0.7</td>
</tr>
<tr>
<td>L. plantarum (10% DH)</td>
<td>13.7 ± 0.3</td>
<td>37.2 ± 0.7</td>
<td>9.4 ± 0.9</td>
<td>91.5 ± 0.4</td>
</tr>
<tr>
<td>B. subtilis (10% DH)</td>
<td>11.6 ± 1.2</td>
<td>34.2 ± 0.6</td>
<td>11.2 ± 0.7</td>
<td>88.1 ± 1.4</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

Abbreviations: SSF (solid-state fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); EA (emulsifying activity); ES (emulsion stability).

The emulsifying properties of hydrolysates such as fermented PPEF are mostly affected by the size and amphiphilic nature of the hydrolyzed peptides. Shröder et al. (2017) reported that bigger peptides above 2 kDa increased the emulsifying activity of whey hydrolysates because of their ability to unfold at the oil-water interface. Smaller peptides are prone to diffusion at the oil-water interface because of their exposed hydrophobic cores. Aspergillus oryzae and Rhizopus oligosporus increased the ES by 1.17 ~ 1.25-times compared to unhydrolyzed sample. Thus, it is suggested that during fermentation of PPEF, the proteolytic activity of Aspergillus oryzae and Rhizopus oligosporus produced relatively larger molecular weight peptides (>2 kDa) that allowed them to migrate more readily to the oil-water interface. Hydrolyzed and partially unfolded fermented PPEF further unfolded at the oil-water interface to expose hydrophobic and hydrophilic protein residues, allowing them to interact with both the aqueous and oil phases of the emulsion. This interaction could stabilize the oil-water interface of emulsions by reducing the interfacial tension, explaining the increased ES as well. The ratio of hydrophobic to hydrophilic residues further determines the stability of the emulsion, and it is possible that Aspergillus oryzae and Rhizopus oligosporus were more effective in this regard. A study by Prinyawiwatkul et al. (1997) on the functional characteristics of fermented cowpea
flour concluded that fungal proteolytic activity may have exposed hydrophobic protein groups which resulted in a change in the hydrophilic-hydrophobic ratio that eventually favoured stable emulsion formation.

In contrast, the reduced EA of PPEF fermented with *R. oryzae, L. plantarum,* and *B. subtilis* suggests the proteolytic activity of *R. oryzae* on PPEF produced peptides that were either too small and diffused out of the interface and/or peptides with reduced amphiphilicity that favoured either more hydrophobic interactions or hydrophilic interactions.

Fermentation effects of fungi such as *A. oryzae, R. oligosporus,* and *R. oryzae* on the emulsifying properties of fermented PPEF are influenced by the extent of growth of the network of hyphae formed on the substrate (Parachin et al., 2011). Different species of fungi can form different types of hyphae such as surface, biofilm, penetrative and aerial hyphae, at different stages of growth. Sugai-Guérios et al. (2015) studied and confirmed the ability of *A. oryzae* and *R. oligosporus* to effectively form penetrative hyphae, thus providing some similarities for these two microorganisms which helps explain why the two had a different effect than *R. oryzae*.

Meanwhile, pea protein-enriched flour (10% DH) fermented with *L. plantarum* and *B. subtilis* also demonstrated a reduced EA. The reduced EA is hypothesized to be caused by a higher hydrophobic-hydrophilic protein ratio caused by enzymatic hydrolysis of PPEF, which consequently decreased protein solubility and its ability to be absorbed at the oil-water interface, ultimately affecting the EA.

As shown on Table 4.5, the ES of fermented PPEF was significantly lower at pH 4.0 compared to at pH 7.0 due to the lower solubility at pH values closer to the pI of pea protein. However, the interaction of microorganism and pH was significant (p<0.05, Table 4.2). At pH 4.0, fermentation of pea protein-enriched flour with *A. oryzae* increased ES by 3.0% from 12.1% to 15.1%; an increase of 6.3% from 86.9% to 93.2% was observed at pH 7.0 (Table 4.5). At pH 4, fermentation of PPEF with *R. oryzae* decreased ES by 1.8% from 12.1% to 10.3%; in contrast an increase of 1.7% from 86.9% to 88.6% was observed at pH 7 (Table 4.5). PPEF fermented with *R. oligosporus* increased by 2.1% and 3.3% at pH 4.0 and 7.0, respectively. PPEF samples fermented with *L. plantarum* and *B. subtilis* demonstrated a lowered ES at pH 4 but an increase at pH 7; a decrease of 2.7% from 12.1% to 9.4% for *L. plantarum* and a decrease of 0.9% from 12.1% to 11.2% for *B. subtilis* at pH 4.0, and an increase of 4.6% from 86.9% to 91.5% for *L. plantarum* and an increase of 1.2% from 86.9% to 88.1% for *B. subtilis* at pH 7.0 (Table 4.5). The stabilities
of the emulsions are dictated by the ability of the proteins to adsorb to the oil-water interface, which highlights the importance of the size of the hydrolyzed protein peptides. Additionally, the ability of the absorbed proteins to conform and reorient by folding or unfolding at the interface to interact with both the oil and water with hydrophobic and hydrophilic side chains and reducing interfacial tension is of great importance. The observed increases in ES at pH 7.0 are primarily attributed to the increased amphiphilicity of the PPEF due to the balanced ratio of hydrophobic and hydrophilic side chains unfolded during partial hydrolysis via fermentation. The observed decreases in some of the fermented samples at pH 4 is primarily attributed to the unpredictable behaviour of the proteins close to the isoelectric precipitation point with reduced solubility.

**Foaming**

The foaming capacity (FC) of a protein is determined from the amount of interfacial area that can be created by mechanical whipping of the protein. Foam stability is measured as the volume of foam retained after 30 minutes. Foam formation and foam stability (FS) are functions of the type of protein, pH, processing methods, viscosity, and surface tension. Foaming capacity has been reported to enhance the consistency, texture, and appearance of foods (Sathe et al., 1982).

A two-way ANOVA was conducted for the FC and FS of fermented pea PPEF samples. The individual effect between fermenting microorganisms was significant for both FC (p<0.001) and FS (p<0.001). In addition, the individual effect of pH was significant (p<0.001) for both FC and FS (Table 4.2). The ANOVA also showed that only the interactions were significant for both FC (p<0.05) and FS (p<0.001).

The FC and FS decreased for all fermented PPEF samples, and FS of fermented PPEF was not detected at all at pH 4 (Table 4.6). At pH 4, the FC of PPEF was significantly lower than at pH 7. These pH-dependencies can be explained by the fact that the isoelectric points of many pulse proteins are at or near pH 4. One of the main factors affecting the foaming properties of proteins is solubility, a decrease in solubility correlates to a decrease in foaming properties. The solubility of fermented PPEF is very low at pH 4.0 due to its proximity to its pl (4.6), which is consistent with reduced foaming properties. The reduced number of soluble proteins resulted in a reduction in protein absorption to the air-water interface, meaning there was less proteins in the air-water interface to reduce the surface tension, which ultimately decreased the FC and FS of fermented PPEF.
Table 4.6 Foaming capacity (%) and foam stability (%) of SSF PPEF at 10% DH vs unfermented PPEF at pH 4.0 and pH 7.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FC (%) at pH 4.0</th>
<th>FC (%) at pH 7.0</th>
<th>FS (%) at pH 4.0</th>
<th>FS (%) at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>147.8 ± 6.9</td>
<td>176.5 ± 4.2</td>
<td>9.3 ± 0.9</td>
<td>86.9 ± 1.3</td>
</tr>
<tr>
<td>A. oryzae (10% DH)</td>
<td>117.1 ± 2.6</td>
<td>146.2 ± 4.6</td>
<td>n.d.</td>
<td>21.7 ± 1.6</td>
</tr>
<tr>
<td>R. oryzae (10% DH)</td>
<td>104.6 ± 3.1</td>
<td>148.2 ± 7.1</td>
<td>n.d.</td>
<td>19.4 ± 1.1</td>
</tr>
<tr>
<td>R. oligosporus (10% DH)</td>
<td>108.2 ± 1.8</td>
<td>155.4 ± 2.1</td>
<td>n.d.</td>
<td>22.7 ± 2.3</td>
</tr>
<tr>
<td>L. plantarum (10% DH)</td>
<td>103.2 ± 0.5</td>
<td>152.3 ± 1.7</td>
<td>n.d.</td>
<td>19.1 ± 0.4</td>
</tr>
<tr>
<td>B. subtilis (10% DH)</td>
<td>99.1 ± 6.3</td>
<td>149.4 ± 2.4</td>
<td>n.d.</td>
<td>18.6 ± 1.3</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

Abbreviations: SSF (solid-state fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); FC (foaming capacity); FS (foam stability); n.d. (none detected).

