SUBMERGED FERMENTATION BIOCONVERSION OF AIR-CLASSIFIED STARCH-RICH PULSE FLOURS TO PROTEIN-RICH PRODUCTS

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ABSTRACT

Pulse starch is a low-value, often underutilized co-product of the pulse industry. This research focuses on the submerged fermentation of starch-rich pulse fractions by generally recognized as safe (GRAS) microbes that result in production of microbial biomass enriched in crude protein, converting low-value starch to higher-value microbial protein. Accordingly, starch-rich pulse fractions of yellow field pea, yellow lentil and faba bean flours were fermented by *Lactobacillus plantarum* or *Aspergillus oryzae* applied as single- and multi-strain cultures. The fermentation process converted starch into microbial protein, increasing protein levels in fermented flour. The protein content of starch-rich yellow pea, yellow lentil and faba bean flours increased from 7.8% to 10.2%, 16.5% to 18.5% and 14.5% to 16.4% respectively. However, the increase in protein content was not sufficient to make the fermented substrates reach the targeted level of >45% protein. This was likely due to the shortage of nitrogen as starch-rich flours have 80% or above carbohydrate. The addition of inexpensive, commonly available nitrogen compounds was tested to increase protein. The starch-rich flours were supplemented with ammonium sulphate, ammonium phosphate or urea at varying concentrations (15 g/L – 35 g/L) over the fermentation time course to aid in *de novo* microbial protein synthesis. It was found that nitrogen supplementation aided microbial growth during fermentation and resulted in higher protein yield than when no additional nitrogen was added. Supplementation of urea at 35 g/L resulted in highest protein yield in all three pulse flours, resulting in final protein levels above 45%. The protein-rich fermented substrates were then further analyzed for proximate composition including starch, ash, lipid and moisture contents and *in vitro* protein digestibility (IVPD). It was found that as the protein content increased, the starch and lipid levels in the fermented substrates decreased. The overall protein digestibility of substrates fermented by *L. plantarum* was also improved and significantly higher ($p<0.05$) compared to *A. oryzae* and *L. plantarum-A. oryzae* co-culture fermented samples. Overall, this research highlights that fermentation by GRAS microbes for single cell protein (SCP) production is a highly efficient method to increase value of under-utilized starch-rich by-products in the pulse industry, as SCP can be used as an alternative for conventional food and feed.

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LIST OF ABBREVIATIONS

A. oryzae	*Aspergillus oryzae*
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
CFU	Colony forming units
d.b.	Dry basis
FAO	Food and Agriculture Organization of the United Nations
FB	Faba bean flour
g	Gravitational force
IVPD	*In vitro* protein digestibility
L. plantarum	*Lactobacillus plantarum*
LE	Yellow lentil flour
MRS	De Man, Rogosa and Sharpe media
PDA	Potato Dextrose Agar
SCP	Single cell protein
Smf	Submerged fermentation
w/v	Weight to volume
w/w	Weight to weight
YP	Yellow pea flour

1. INTRODUCTION

1.1 Overview

The rise in global population has increased demand for sources of protein (Delgado, 2003; Popkin *et al*., 2012). Although alternative protein markets including plant-based proteins have seen increased growth, meat, dairy, and other animal products currently dominate the global protein supply (Delgado, 2003; Henchion *et al*., 2014). This leads to a rising livestock production that would need to be supplied with proper protein-rich animal feed to ensure production of healthy livestock. Although soy protein dominates the global supply of plant-based protein (Rizzo & Baroni, 2018; Sui *et al*., 2021), pulses have been of increasing interest as an economically viable source of this nutrition (Richter *et al*., 2015; Päivärinta *et al*., 2020). Pulse proteins can be enriched through dry or wet fractionation. In dry fractionation, pulse flour is fractionated into two fractions: a highly desirable protein-rich light fraction and low-value, starch- rich heavy fraction. The protein-rich fraction has a lysine-rich amino acid profile that makes them a good complementary protein source for use as a plant-based food ingredients, especially for animal feed (Boye *et al*., 2010). However, the starch-rich fraction is often under-utilized. One effective way to increase the value of starch-rich fractions would be to utilize them as substrates for production of single cell protein (SCP) that could be used as direct protein supplements in human and animal feed ingredients, which would be of higher economic value. This thesis research focused on the microbial fermentation of starch-rich pulse fractions of yellow field pea, yellow lentil and faba bean flours to increase production of microbial biomass enriched in crude microbial protein.