A 2011 study by Adebowale and Maliki observed a similar decrease in FC and FS of pigeon pea flour with increasing periods of fermentation. Interestingly, Oloyede et al. (2015) found the FC of fermented Moringa flour significantly increased in the first 12 hours of fermentation, but subsequently decreased during the 24- and 72-hour fermentation periods. Fermented sorghum flour showed no foaming capacity at all according to Elkhalifa et al. (2005). In contrast, Chawla et al. (2017) determined that fermentation of black-eyed pea flour increased in FC and FS. Results from these previous studies and this study on the FC and FS behaviours of various flours suggests the degree of protein hydrolysis is a significant factor. Generally, partial denaturation of a globular proteins achieved by either heat exposure or fermentation will increase foaming properties. As the protein structure unfolds and exposes hydrophobic residues, it may be able to adsorb more readily to air-water interfaces and lower the interfacial tension, thereby trapping more air and increasing the foaming capacity. Extensive denaturation of proteins will decrease their ability to form foams. Therefore, the reduced FC and FS of fermented PPEF samples may suggest that at 10% DH, hydrolysis of pea protein-enriched flour perhaps denatured the protein too extensively to the point
it cannot enhance foaming properties.

**Water Hydration Capacity and Oil-holding Capacity**

Water hydration capacity (WHC) and oil-holding capacity (OHC) are important functional properties in the food industry, it affects most food preparation processes as it influences other functional and sensory properties (Boye *et al.*, 2010). WHC can be defined as the ability of a protein matrix to absorb and retain bound water against external forces. WHC is an important protein-water interaction that is prominent in various food systems. Similarly, OHC is the ability of proteins to absorb and retain bound oil against external forces such as centrifugation. The range of applications of fermented pea protein as a food ingredient is largely dependent on how well it interacts with water and/or oil.

An ANOVA was performed to test the significance of the effect of the fermenting microorganisms on the WHC and OHC of fermented PPEF samples (Table 4.2). The effect of microorganisms was significant on WHC (p<0.001) and on OHC (p<0.001). At 10% DH, WHC and OHC of fermented PPEF were found to have increased compared to those of unfermented PPEF (0% DH). Unfermented samples showed about 1.2 g/g WHC for all samples; whereas PPEF fermented with *A. oryzae* resulted in an increase of its WHC by 1.2 g/g, which was the highest observed WHC increase (Figure 4.2). A similar increase in WHC was reported in black-eyed pea flour fermented with *A. oryzae*, where WHC increased by up to 1.3 g/g with increasing time of fermentation (Chawla *et al.*, 2017). Fermentation likely caused the denaturing and subsequent unfolding of protein complexes (Oloyede *et al.*, 2015). This unfolding of pea protein exposed hydrophilic regions and amino acid residues of proteins that resulted in a higher affinity for interactions with water.
Figure 4.2 Water hydration capacity (g/g) of SSF PPEF at 10% DH vs unfermented PPEF. Data represents the mean values from triplicate batches of composite blend PPEF samples ± standard deviation (n=3). Abbreviations: SSF (solid-state fermentation); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); PPEF (pea protein-enriched flour); WHC (water-holding capacity).

In comparison, solid-state fermentation with the other filamentous fungi (R. oligosporus and R. oryzae) improved WHC of PPEF by 0.9 g/g and by 0.7 g/g, respectively. The observed differences in WHC between these fungal microorganisms may be due to the different levels of exposure of hydrophilic peptides as a result of protein denaturation and unfolding.

Another important factor to consider is the effect of the microorganism itself. Kyanko et al. (2012) studied dietary fiber and β-glucan content as well as the fungal hydration properties such as WHC of different fungi, including R. oryzae. They identified a positive correlation between increasing levels of β-glucan and increasing WHC of fungi; isolates derived from filamentous fungi such as R. oryzae had greater water holding capacities. Therefore, it is reasonable to assume that fungal fiber such as β-glucan also contributed to the improved WHC of fermented PPEF. Due to the structures of filamentous fungi such as A. oryzae, R. oryzae, and R. oligosporus containing high levels of proteins and polysaccharides, they could enhance the functional properties such as WHC of fermented PPEF for food applications.

Fermenting PPEF with bacterial strains yielded similar increases in WHC. PPEF samples
fermented with *L. plantarum* demonstrated an increased WHC by 0.7 g/g. The influence of *L. plantarum* fermentation on the functional properties of soybean protein meal was reported by Amadou *et al.* (2011). Their results showed a reduced WHC, which contradicts the results observed from fermented PPEF. This highlights the importance of substrate content and the degree of protein denaturation; the extent of protein denaturation significantly affects whether the protein is amphiphilic, hydrophilic, or hydrophobic. Furthermore, Das *et al.* (2014) explored α-D-glucan produced by *L. plantarum* for *in vitro* prebiotic activities. The α-D-glucan polymer demonstrated a 316.9% water holding capacity. The authors claim this observed property is associated with the porous matrix structure formed by polysaccharide chains which can hold large amounts of water through hydrogen bonds. Thus, like fungal β-glucans, the production of α-D-glucan by *L. plantarum* may have contributed to the increase in WHC of fermented PPEF.

Lee *et al.* (2014) investigated the functionality of poly-γ-glutamic acid (γ-PGA), which is produced by *B. subtilis*, for its potential applications in medicine and cosmetics. Among its various observed functionalities, the γ-PGA showed water-holding capacity and hygroscopicity. In addition to hydrolysis being effective in protein denaturation and unfolding to reveal more hydrophilic sites in the protein matrix, γ-PGA could partially explain why *B. subtilis* fermentation of PPEF resulted in an increase in WHC by 0.9 g/g. Additionally, Liu *et al.* (2018) assessed the crosslinking characteristics of *B. subtilis* transglutaminase (BTG) and how it affects the functional properties of whey protein. In food proteins, a covalent crosslinking of ε-(γ-glutamine)-lysine isopeptidic bonds can be formed between a ε-amino group in a protein-bound lysine residue and a γ-carboxyamide group of a protein-bound glutamine residue by the transglutaminase through acyl-transfer reaction. A notable increase of WHC was reported in the whey protein samples treated with BTG; they suggested that the fine and dense networks might have been formed by the smaller aggregates and pore sizes in the matrix produced by BTG cross-linking, resulting in a greater capillary action to enhance the entrapment of water and thereby enhance WHC.

Furthermore, increases in WHCs of various legume flours as a result of fermentation were previously reported by Akubor and Chukwu (1999), Reyes-Moreno *et al.* (2004), Chandra-Hioe *et al.* (2016), Xiao *et al.* (2018) and Kumitch *et al.* (2020). The WHC increases may be explained by the microbial proteolytic activity leading to increased numbers of highly hydrophilic low-molecular weight proteins and/or increased surface availability of hydrophilic amino acids in fermented samples. OHCs of various legume flours were also previously reported to be increased.
as a result of fermentation (Chandra-Hioe et al., 2016; Xiao et al., 2015, 2018; Kumitch et al., 2020). Increased surface availability of hydrophobic amino acids in fermented samples can explain the OHC increases.