2. LITERATURE REVIEW

2.1 Introduction

The global population is expected to rise from its current 8 billion to reach approximately 9.7 billion people by the year 2050 (World Population Prospects 2022: Summary of Results, 2022) (United Nations, 2022). As a result of this, the global demand for food will increase by almost 70% (Lutz & Kc, 2010; Gerland *et al*., 2014). In particular, the total protein demand is estimated to increase to 943.3 million metric tons by the year 2054 (Henchion *et al*., 2017; Ismail *et al*., 2020). Meeting the continuously growing demand for protein, within environmental limits, has become one of the major challenges for the global food system in the 21st century (Weindl *et al*., 2020). Several animal-origin protein sources require lots of land and water that creates increasing pressure on such scarce resources. This urges for development of protein alternatives that can sustainably meet global demand (Sabaté & Soret, 2014; Joseph et al., 2020).

The major protein sources that currently dominate the global protein supply are meat, dairy and other animal products (Henchion et al., 2014, 2021). The total global meat consumption has been rising by almost 2% a year over the past 10 years (Michele, 2021). The growing consumption of animal-based products will have a huge impact on livestock production systems around the world over the coming decades (Baldi & Gottardo, 2017). Between 2000 and 2050, the global cattle population could rise from 1.5 to 2.7 billion (Smith *et al*., 2018). The global goat and sheep population is also expected to rise from 1.5 to 2.8 billion (Ha, 2018; Miller & Lu, 2019). These rising livestock populations would need to be supplied with proper protein-rich animal feed to ensure production of healthy livestock.

Growth in demand for animal-based products will create a similar high demand for animal feed. The animal feed market has reached a value of 482 billion USD in 2021 and is projected to

exhibit a compound annual growth rate (CAGR) of 3.5% by the year 2027 (Coffey *et al*., 2016; Chaudhary, 2021). The growing demand for feed supply leads to increased demand for protein supply which is provided in the form of both plant-based and animal-based ingredients. To make this protein supply sustainable, it is essential to use non-animal-based protein sources, *e.g.*, plant-based proteins. Currently, the major plant-based protein source in the animal feed market is soy (Dei, 2011). The global growth in production of soy has continued to increase since 2000 (Stein *et al*., 2008). Much of this increase is for soybean oil production, and the by-product of oil extraction is soybean meal. Soybean meal is a rich source of high-quality protein and amino acids (Willis, 2004) thus it is a preferred feedstock for livestock feed. However, there has been a growing interest in searching for alternatives to use of soy in the animal feed market.

Pulses such as peas, lentil and faba bean have drawn increasing interest as alternatives to soy as novel feed ingredients recently. Pulses are edible seeds from the legume family that are nutritionally and economically viable sources of protein. In addition to protein, pulses also contain high contents of vitamins, fiber and minerals including potassium, folate, iron, and manganese (Tosh & Yada, 2010; Mudryj *et al*., 2012). The functional properties of pulses are also comparable to soy with the advantage of lower allergy-causing effects and are useful in formulation and processing of plant-based proteins (Patrascu *et al*., 2017). Canada is one of the largest producers and exporters of pulses worldwide. The province of Saskatchewan is responsible for the production of more than 80% of lentils grown in Canada (Dade et al., 2017).

2.2.1 Field peas

Field peas (*Pisum sativum* L.) are predominantly used for human consumption or as livestock feed. Field pea is one of the most common food legumes grown throughout the world on over 25 million acres, with 3.6 million acres of field pea grown in Canada (Wang, 2020). Pea protein has been increasingly used in animal feed for ruminants, poultry, and swine, as an alternative to soy protein due to its lower allergic effects according to the perception of consumers (Shanthakumar et al., 2022). Previous studies have shown that field peas contain 5% to 20% lower

levels of trypsin inhibitors than soybeans (Vidal-Valverde *et al*., 2003). Field peas are a rich source of protein, carbohydrates, dietary fibre, vitamins, and minerals with less fat or cholesterol. The protein content of field pea is generally about 20% to 25% with higher levels of lysine and tryptophan when compared to cereal grains (Vidal-Valverde *et al*., 2003).