The improved OHC of fermented PPEF is primarily a result of protease activity secreted by the microorganisms. The microbial enzymes hydrolyzed the pea protein and exposed previously buried hydrophobic sites, and this action subsequently promoted the aggregation of these exposed hydrophobic protein moieties. The aggregation of these hydrophobic protein moieties created micro-capillaries that can physically entrap oil bodies and retain the oil through hydrophobic interactions (Avramenko et al. 2013). The different degrees of enhancement of OHC observed from different microorganisms used may shed light on the different metabolisms and characteristics of the microorganisms.

Fermentation with B. subtilis and R. oryzae led to the highest OHCs, each with an increase of 1.1 g/g; however, the OHC increases of 1.0 g/g and 0.9 g/g in fermented PPEF fermented with L. plantarum and R. oligosporus, respectively, were not much lower in comparison. The OHC of fermented PPEF fermented with A. oryzae had the lowest increase at 0.8 g/g (Figure 4.3). As mentioned previously, the B. subtilis transglutaminase (BTG) produced during fermentation can cause the cross-linking of proteins to form capillary networks that would physically entrap water or oil. In this case, oil would also be entrapped and retained via hydrophobic interactions, thus BTG may have also contributed to the increased OHC.

Omosebi and Otunola (2013) studied tempeh flour produced from three different Rhizopus species, among which R. oryzae and R. oligosporus demonstrated comparable OHCs of 0.96 g/g and 0.98 g/g. Soybean protein meal was also reported to show an increased OHC when fermented with L. plantarum (Amadou et al., 2011). Aspergillus oryzae showed the lowest increase in OHC, which is interesting because it also showed the highest increase in WHC, which suggests that protein unfolding caused by fermentation with A. oryzae liberated more hydrophilic protein moieties and less hydrophobic ones.
Figure 4.3  Oil-holding capacity (g/g) of SSF PPEF at 10% DH vs unfermented PPEF.

Data represents the mean values from triplicate batches of composite blend PPEF samples ± standard deviation (n=3). Abbreviations: SSF (solid-state fermentation); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); PPEF (pea protein-enriched flour); OHC (oil-holding capacity).

Findings of OHC indicated that SSF of PPEF exposed the hydrophobic interaction sites of fermented samples compared with unfermented samples. Similar observations were made by Xiao *et al.* (2015) and Elkhalifa and Bernhardt (2005) for SSF chickpea and sorghum flour, respectively. They stated that the binding of oil correlated with hydrophobic binding sites of amino acids present on the surface of the product, and higher OHC could be attributed to the unfolding of non-polar amino acids from the interior of the protein molecule. In addition, improvement in OHC after fermentation would be caused by physical entrapment of oil on the surface of fermented flour samples.

4.1.5. Protein Quality and Digestibility

The protein quality of peas was shown to have a high lysine content and a relative deficiency in tryptophan and the sulphur-containing amino acids methionine and cysteine. Pea protein concentrates contained adequate levels of all the essential amino acids, except, as expected, cysteine and methionine. Gatel (1994) demonstrated the *in vivo* protein digestibility of pea protein
in pigs is slightly lower compared to soybean meal (0.74 in peas vs 0.80 in soybean meal). The author attributed the lower digestibility of peas to the presence of anti-nutritional factors (ANF, e.g. protease inhibitors, lectins or tannins) and/or fibrous material, leading to low accessibility of legume seed protein to digestive enzymes.

The *in vitro* protein digestibility (IVPD) of PPEF used in the current study was 75.2%, a comparable value to the *in vivo* protein digestibility of 74% found using pigs as a model system as reported by Gatel (1994). In other studies, the IVPD of moth bean was 58.75% (Khokhar & Chauhan, 1986), 80.20% for mung bean (Mubarak, 2005), 71.10% for breadnut flour, 75.60% for cashew nut flour, and 78.70% for pumpkin flour (Fagbemi *et al.*, 2005). IVPD of peas was shown to increase from 78.4 to 81.39 after fermentation (Khattab *et al.*, 2009).

In the present study, the limiting amino acids in PPEF were shown to be methionine and cysteine with an amino acid score of 0.866. Therefore, the *in vitro* protein digestibility-corrected amino acid score (IV-PDCAAS) of the control sample of pea protein was 65.1%. A two-way ANOVA showed the effects of fermentation and the fermenting microorganism on IVPD and IV-PDCAAS were all statistically significant (Table 4.7).

Fermentation (10% DH) increased the IVPD of pea protein in all samples; however, it did not affect the limiting amino acid score, hence the IV-PDCAAS also increased as a result (p<0.05) (Table 4.8). This increase in digestibility can be attributed to an increase in microbial protease activity, and the consequent hydrolysis of proteins into smaller peptides which allows for easier digestion. Also, in a previous study by Kumitch *et al.* (2019), a decrease in anti-nutritional factors was shown as fermentation time increased. The authors discussed that the decrease in anti-nutritional factors allowed for protein cross-linkages to become more susceptible to proteolytic attack, which ultimately contributed to an increase in digestibility of pea protein. PPEF fermented with *B. subtilis* had the largest increase in IV-PDCAAS, by 5.4% to 70.5%, closely followed by PPEF fermented with *R. oligosporus* by 4.4% to 69.5%, followed by an increase of 4.0% to 69.1% and by 3.1% to 68.2% for PPEF fermented with *A. oryzae* and *R. oryzae*, respectively. Pea protein hydrolyzed with *L. plantarum* resulted in the smallest increase to 67%, by 1.9% (Table 4.8).
Table 4.7 Analysis of variance of the in vitro protein digestibility (IVPD) and in vitro protein digestibility-corrected amino acid score (IVPDCAAS) of SSF PPEF.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Protein Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IVPD</td>
</tr>
<tr>
<td>Microorganism</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Fermentation (10% DH)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Interactions</td>
<td></td>
</tr>
<tr>
<td>Microorganism x Fermentation</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.8 in vitro protein digestibility (IVPD) and in vitro protein digestibility-corrected amino acid score (IVPDCAAS) of SSF PPEF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Limiting amino acid</th>
<th>Limiting amino acid score</th>
<th>IVPD (%)</th>
<th>IV-PDCAAS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MET + CYS</td>
<td>0.866</td>
<td>75.16±0.31</td>
<td>65.09%</td>
</tr>
<tr>
<td>A.oryzae</td>
<td>MET + CYS</td>
<td>0.876</td>
<td>78.84±0.37</td>
<td>69.06%</td>
</tr>
<tr>
<td>R.oryzae</td>
<td>MET + CYS</td>
<td>0.853</td>
<td>79.92±0.25</td>
<td>68.17%</td>
</tr>
<tr>
<td>R.oligosporus</td>
<td>MET + CYS</td>
<td>0.864</td>
<td>80.41±0.42</td>
<td>69.47%</td>
</tr>
<tr>
<td>L.plantarum</td>
<td>MET + CYS</td>
<td>0.844</td>
<td>79.38±0.29</td>
<td>67.00%</td>
</tr>
<tr>
<td>B.subtilis</td>
<td>MET + CYS</td>
<td>0.869</td>
<td>81.16±0.41</td>
<td>70.53%</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

Abbreviations: SSF (solid-state fermentation); PPEF (pea protein-enriched flour); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); IVPD (in vitro protein digestibility); IVPDCAAS (in vitro protein digestibility-corrected amino acid score); MET (methionine); CYS (cysteine).

An improvement in the digestibility of legumes after fermentation with B. subtilis was reported by Kiers et al. (2000a). In a solid-state fermentation experiment by Shi et al. (2017), corn-soybean meal fermented using B. subtilis demonstrated an 8% increase in protein digestibility. The authors also suggested the secretion of metabolites by B. subtilis reduced anti-nutritional factors that would tend to inhibit proteolytic activity. In addition, Kiers et al. (2000b) reported that the digestibility of B. subtilis-fermented soybeans were higher than that of tempeh fermented with R.
oryzae, R. oligosporus, and L. plantarum. In their study, IVPD of soybean, maize and cowpea fermented with R. oryzae and R. oligosporus were reported to have increased by similar values. The authors said the large observed increase in total digestibility of maize and cowpea was most likely due to the presence of large amounts of starch becoming gelatinized making it more readily available for the enzymatic degradation (Kiers et al., 2000a).

The reason why PPEF fermented with filamentous fungi (i.e., A. oryzae, R. oryzae and R. oligosporus) in the present study had lower IVPD values than the bacterium, B. subtilis, may be due to different properties of fungi with lower levels of antinutritional factor degrading activity. Kiers et al. (2000a) also stated although total digestibility of cowpeas and soybeans did not increase to a large extent, the amount of absorbable matter without enzymatic degradation (absorbability) increased significantly due to the increase in fungal biomass, and from the ratio between absorbability and digestibility they showed that fungal fermentation (biomass) contributed up to 60% of total digestibility. Thus, the authors concluded that fungal fermentation can pre-digest the material to a significant extent (Kiers et al., 2000a). Kumitch et al. (2019) also reported a similar increase in IVPD of fermented pea protein-enriched flour fermented with A. oryzae. The increase in IVPD of L. plantarum fermented PPEF is also primarily associated with hydrolysis of protein; however, the difference from PPEF fermented with the other four microorganisms may have been due to the different levels, or lack thereof, of certain anti-nutritional factor-degrading activity. Mugula et al. (2003) reported that L. plantarum had proteolytic activity as well as tannase activity; tannase degrades tannin complexes with protein, allowing for the hydrolysis of protein into smaller peptides and making them available for pepsin digestion, and hence contributing to the increase of IVPD (Duodu et al., 2003).

4.2. Submerged Fermentation of Pea Protein

4.2.1. Optimized SmF Parameters for Fermenting Microorganisms

Submerged fermentation (SmF) of PPEF was conducted at two different temperatures with each microorganism to optimize fermentation conditions to achieve a partial degree of protein hydrolysis of 10%. The fungal strains were evaluated under 24°C and 30°C. Fungal strains R. oryzae and R. oligosporus achieved 10% DH faster at room temperature (24°C) compared to at 30°C, whereas A. oryzae fermentation achieved 10% DH faster at 30°C. The bacterial strains were
evaluated at 30°C and 37°C. PPEF fermented with *L. plantarum* achieved 10% DH sooner at 30°C, while PPEF fermented with *B. subtilis* was optimal at 37°C. Figure 4.4 shows the degree of hydrolysis of PPEF as a function of fermentation time for each microorganism. PPEF fermented with *L. plantarum* achieved 10% DH after 24 hours, PPEF fermented with *A. oryzae, R. oryzae,* and *B. subtilis* all achieved 10% DH after 48 hours. Meanwhile, PPEF fermented with *R. oligosporus* achieved 10% DH after 120 hours. Accordingly, the optimized conditions for SSF for each microorganism to achieve partially-hydrolyze PPEF to 10% DH were subsequently used for further digestibility and functional properties assays.

![Figure 4.4](image_url)

**Figure 4.4** Degree of hydrolysis (%) of SmF PPEF over a 120-hour fermentation time inoculated with (a) *A. oryzae* NRRL 5590, (b) *R. oryzae* NRRL 395, (c) *R. oligosporus* NRRL 2710, (d) *L. plantarum* NRRL B4496 and (e) *B. subtilis* ATCC 6051.

Data represents the mean values from triplicate PPEF samples ± standard deviation (n=3).

**Abbreviations**: SmF (submerged fermentation); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); PPEF (pea protein-enriched flour).
4.2.2. **SmF Effect on Proximate Composition of Pea Protein**

The protein, ash, and lipid contents of fermented PPEF under SmF at 10% degree of hydrolysis increased significantly (p<0.001) for all fermented samples (Table 4.9). Under SmF, *B. subtilis* increased the protein content of PPEF the most by 6.0% from 46.3% to 52.3%, *R. oligosporus* increased protein content of PPEF by 5.2% from 46.3% to 51.5% (which was the highest increase among fungal strains), *A. oryzae* increased protein content of PPEF by 3.4% from 46.3% to 49.7%, *L. plantarum* increased protein content of PPEF by 3.1% to 49.5%, while *R. oryzae* increased protein content of PPEF by 2.8% to 49.1% (Table 4.10). The increase in protein content of PPEF is hypothesized to primarily be due to the increased microbial biomass protein as a result of submerged fermentation; a similar observation of increased protein content under solid-state fermentation was reported previously in this study.

Under SmF, *A. oryzae* increased ash content of PPEF the most by 1.2% from 4.4% to 5.6%, *R. oligosporus* increased ash content of PPEF by 1.1% to 5.5%, *R. oryzae* increased ash content of PPEF by 0.8% to 5.2%, *B. subtilis* increased the ash content of PPEF by 0.7% to 5.1%, while *L. plantarum* increased ash content of PPEF the least by 0.5% to 4.9% (Table 4.10). The increased ash content of PPEF under submerged fermentation is hypothesized to be from the increased microbial biomass secreting exogenous minerals and/or minerals being released from the protein-carbohydrate complexes as a result of proteolytic or enzymatic activity; lipid contents increased, therefore, it is likely the microorganisms used carbohydrates as energy source instead of lipids (all five microorganisms employed are capable of producing amylases and lipases to use either lipids or carbohydrates as fuel). Jin *et al.* (1998) used starch processing wastewater for production of microbial biomass protein and fungal amylase by *Aspergillus oryzae*. The PPEF contains starch, which the microorganisms were able to break down with amylase to use as fuel for growth. The utilization of carbohydrates resulted in an increase in mineral content and thus ash content was increased as a result of submerged fermentation.
<table>
<thead>
<tr>
<th></th>
<th>Composition</th>
<th>Surface properties</th>
<th>Functional properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Ash</td>
<td>Lipid</td>
</tr>
<tr>
<td><strong>Main effects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microorganism</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Interactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microorganism x pH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Composition and Surface properties were conducted in triplicate on the composite sample blend from three batches of fermentation (n=3). Functional properties data represents the mean of triplicate measurements on PPEF ± one standard deviation (n=3).

Microorganism (*Aspergillus oryzae, Rhizopus oryzae, Rhizopus oligosporus, Lactobacillus plantarum, Bacillus subtilis*); pH (4.0 and 7.0).

**Abbreviations:** EA (emulsifying capacity); ES (emulsion stability); FC (foaming capacity); FS (foam stability); WHC (water hydration capacity); OHC (oil-holding capacity); SmF (submerged fermentation); PPEF (pea protein-enriched flour); NS (not significant).
Table 4.10 Proximate composition of SmF PPEF at 10% DH vs unfermented PPEF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (%)</th>
<th>Ash (%)</th>
<th>Lipid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>46.31 ± 0.59</td>
<td>4.41 ± 0.21</td>
<td>1.62 ± 0.17</td>
</tr>
<tr>
<td>A. oryzae (10% DH)</td>
<td>49.73 ± 0.89</td>
<td>5.62 ± 0.42</td>
<td>2.31 ± 0.17</td>
</tr>
<tr>
<td>R. oryzae (10% DH)</td>
<td>49.12 ± 1.07</td>
<td>5.21 ± 0.16</td>
<td>2.64 ± 0.28</td>
</tr>
<tr>
<td>R. oligosporus (10% DH)</td>
<td>51.49 ± 1.26</td>
<td>5.49 ± 0.31</td>
<td>3.22 ± 0.25</td>
</tr>
<tr>
<td>L. plantarum (10% DH)</td>
<td>49.45 ± 1.29</td>
<td>4.88 ± 0.09</td>
<td>2.83 ± 0.18</td>
</tr>
<tr>
<td>B. subtilis (10% DH)</td>
<td>52.34 ± 1.16</td>
<td>5.05 ± 0.11</td>
<td>3.31 ± 0.12</td>
</tr>
</tbody>
</table>

Data represents the mean values from triplicate batches of composite PPEF samples ± one standard (n=3).