2.2.2 Lentil

Canada is the leading producer of lentil (*Lens culinaris*) and is the largest exporter in the world accounting for over 80% of lentil world trade. The province of Saskatchewan accounts for almost 95% of lentil production in Canada. Lentil is a rich source of nutrients, including protein, fibre, carbohydrates, and micronutrients (Thavarajah *et al*., 2013). Lentil contains all essential amino acids and is especially rich in aspartic, arginine, lysine, leucine, and glutamic acid (Khazaei *et al*., 2019). However, tryptophan and sulfur-containing amino acids such as cysteine and methionine are limited (Joehnke *et al*., 2021).

2.2.3 Faba bean

Faba bean (*Vicia faba* L.) is a nutrient-rich legume grown widely throughout the world. Faba beans are rich in proteins (26.1%) and carbohydrates (58.3%), and also contain a variety of bioactive compounds such as phenolics and flavonoids (Dhull *et al*., 2022). Faba bean has higher amounts of the essential amino acid, lysine, than other pulse crops. However, presence of different antinutritional factors such as phytic acids, condensed tannins and lectins can negatively affect its nutritional value (Valente et al., 2018). One way to address this could be fermentation which has been previously studied to decrease levels of antinutritional factors in cereals (Singh et al., 2012).

2.3 Fractionation of pulses

Fractionation of pulses into protein, starch, and fibre to be used as ingredients in processed foods is a common value-added process. Pulse fractionation can be achieved by two methods – wet and dry processing. The most commonly used wet processing method is aqueous alkaline extraction followed by isoelectric precipitation (Boye *et al*., 2010). In this process, protein is separated from starch and fibre at an alkaline pH, which results in protein dispersion in an aqueous phase. Following this, the pH is adjusted to the iso-electric point at which the proteins precipitate. This removes impurities left in solution, then, protein is resuspended in water and the pH re-adjusted to neutrality (Schutyser & van der Goot, 2011). Although wet fractionation yields up to 80% to 90% protein, this method has some major drawbacks such as high processing costs, inefficiency in terms of high-water usage, and generation of large amounts of effluents.

A relatively simple method of pulse fractionation is dry processing followed by air classification (Pelgrom *et al*., 2013). During air-classification, pulse fractions are separated based on shape, size, density, and physicochemical properties. Raw material is milled into fine particles. The fine-milled flour is then taken up into the air-classifier chamber by air flow to separate and move light and small particles higher up than large particles that collect at the bottom based on weight (Swanson, 1990). The light fractions are high in proteins, and the heavy, coarse fractions are mainly with starch. The dry processing method uses less energy and water than the wet processing method and has lower operation costs. Due to this, air classification is an increasingly popular fractionation method and has resulted in generation of the majority of Canada's pulse starches in the form of air-classified starch-rich coarse flours.

The protein-rich fractions generated by either type of fractionation are highly desirable as food or feed ingredients. However, the starch-rich fractions are often under-utilized and have poor market values due to their beany flavor and high content of antinutritional factors (De Angelis *et al*., 2021).

2.4 Pulse protein

The protein content and amino acid composition of pulses can vary depending on several factors – type of pulse, genetic factors, growth environmental conditions and application of fertilizers. This can also lead to differences in functional traits (Hood-Niefer *et al*., 2012). Generally, the protein content of pulses is between 18% to 32%. Pulse proteins include two major fractions and two minor fractions that include: i) albumin and globulin and ii) prolamins and glutelins respectively (Boye *et al*., 2010). Globulins are salt-soluble proteins that constitute 70% to 80% of total protein and albumins are water-soluble proteins that constitute of 20% to 30% of total protein. Albumins encompass structural proteins and enzymes such as protease inhibitors. The overall molecular mass of albumins range from 5,000 Da to 80,000 Da (Marquez & Lajolo, 1981). The amino acid profile of albumins generally contains a high level of lysine and thiol-containing amino acids, such as cysteine and methionine. On the other hand, globulins act as major storage proteins and include legumin (11*S*) and vicilin (7*S*) proteins (Gupta *et al.*, 2010). A third type of globulins, known as convicilin, is also present but in smaller amounts (Barac *et al*., 2010). Globulins have a high amount of aspartic acid, glutamine, and arginine. Prolamins are alcohol-soluble and glutelins are soluble in dilute alkali solutions. Prolamins have a higher proportion of proline and glutamine. Glutelins have a similar amino acid profile to globulins as they also have high levels of methionine and cysteine. Previous reports have shown that modification of pulses to achieve higher levels of glutelin would improve overall protein quality (Gasim *et al*., 2015).