**Abbreviations:** SmF (submerged fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); d.b. (dry weight basis).

Under SmF, *B. subtilis* increased the lipid content of PPEF the most by 1.7% from 1.6% to 3.3%, the increase in lipid content of PPEF with *R. oligosporus* was 1.6% from 1.6% to 3.2%, while *L. plantarum* increased lipid content of PPEF by 1.2% to 2.8%, *R. oryzae* increased lipid content of PPEF by 1.0% to 2.6%, and *A. oryzae* increased lipid content of PPEF the least by 0.7% from 1.6% to 2.3% (Table 4.10). As mentioned above, it is hypothesized that the microorganisms preferred using carbohydrates for growth in a SmF system as lipids are hydrophobic and not accessible to the microorganisms in an aqueous environment. The microorganisms were allowed more mobility due to being submerged in water which gave them more access to the full available nutrients of the substrate PPEF, thus allowing microbial biomass to grow. The increase in lipid content can be attributed to protein hydrolysis exposing hydrophobic cores of the proteins which may have had bound lipids that are now liberated. In addition, lipid by-products of microbial metabolism as a function of fermentation may have increased the lipid content of PPEF. Muniraj *et al.* (2013) studied the production of microbial lipids from *Aspergillus oryzae* from potato processing wastewater and reported a yield of 3.5 g/L of potato wastewater (which is high in starch as with PPEF).
4.2.3. SmF Effect on Surface Properties of Pea Protein

Surface charge (zeta potential)

The surface properties of fermented pea protein-enriched flour (PPEF) under submerged fermentation (SmF) at 10% degree of hydrolysis were affected significantly (p<0.001) for all fermented samples (Table 4.9). The surface charge increased for all fermented PPEF samples under SmF, *B. subtilis* increased the zeta potential of PPEF the most by 9.9 mV to -24.8 mV, *R. oryzae* increased zeta potential of PPEF by 8.5 mV to -23.4 mV, *R. oligosporus* increased zeta potential of PPEF by 5.9 mV to -20.8 mV, while *L. plantarum* increased zeta potential of PPEF by 4.8 mV to -19.6 mV, and finally *A. oryzae* increased zeta potential of PPEF by 4.0 mV to -18.9 mV (Table 4.11). The zeta potential (ZP) of proteins is used to determine its stability in a solution based on the repulsiveness or attractiveness of the proteins; proteins in solution at a high or low pH (further from pI value of the protein) carry a charge which can cause electrostatic repulsion between them. According to Wu *et al.* (2015), if the absolute value of the ZP is greater than 30 mV, electrostatic repulsion will be effective in inhibiting protein-protein interactions and thus prevent the aggregation of proteins which helps stabilize the solution.

Table 4.11 Surface properties of SmF PPEF at 10% DH vs unfermented PPEF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta Potential (mV)</th>
<th>Surface Hydrophobicity (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>-14.86 ± 0.74</td>
<td>16.24 ± 0.45</td>
</tr>
<tr>
<td><em>A. oryzae</em> (10% DH)</td>
<td>-18.93 ± 0.42</td>
<td>7.82 ± 0.84</td>
</tr>
<tr>
<td><em>R. oryzae</em> (10% DH)</td>
<td>-23.35 ± 0.68</td>
<td>8.92 ± 0.17</td>
</tr>
<tr>
<td><em>R. oligosporus</em> (10% DH)</td>
<td>-20.79 ± 0.14</td>
<td>8.23 ± 0.11</td>
</tr>
<tr>
<td><em>L. plantarum</em> (10% DH)</td>
<td>-19.64 ± 0.47</td>
<td>9.29 ± 0.55</td>
</tr>
<tr>
<td><em>B. subtilis</em> (10% DH)</td>
<td>-24.76 ± 0.48</td>
<td>8.78 ± 0.21</td>
</tr>
</tbody>
</table>

Data represents the mean values from triplicate batches of composite PPEF samples ± one standard (n=3).

Abbreviations: SmF (submerged fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); mV (millivolts); a.u. (arbitrary units).

The pI of pea protein (4.6) is lower than the pH 7 solution of the samples; therefore, the fermented PPEF samples will carry a net-negative charge. The partial hydrolysis of proteins under submerged
fermentation caused the liberation of some proteins from larger protein-complexes and exposed acidic protein moieties which formed negatively charged clusters. Tirgar \textit{et al.} (2017) reported that high negative ZP are the result of a high ratio of acidic to basic protein moieties. However, despite the increase in ZP seen in this thesis research, it was still not high enough to stabilize the protein solution due to the repulsive forces being too weak to prevent protein aggregation. Therefore, this result indicates a pH 7 solution was not ideal for stabilizing fermented PPEF and the pH of the solution must be further away from the pI of pea protein. This increase in charge density also indicates that both positively- and negatively charged protein moieties were exposed; the mobility of microorganisms in SmF allowed for a greater access to protein surfaces for better hydrolysis of protein-complexes to expose both positively- and negatively charged protein moieties.

**Surface hydrophobicity**

The surface hydrophobicity decreased for all fermented PPEF samples under SmF, \textit{A. oryzae} decreased surface hydrophobicity of PPEF by 8.4 a.u., \textit{R. oligosporus} decreased surface hydrophobicity of PPEF by 8.0 a.u., \textit{B. subtilis} decreased surface hydrophobicity of PPEF by 7.5 a.u., while \textit{R. oryzae} decreased surface hydrophobicity of PPEF by 7.3 a.u., and finally \textit{L. plantarum} decreased surface hydrophobicity of PPEF by 7.0 a.u. (Table 4.11). These observations suggest that same proteins could have different conformations: proteins in SmF samples were surrounded by water and therefore likely tended to have more hydrophilic surfaces; respectively, these proteins could be expected to have greater surface charges than those from SSF samples.

**4.2.4. SmF Effect on Functional Properties of Pea Protein**

**Solubility**

A two-way ANOVA was performed on the solubility of fermented PPEF under SmF at 10% degree of hydrolysis as a function of fermenting microorganism and pH was significantly different for the individual effects (p<0.001) for all fermented samples, but their interaction was not significant (Table 4.9). The solubility decreased for all fermented PPEF samples under SmF. At pH 7, \textit{R. oryzae} decreased solubility of PPEF by 18.3% to 41.5%, \textit{R. oligosporus} decreased solubility of PPEF by 15.5% to 44.3%, \textit{A. oryzae} decreased solubility of PPEF by 14.5% from 59.8% to 45.3%, while \textit{Bacillus subtilis} decreased solubility of PPEF by 14.1% to 45.7%, and
finally *L. plantarum* decreased solubility of PPEF by 12.6% to 47.2% (Table 4.12). At pH 4, the solubility of PPEF under submerged fermentation decreased by an average of 34.6%.