In addition to supplying essential amino acids, pulses are also a rich source of bioactive compounds such as enzyme inhibitors including trypsin inhibitors, chymotrypsin inhibitors, and α-amylase inhibitors, as well as lectins, oligosaccharides, and phenolic compounds such as tannins (Patterson *et al*., 2017). Although these bioactive compounds modulate several metabolic processes with health-promoting effects, they can still act as antinutritional factors (Patterson *et al*., 2017). Previous reports on lectin and certain enzyme inhibitors in pulses have shown their ability to bind micronutrients and thereby reduce digestibility of macronutrients (Samtiya *et al*., 2020). This negative nutritional aspect can result in low protein digestibility. The nutritional

quality of pulse proteins also depends on their amino acid composition and overall digestibility. Pulse proteins that are highly digestible are more useful as higher digestibility leads to increased absorption of amino acids following proteolysis in the digestive system.

2.5 Pulse starch

Starch is the major component of pulse flours which accounts for 40-50% of dry weight. Starch is generally present in a granular form made up of glucose units linked together forming a polymer. The diameter of granules in different starches varies from 0.5 to 170 µg and can occur in different shapes such as spheres, ellipsoids, or irregular tubules (Schenck and Hebeda., 1992). Differences in their linkages and structural organization can lead to differences in functionality and food applications (Copeland et al., 2009).

Starch is composed of two molecular components - amylose and amylopectin (Jenkins and Donald., 1995). The molecular weight of amylose is 100kDa while that of amylopectin is much higher at 10^4 (Svihus and Uhlen., 2005). The largest component of starch is amylopectin which accounts for about 80% with highly branched structures. In contrast, amylose content in starch is about 20-30% and has little or no branches formed. For example, the amylose content of pea is 31-49%, lentil is 29% and faba bean is 31-40%. Pulse starches have been used as thickeners and gelling agents due to their high amylose content such as pea starch (Svihus and Uhlen., 2005). Pulse starches with higher levels of amylopectin have also been used as stabilizers as amylopectin causes restricted swelling and increases overall stability during processing (Thomas and Atwell., 1999).

Generally, the amylose:amylopectin ratio in starch is of high importance as it can influence the properties and characteristics of starch (Copeland et al., 2009). One such important characteristic of starch is gelatinization. Gelatinization is a process in which the starch granules are broken down in presence of heat and sufficient water which causes an irreversible disruption of the structure and properties of starch granules (Eliasson., 2004). Gelatinized starch is referred

to as “pasted” due to its high viscosity. When heated, the starch granules swell, absorbing water into the amorphous regions of the granules (Gallant et al., 1997). This disrupts the crystalline structure causing amylose to leach out, increasing viscosity.

Although starch-rich pulse fractions can be used in several food applications, it still has poor market values compared to pulse proteins largely due to the beany flavour and significant content of anti-nutritional factors. Therefore, ways to improve the value of the starch-rich pulse flours are desired. One method is conversion of starch into alternate high-value products. This can be achieved through fermentation of air-classified fractions to increase overall protein content (Massmann *et al*., 2022). This way, in addition to pulse proteins (7-13%) already present in the starch-rich fractions, through fermentation with suitable microorganisms, microbial proteins can be added to further enhance the nutritional value of the starch-rich pulse fractions. Moreover, using gelatinized pulse starch would be more effective because gelatinization would increase the susceptibility of starch in the starch-rich flour to microbial enzymatic digestion during fermentation. Improved digestion of starch will result in increased amounts of biomass with microbial protein produced.

2.6 Fermentation

Fermentation is an ancient food processing technique that has been accessible to, and used by, developing and underdeveloped countries for thousands of years (Ray & Joshi, 2014). Fermentation uses microbial growth and enzymatic reactions of microbes to convert complex substrates such as carbohydrates into simple compounds and new beneficial products. Fermentation produces alcohol by conversion of sugars into ethanol and carbon dioxide (Maicas, 2020). It can also lead to production of organic acids, lowering pH and forming complexes that preserve and stabilize final products (Rosenberg *et al*., 2013). Fermentation is also useful in improvement of diverse flavors and textures in final products (Sharma *et al*., 2020).

In recent times, fermentation has seen increasing interest in the food and feed industries for improving acceptability, digestibility and nutrient content of various foods (Xiang *et al*., 2019;

Gänzle, 2020; Zhang *et al*., 2022). Previous studies have shown an increase in essential amino acid content and increase in vitamins and mineral content by fermentation (Sanjukta & Rai, 2016). In addition to this, fermentation has been widely used to produce SCP, which are dried cells of microorganisms that can be used as protein supplements in animal feed (Ritala *et al*., 2017).

Solid-state fermentation (SSF) and submerged fermentation (SmF) are the two principal methods for fermentation of foods. The major difference between the two is the type of media used. SSF involves the growth of microorganisms on substrates in limited free flow of water and low moisture content, whereas SmF involves growth of microorganisms in a free-flowing liquid medium with more than 95% water content (Soccol *et al*., 2017).

Selection of SSF or SmF depends on the microorganisms that will be used. This is because providing an environment that is similar to the selected microorganism's natural habitat would ensure optimal growth and enzyme production by the microorganisms during fermentation. For instance, SSF would be more suitable for growth of fungi that do not require a high moisture content (Novelli *et al*., 2016). In contrast, SmF would be more suitable for cultivation of microorganisms that require high moisture content for their growth such as bacteria (Chen *et al*., 2022).

SSF has been well studied in several fermentation applications due to its low operation cost and high-volume productivity. SSF of substrates has shown that microorganisms produce bioactive compounds such as phenolic acids and flavonoids that can modify and improve functional properties (Emkani *et al*., 2022). Previous studies have also shown that SSF can cause a reduction in antinutritional factors and increase bioavailability of minerals (Gupta *et al*., 2015). However, there are certain drawbacks to SSF that include difficulty in maintaining adequate moisture content, uniform particle distribution, aeration, diffusional limitations, and removal of metabolic heat. Although low humidity can aid in the prevention of contamination by other microorganisms, if not maintained at an optimal level, it can also restrict the growth of the desired microorganism (Fonseca *et al*., 2018). Also, since SSF does not have free-flowing nutrients and continuous processing, it must be run in batches.

Submerged fermentation has been widely employed for large scale production of ethanol, essential amino acids, and antibiotics. The liquid medium ensures homogenous suspension of organisms with equal access to substrates. This allows easier maintenance of the culture apparatus, more predictable growth kinetics and easier control of temperature and moisture. However, in submerged fermentation, a continuous supply of nutrients is required since substrate utilization is rapid. Its greatest applications are for products where the desired product is excreted from the cells, and thus becomes concentrated in the liquid phase, *i.e.*, ethanol.

2.6.1 Single cell protein

SCP refers to dried cells of microorganisms with a high content of protein. SCPs have been widely considered as an alternative to conventional food and feed ingredients as they show very beneficial features as a nutrient supplement (Ritala *et al*., 2017). SCPs have a high protein content of about 60% - 80% of dry cell weight and are rich in certain essential amino acids such as cysteine and methionine (Suman *et al*., 2015). In addition to this, SCPs also contain vitamins such as riboflavin, biotin, folic acid, pantothenic acid and ascorbic acid and minerals and nucleic acids. SCPs also have a low-fat content and a high protein: carbohydrate ratio (Srividya *et al.*, 2014). Common substrates for SCP production include industrial waste streams and raw materials such as starch, molasses, and fruit and vegetable wastes (Raziq, 2020; Thiviya *et al*., 2022). Utilizing waste for SCP production has several advantages including conversion of low-cost organic waste to more desirable products and reduced environmental pollution. However, waste materials must meet certain criteria to be a useful substrate for SCP production. These include being nontoxic, regenerable, abundant, and inexpensive. An excellent substrate for SCP production is agricultural waste (Yunus *et al*., 2015). Agricultural wastes are abundant in raw material containing high levels of starch which is well-suited for the growth of microorganisms and SCP production. Generally, carbohydrate-rich substrates are preferred for SCP production as the microorganisms would readily utilize the mono- and disaccharides for energy and multiplication (Ritala *et al*., 2017).