### Table 4.12 Solubility (%) of SmF PPEF at 10% DH vs unfermented PPEF at pH 4.0 and pH 7.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solubility (%) at pH 4.0</th>
<th>Solubility (%) at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>25.4 ± 0.8</td>
<td>59.8 ± 0.6</td>
</tr>
<tr>
<td>A. oryzae (10% DH)</td>
<td>10.7 ± 0.4</td>
<td>45.3 ± 0.2</td>
</tr>
<tr>
<td>R. oryzae (10% DH)</td>
<td>10.7 ± 0.1</td>
<td>41.5 ± 0.7</td>
</tr>
<tr>
<td>R. oligosporus (10% DH)</td>
<td>9.6 ± 0.1</td>
<td>44.3 ± 0.6</td>
</tr>
<tr>
<td>L. plantarum (10% DH)</td>
<td>9.2 ± 0.1</td>
<td>47.2 ± 0.7</td>
</tr>
<tr>
<td>B. subtilis (10% DH)</td>
<td>10.4 ± 0.7</td>
<td>45.7 ± 0.3</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

*Abbreviations:* SmF (submerged fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*).

The higher solubility at pH 7 was due to it being further away from the pI of pea protein (4.6), with greater electrostatic repulsive forces allowing protein-water interactions to be favoured over protein-protein interactions, which typically would result in higher solubility of the protein. A decrease in solubility would indicate that the liberation of hydrophobic protein moieties due to the fermenting microorganisms’ proteolytic activities allowed for protein-protein interactions and causing protein aggregation. The increase in biomass protein with a presumed lower solubility than that of PPEF also contributed to the decrease in solubility of fermented PPEF.

**Emulsification**

The EA and ES of fermented PPEF as a function of fermenting microorganism and pH are given in Table 4.13. Analysis of variance demonstrated the levels of significance of the individual effects and their interactions and are reported in Table 4.9. The EA decreased for all fermented PPEF samples under SmF. The EA of fermented PPEF at 10%DH was greater at pH 7 compared to pH 4 (p<0.001) regardless of fermenting microorganism, an observation due to fermented PPEF at pH 4 being too close to the isoelectric precipitation point of pea protein (4.6) which would prevent stability by allowing protein coagulation and preventing emulsion formation. At pH 7, *R.*
*oligosporus* decreased EA of PPEF by 5.2% from 38.4% to 33.2%, *L. plantarum* decreased EA of PPEF by 3.7% to 34.7%, *A. oryzae* decreased EA of PPEF by 3.3% from 38.4% to 35.1%, while *B. subtilis* decreased EA of PPEF by 2.8% to 35.6%, and finally *R. oryzae* decreased EA of PPEF by 1.5% to 36.9% (Table 4.13). The EA of SmF fermented PPEF decreased possibly due to the observed weak repulsive forces (ZP) at pH 7 not being strong enough to prevent flocculation and protein-protein interactions and thus reducing solubility and stability, and ultimately affecting its emulsion forming ability (Çabuk et al., 2018).

### Table 4.13 Emulsifying activity (%) and emulsion stability (%) of SmF PPEF at 10% DH vs unfermented PPEF at pH 4.0 and pH 7.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EA (%) at pH 4.0</th>
<th>EA (%) at pH 7.0</th>
<th>ES (%) at pH 4.0</th>
<th>ES (%) at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td>15.8 ± 0.5</td>
<td>38.4 ± 0.9</td>
<td>12.1 ± 1.1</td>
<td>86.9 ± 1.3</td>
</tr>
<tr>
<td><em>A. oryzae</em> (10% DH)</td>
<td>10.7 ± 0.7</td>
<td>35.1 ± 0.4</td>
<td>18.6 ± 0.3</td>
<td>39.3 ± 0.4</td>
</tr>
<tr>
<td><em>R. oryzae</em> (10% DH)</td>
<td>9.8 ± 0.5</td>
<td>36.9 ± 0.3</td>
<td>18.1 ± 0.6</td>
<td>40.2 ± 0.5</td>
</tr>
<tr>
<td><em>R. oligosporus</em> (10% DH)</td>
<td>15.2 ± 0.2</td>
<td>33.2 ± 0.4</td>
<td>20.8 ± 1.1</td>
<td>38.4 ± 0.2</td>
</tr>
<tr>
<td><em>L. plantarum</em> (10% DH)</td>
<td>6.9 ± 0.2</td>
<td>34.7 ± 0.6</td>
<td>21.2 ± 0.6</td>
<td>25.6 ± 0.4</td>
</tr>
<tr>
<td><em>B. subtilis</em> (10% DH)</td>
<td>7.8 ± 0.2</td>
<td>35.6 ± 0.6</td>
<td>17.9 ± 0.3</td>
<td>23.9 ± 0.2</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

**Abbreviations:** SmF (submerged fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); EA (emulsifying activity); ES (emulsion stability).

An analysis of variance on the effect of fermenting microorganism and pH on the ES of fermented PPEF under SmF at 10% degree of hydrolysis has shown significant changes for the individual effects for all fermented samples as well as their interactions (Table 4.9). The ES decreased for all fermented PPEF samples under SmF at pH 7, *B. subtilis* decreased ES of PPEF by 63.0% from 86.9% to 23.9%, *L. plantarum* decreased ES of PPEF by 61.3% to 25.6%, *R. oligosporus* decreased ES of PPEF by 48.5% to 38.4%, while *A. oryzae* decreased ES of PPEF by 47.6% to 39.3%, and finally *R. oryzae* decreased ES of PPEF by 46.7% to 40.2% (Table 4.13).
The observed decline in ES could be attributed to hydrolyzed hydrophobic protein moieties affecting the oil-water interface and preventing protein-water interactions while favouring the aggregation of released peptides and unhydrolyzed proteins (Çabuk et al., 2018).

At pH 4, the ES of unfermented PPEF was significantly low (12.1%), reflecting its unstable nature. The ES of PPEF following SmF increased for all samples; *L. plantarum* increased ES of PPEF by 9.1% from 12.1% to 21.2%, *R. oligosporus* increased ES of PPEF by 8.7% to 20.8%, *A. oryzae* increased ES of PPEF by 6.5% from 12.1% to 18.6%, *R. oryzae* increased ES of PPEF by 6.0% to 18.1%, and *B. subtilis* increased ES of PPEF by 5.8% to 17.9% (Table 4.13). Emulsion stability is the ability of the emulsion to hold its structure and resist changes over time. SSF samples formed more stable emulsions at pH 7 (86.9%) compared to at pH 4 (12.1%); SmF samples’ ES was likewise greater at pH 7 than at pH 4 as expected, but it was significantly less stable than SSF samples at 38.6%, this difference may be attributed to the loss of hydrophilic proteins in the supernatant during an extra centrifugation step in sampling under SmF platform. This would lead to an imbalance in hydrophilic to hydrophobic proteins, thereby destabilizing the emulsion.

### Foaming

A two-way ANOVA revealed that the effects of pH and microorganisms were significant, and their interaction was significant as well (Table 4.9). The FC decreased for all SmF samples. At pH 4, the FC of PPEF samples decreased by 28.7%; this was due to the proximity of the PPEF’s isoelectric precipitation point (pI = 4.6) and the foam being less stable due to the low solubility of proteins. At pH 7, PPEF fermented with *L. plantarum* decreased FC the most, by 110.9% to 65.6%, PPEF fermented with *A. oryzae* decreased FC by 109.7% to 66.8%, PPEF fermented with *R. oryzae* decreased FC by 104.7% to 71.8%, PPEF fermented with *R. oligosporus* decreased FC by 102.8% to 73.7%, and finally PPEF fermented with *B. subtilis* decreased FC the least, by 102.1% to 74.4%.