SCP and pulse proteins are similar in that both are rich in lysine, vitamins, and minerals. Use of pulse proteins as food for human consumption has been widely explored and considered safe whereas only a few SCP products have been reported as suitable for human consumption ("Food out of Thin Air," 2020). The major limitation in use of SCP for human consumption is the presence of high amounts of microbial nucleic acid, which is undesirable in humans (Akin & Chao, 1974; Abu-Ruwaida *et al*., 1988). When SCP is ingested as foods, a high amount of nucleic acid is also ingested. Nucleic acids are made up of nucleotides and store the genetic material (DNA/RNA) in the microbial cells. Consumption of high nucleic acid-containing foods leads to production of high amounts of uric acid in blood which causes gout and kidney stones (Alvarez & Enriquez, 1988). However, the presence of high amounts of nucleic acid does not affect domesticated animals because uric acid can be converted to allantoin which can then be excreted in urine (Pizzichini *et al*., 1996). Therefore, SCP can be used in animal feed.

The nutritive value of SCP also depends on the microorganism used. Various microorganisms such as bacteria, fungi, yeast, and algae have been previously examined for SCP production utilizing inexpensive feedstock and waste. Use of each type of microorganism has its own advantages and disadvantages. But mainly, the microorganisms used for SCP production must be safe, toxin-free, and non-pathogenic. Therefore, GRAS microbes are preferred for SCP production. Microorganisms that are fast-growing and can tolerate scale-up processes are usually utilized (Ritala *et al*., 2017).

2.6.2 Bacteria

SCP production by bacteria generally yields 50% - 80% protein (Garimella *et al*., 2017). Bacteria have a smaller cell size, low density and fast growth and replication and can utilize a wide range of substrates such as sugar, starch, and waste materials (Kurbanoglu & Algur, 2002). Certain bacteria such as *Methyloccus capsulatus* can also utilize methane for SCP production (Bothe *et al*., 2002; Xu *et al*., 2021). In order to be suitable for SCP, bacteria must possess certain characteristics

such as providing good yield, being genetically stable, being tolerant to heat and foam generation during fermentation, and allowing a good recovery rate (Gęsicka *et al*., 2021).

Previous reports have shown that batch fermentation of ram horn hydrolysate by *Bacillus subtilis, Bacillus cereus* and *Escherichia coli* resulted in SCP production with a protein content of 71% for *B. subtilis*, 68% for *B. cereus* and 66% for *E. coli*. (Kurbanoglu & Algur, 2002). *Rhodopseudomonas sp.* and *Rhodocyclus sp.,* which are photosynthetic non-sulfur bacteria have been shown to produce approximately 71% crude protein when cultivated on industrial wastewater (Garimella *et al*., 2017). Waste potato effluent has been used as a substrate for SCP production by *Bacillus licheniformis* with a protein content of 30% (Arenas Santiago, 1981). Other studies on the amino acid profiles of SCP have shown that they had similar amino acid compositions to soybean protein (Yousufi, 2012; Hardy *et al*., 2018). Various bacterial species have been previously studied to be included in animal feed for SCP production, including *Cellulomonas sp., B. subtilis*, *Acinetobacter calcoaceticus*, *Brevibacterium sp.*, *Bacillus megaterium*, and *Aeromonas hydrophila* (Bough *et al*., 1972; Hitchner & Leatherwood, 1980; Rajoka *et al*., 2012).