FS of fermented PPEF decreased significantly as a result of fermentation. At pH 4, the FS of PPEF decreased by 77.6% to 9.3%, which was again due to the low solubility of pea protein at pH values close to its pI. At pH 7, bacterial strains decreased FS the most; PPEF fermented with *B. subtilis* decreased FS by 70.2% to 16.7%, while PPEF fermented with *L. plantarum* decreased FS by 69.2% to 17.7%. PPEF fermented with *R. oligosporus* decreased FS by 67.7% to 19.2%,
PPEF fermented with *A. oryzae* decreased FS by 67.3% to 19.6%, PPEF fermented with *R. oryzae* decreased FS by 65.3% to 21.6% (Figure 4.14).

**Table 4.14** Foaming capacity (%) and foam stability (%) of SmF PPEF at 10% DH vs unfermented PPEF at pH 4.0 and pH 7.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FC (%) at pH 4.0</th>
<th>FC (%) at pH 7.0</th>
<th>FS (%) at pH 4.0</th>
<th>FS (%) at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented (0% DH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. oryzae</em> (10% DH)</td>
<td>96.1 ± 0.3</td>
<td>66.8 ± 0.4</td>
<td>20.2 ± 0.5</td>
<td>19.6 ± 0.1</td>
</tr>
<tr>
<td><em>R. oryzae</em> (10% DH)</td>
<td>95.9 ± 0.6</td>
<td>71.8 ± 0.5</td>
<td>23.5 ± 0.4</td>
<td>21.6 ± 0.1</td>
</tr>
<tr>
<td><em>R. oligosporus</em> (10% DH)</td>
<td>98.2 ± 0.3</td>
<td>73.7 ± 0.8</td>
<td>18.6 ± 0.7</td>
<td>19.2 ± 0.8</td>
</tr>
<tr>
<td><em>L. plantarum</em> (10% DH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em> (10% DH)</td>
<td>82.5 ± 0.4</td>
<td>74.4 ± 0.4</td>
<td>19.6 ± 0.8</td>
<td>16.7 ± 0.2</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3).

**Abbreviations**: SmF (submerged fermentation); PPEF (pea protein-enriched flour); DH (degree of hydrolysis); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); FC (foaming capacity); FS (foam stability).

The observed decreases in FC and FS of fermented PPEF are primarily attributed to the decreased solubility; solubility directly influences the emulsifying and foaming properties of a protein because it must be soluble and in small molecular size in order to get adsorbed to the air-water interface to form and stabilize foams. Solubility decreased due to hydrophobic protein moieties increasing as a result of microbial proteolytic activity, which also affected and decreased the foaming properties of PPEF. Similar findings were previously reported by some authors who studied functional properties of various fermented legume flours. Particularly, Elkhalifa *et al.* (2005) found that sorghum flour had decreased FC as a result of fermentation. Fermented faba bean, desi, and kabuli chickpea flours were also found to have lower FSs compared to those of unfermented counterparts (Chandra-Hioe *et al*., 2016). However, Xiao *et al.* (2018) who studied the effects of fermentation on the properties of red bean flour (RBF), reported no significant difference between FCs of fermented and unfermented RBFs; at the same time, FS of fermented
RBF was found to be higher than FS of unfermented RBF. Some discrepancies in findings of different authors who studied properties of fermented legume flours suggest that the outcomes of fermentation, in terms of the functional properties of the products, may depend on the types of studied legumes and on the processing conditions used during preparation of legume flours (Lam et al., 2016).

**Water Hydration Capacity / Oil Holding Capacity**

An analysis of variance determined the effect of microorganisms were significantly different on the WHC and OHC of SmF PPEF (Table 4.9). WHCs of SmF PPEF samples were higher by 0.4 – 1.1 g/g as shown below on Figure 4.5, and OHCs of SmF PPEF samples were higher by 2.1 – 2.6 g/g, compared to those of respective unfermented samples (Figure 4.6). Fermentation with A. oryzae led to highest WHC increase, by 1.1 g/g. PPEF fermented with R. oligosporus increased WHC by 0.7 g/g, while PPEF fermented with R. oryzae increased WHC by 0.5 g/g. Bacterial strains L. plantarum and B. subtilis increased WHC of fermented PPEF the least, each by 0.4 g/g. Fermentation with A. oryzae also led to the highest OHC increase; however, the OHC increases in samples fermented with B. subtilis and R. oligosporus were not much lower than those achieved with A. oryzae (2.6 g/g vs. 2.3 g/g increases; Figure 4.6). PPEF fermented with L. plantarum and R. oryzae increased OHC by 1.7 g/g and 1.9 g/g, respectively.

Increases in WHCs of various legume flours as a result of fermentation were previously reported by Akubor and Chukwu (1999), Reyes-Moreno et al. (2004), Chandra-Hioe et al. (2016), Xiao et al. (2018) and Kumitch et al. (2020). The WHC increases may be explained by the microbial proteolytic activity leading to increased numbers of highly hydrophilic low-molecular-weight proteins and/or increased surface availability of hydrophilic amino acids in fermented PPEF samples. OHCs of various legume flours were also previously reported to be increased as a result of fermentation (Chandra-Hioe et al., 2016; Xiao et al., 2015, 2018; Kumitch et al., 2020). Increased surface availability of hydrophobic amino acids in fermented PPEF samples provide an explanation for the observed OHC increases.
Figure 4.5  Water hydration capacity (g/g) of SmF PPEF at 10% DH vs unfermented PPEF.

Data represents the mean values from triplicate batches of composite blend PPEF samples ± standard deviation (n=3). Abbreviations: SmF (submerged fermentation); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); PPEF (pea protein-enriched flour); WHC (water-holding capacity).

Figure 4.6  Oil-holding capacity (g/g) of SmF PPEF at 10% DH vs unfermented PPEF.

Data represents the mean values from triplicate batches of composite blend PPEF samples ± standard deviation (n=3). Abbreviations: SmF (submerged fermentation); DH (degree of hydrolysis); A. oryzae (Aspergillus oryzae); R. oryzae (Rhizopus oryzae); R. oligosporus (Rhizopus oligosporus); L. plantarum (Lactobacillus plantarum); B. subtilis (Bacillus subtilis); PPEF (pea protein-enriched flour); OHC (oil-holding capacity).
4.2.5. Protein Quality and Digestibility

Fermentation (10% DH) increased the IVPD of pea protein in all samples, but it decreased the limiting amino acid score as it resulted in lower methionine and cysteine levels, thus the IV-PDCAAS decreased as a result (p<0.01) (Table 4.15). The increase in IVPD can again be attributed to an increase in microbial proteolytic activity on proteins, which resulted in smaller peptides that were easier to digest. The decrease in anti-nutritional factors allowed for protein cross-linkages to become more susceptible to proteolytic attack, which ultimately contributed to an increase in digestibility of pea protein. PPEF fermented with *R. oryzae* decreased IV-PDCAAS by 9.0% to 56.1%, PPEF fermented with *A. oryzae* decreased by 8.8% to 56.3%, while PPEF fermented with *R. oligosporus* decreased by 8.3% to 56.8%. PPEF fermented with *B. subtilis* decreased IV-PDCAAS by 7.7% to 57.4%, while PPEF fermented with *L. plantarum* decreased the least, by 4.2% to 60.9% (Table 4.16). Lower levels of methionine and cysteine in SmF samples led to lower amino acid scores which ultimately lowered the IV-PDCAAS values, this may have been due to a loss of amino acids during the initial extra centrifugation step involved in sampling fermented PPEF to freeze dry. Some solubilized amino acids (methionine and cysteine) may be lost in the supernatant prior to freeze-drying.

**Table 4.15** Analysis of variance of the *in vitro* protein digestibility (IVPD) and *in vitro* protein digestibility-corrected amino acid score (IVPDCAAS) of SmF PPEF.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Protein Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microorganism</em></td>
<td>IVPD</td>
</tr>
<tr>
<td><em>Fermentation (10% DH)</em></td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><em>Interactions</em></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td><em>Microorganism x Fermentation</em></td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

The IVPD of all fermented PPEF samples increased significantly (Table 4.16), bacterial strains increased IVPD the most; PPEF fermented with *B. subtilis* increased IVPD the most, by 4.4% to 79.6%, PPEF fermented with *L. plantarum* increased IVPD by 4% to 79.2%, PPEF fermented with *R. oryzae* increased by 3.7% to 78.8%, PPEF fermented with *R. oligosporus* increased by 3.5% to 78.7%, while PPEF fermented with *A. oryzae* increased IVPD the least, by
1.8% to 77%. The increase in IVPD due to SmF was mainly because of the proteolytic activity of the microorganisms cleaving larger proteins into smaller sizes that were easier to digest. Although the increased protein digestibility as a result of fermentation is promising and helps improve the quality by ANF degradation, the limiting amino acids of pea protein (as shown by the IV-PDCAAS values in Table 4.16) does not make it a complete protein and must thus be supplemented with other complimentary proteins such as those from cereal grains.

**Table 4.16 in vitro** protein digestibility (IVPD) and *in vitro* protein digestibility-corrected amino acid score (IVPDCAAS) of SmF PPEF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Limiting amino acid</th>
<th>Limiting amino acid score</th>
<th>IVPD (%)</th>
<th>IV-PDCAAS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MET + CYS</td>
<td>0.866</td>
<td>75.16±0.3</td>
<td>65.09%</td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td>MET + CYS</td>
<td>0.732</td>
<td>76.97±0.2</td>
<td>56.34%</td>
</tr>
<tr>
<td><em>R. oryzae</em></td>
<td>MET + CYS</td>
<td>0.712</td>
<td>78.83±0.3</td>
<td>56.13%</td>
</tr>
<tr>
<td><em>R. oligosporus</em></td>
<td>MET + CYS</td>
<td>0.722</td>
<td>78.68±0.3</td>
<td>56.81%</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>MET + CYS</td>
<td>0.769</td>
<td>79.15±0.3</td>
<td>60.87%</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>MET + CYS</td>
<td>0.722</td>
<td>79.56±0.4</td>
<td>57.44%</td>
</tr>
</tbody>
</table>

Data represents the mean values of triplicate measurements on PPEF samples ± one standard (n=3). 

*Abbreviations:* SmF (submarged fermentation); PPEF (pea protein-enriched flour); *A. oryzae* (*Aspergillus oryzae*); *R. oryzae* (*Rhizopus oryzae*); *R. oligosporus* (*Rhizopus oligosporus*); *L. plantarum* (*Lactobacillus plantarum*); *B. subtilis* (*Bacillus subtilis*); IVPD (*in vitro* protein digestibility); IVPDCAAS (*in vitro* protein digestibility-corrected amino acid score); MET (methionine); CYS (cysteine)
5. OVERALL CONCLUSIONS

Pulse crops are nutrient-rich and versatile in their functionality. However, while pulses contain double the amount of protein compared to cereal grains, their digestibility is lower, and they tend to be limiting in either sulfur amino acids or tryptophan. In addition, pulses contain antinutritional factors that affect their nutrient bioavailability. Therefore, pulses in their native state are limiting and must be modified to improve the protein quality and functionality prior to utilization and consumption (Cargo-Froom et al., 2020).

Degree of hydrolysis (DH) is an important marker of individual protein functionalities (Zink et al., 2016). Prior research has reported evidence that partial enzymatic hydrolysis of a protein contributes to the selective improvement of functional properties of protein including solubility, emulsification, and foaming (Barac et al., 2012; Ghribi et al., 2015; Panyam & Kilara, 1996). However, differing results have also been reported. Lamsal et al. (2006) reported reduced emulsification properties of soy protein that was partially hydrolyzed. Foaming properties of ethanol-washed soy protein concentrate also was lowered after partial hydrolysis (Jung et al., 2005). Yust et al. (2010) showed that limited chickpea protein hydrolysates had a lower EA compared to unmodified proteins. These results imply that the specific effect of proteolysis on the functional properties varies with the nature of the substrate; Acidic subunits of 11S legumin of pea protein were more susceptible to proteolysis compared to basic ones because of their orientation at the molecular surface (Barac et al., 2012).

In this research study, the protein quality and the physicochemical and functional properties of PPEF fermented with five different GRAS microorganisms over a 120-hour fermentation was evaluated under optimal conditions for both solid-state fermentation and submerged fermentation platforms. Protein contents of fermented PPEF samples at 10% DH increased under both fermentation platforms, due in part to increased microbial biomass. Lipid contents decreased for SSF samples, indicating the microorganisms utilized lipids as fuel for growth and metabolism using lipases. The lipid contents increased for SmF samples, which suggests the microorganisms more effectively used carbohydrates as fuel under submerged conditions and excreted lipases as a metabolite. The globular structure of pea protein was partially hydrolyzed and altered by microbial proteolytic enzymes, which significantly affected the physicochemical and functional properties.
of PPEF. As a result of protein hydrolysis by both SSF and SmF, hydrophobic protein moieties were liberated and caused protein-protein aggregation in favour of protein-water interactions, which led to the decrease in the solubility of PPEF. Zeta potential of fermented PPEF increased but it did not create strong enough electrostatic force to prevent protein-protein interactions. The decreased solubility affected other functional properties as well, as emulsifying, and foaming properties of fermented PPEF were not improved at 10% DH. The solubility, emulsifying, and foaming properties of fermented PPEF were better at pH 7 compared to at pH 4 due to it being further away from the isoelectric precipitation point of pea protein. Under both SSF and SmF platforms, fermented PPEF demonstrated an improved WHC and OHC; this was attributed to the microbial proteolytic activity hydrolyzing and liberating both hydrophilic and hydrophobic small-molecular-size peptides, allowing them to absorb the oil or water body.

The protein quality of pea protein was limited by low levels of methionine and cysteine. Fermentation did not improve the quality of PPEF but it did, however, improve IVPD; the improved digestibility of PPEF was attributed to the reduction of anti-nutritional factors as well as hydrolyzing larger proteins into small-molecular-weight peptides that were easier for digestion.

In conclusion for this thesis research, protein content was increased under all parameters, but SSF with L. plantarum at pH 7.0 increased it the most. SSF with A. oryzae at pH 7.0 resulted in the highest increase of EA and ES. SSF with B. subtilis improved IVPD and IVPD-CAAS the most. Other properties were either reduced or affected negatively under SSF at 10% DH. Under SmF, surface charge was increased the most with B. subtilis at pH 7.0; surface hydrophobicity was decreased the most by A. oryzae at pH 7.0; WHC and OHC were both enhanced the most with A. oryzae at pH 7.0. These results may suggest that the specific effect of enzymatic hydrolysis via microbial fermentation on the functional properties of PPEF varies with the microorganism and fermentation platform. Thus, the effects of combined interactions of various parameters such as pH, enzyme type, fermenting microorganism, and DH on protein quality and functionality must be considered.

Pulse ingredients if modified or hydrolyzed to the right DH, can provide viscosity, water-holding ability, oil-holding ability, gelation, emulsification, foaming, adhesion, and film formation. They can be utilized in different types of novel food products to enhance protein content and replace soy, dairy, and meat proteins. Further research to improve on this thesis work may focus on the effect of varying DH (perhaps greater than 10% DH) on the functional properties and protein...
quality of PPEF. Another opportunity to enhance this study would be to narrow the scope of work and focus on specific functional properties to optimize the improvement based on the application needs. In addition, the impact of microbial fermentation on the flavour profile of PPEF may be of interest for its use as a food ingredient. Understanding the application needs and pulse protein functionality and developing function-specific fermentation approaches will allow product developers to successfully utilize pulse ingredients such as PPEF.
REFERENCES