2.6.3 Fungi

Fungi contain up to 63% protein and several fungal species have been used for SCP production (Ravindra, 2000). Fungal proteins have a high content of lysine and threonine but lack sulfur-containing amino acids such as cysteine and methionine (Willetts & Ugalde, 1987). Fungi are also rich in vitamins such as biotin, choline, pantothenic acid, pyridoxine, and thiamine. Some of the common fungal sources for SCP production include *Aspergillus niger*, *Aspergillus oryzae* and *Fusarium venenatum*. Previous studies show that SSF of banana waste by *A. niger* resulted in increased crude protein content by 23% (Baldensperger, 1985). *Aspergillus niger* has also been studied to increase protein content of potato starch processing waste (Liu *et al*., 2014). The protein content of pectin-extracted apple pomace was found to be increased by 20% when fermented by a co-culture of *Candida utilis* and *A. niger* (Bhalla & Joshi, 1994). Recently, products from *Saccharomyces, Fusarium,* and *Torulopsis* spp. have been made commercially available.

2.6.4 GRAS microorganisms

Bacteria, fungi, and yeast are generally recognized as safe (GRAS) if used in accordance with good manufacturing practices and have a history of similar applications (EFSA Panel on Biological Hazards (BIOHAZ, 2017). Some common GRAS microorganisms used in fermentation and production of SCP include *A. oryzae* and *Lactobacillus plantarum* (Ravinder *et al*., 2003). Fermentation of starch-rich pulse fractions with these microorganisms, either alone or in combination, can utilize the less-desirable starch as substrates for production of microbial biomass rich in SCP. The enzymes secreted by these microorganisms also aid in the modification of the pulse protein substrates. For instance, proteases secreted by these microorganisms are responsible for the partial degradation of proteins, resulting in the formation of smaller peptides with potential bioactive properties (Pessione & Cirrincione, 2016).

2.6.5 *Lactobacillus plantarum*

Lactobacillus plantarum is a Gram-positive, non-motile bacterium that belongs to the genus *Lactobacillus*. *Lactobacillus plantarum* cells have a straight rod shape and can occur as single cells, or in pairs or short chains. *Lactobacillus plantarum* comprises one of the largest genomes compared to other *Lactobacillus* species and has been proven to be a metabolically versatile species (Zhang *et al*., 2018). *Lactobacillus plantarum* can grow at 15°C, is aerotolerant and can withstand up to 4% NaCl. *Lactobacillus plantarum* is commonly found or utilized in meats, dairy products and several vegetable fermentations including sauerkraut, pickles, and olives, which is why it is sometimes called as a plant bacterium (Behera *et al*., 2018).

Lactobacillus plantarum has been widely used in industrial fermentation and has been known to contribute beneficially to food processing industry as the quality of end products such as shelf life and safety is high. One of the most well characterized properties of *L. plantarum* is its ability to produce antimicrobial peptides and bacteriocins that can act against foodborne pathogens or bacteria that cause food spoilage (Muhammad *et al*., 2019). *Lactobacillus plantarum* strains have also been involved in production of novel functional foods with enriched nutrients such as

exopolysaccharides that have potential in cholesterol lowering activities (Yang *et al*., 2016). The use of *L. plantarum* as probiotics and starter cultures in the food industry have also paved the way for the use of these bacteria in the production of value-added food ingredients (Yilmaz *et al*., 2022). *Lactobacillus plantarum* has also been widely used for fermentation of several carbohydrate-derived foods. In particular, α-amylase produced by *L. plantarum* was found to be useful for modification of starch properties and structure and to improve bread making (Woo *et al*., 2020). *Lactobacillus plantarum* isolated from maize flour was reported to have good starch degradation ability by production of high levels of extracellular amylase (Giraud *et al*., 1994).

Lactobacillus plantarum requires nitrogen for amino acid synthesis, however, *L. plantarum* cannot synthesize all amino acids on its own and thus requires an external source of preformed amino acids for growth and survival. In order to fulfill this nitrogen requirement and to obtain amino acids from peptides, *L. plantarum* has developed a complex combination of peptidases and proteinases that form a proteolytic system (Savijoki *et al*., 2006). This gives *L. plantarum* the ability to hydrolyze extracellular protein into peptides and amino acids, that are useful in industrial production of amino acids which can be used as health supplements and animal feed supplements (Toe *et al*., 2019). The proteolytic system of *L. plantarum* contains three main components: cell wall-bound proteinases, peptide transporters and various intracellular peptidases. The cell wall-bound proteinases initiate degradation of extracellular proteins into oligopeptides ranging from 5 to 25 amino acids, which are then released into the extracellular media (Courtin *et al*., 2002). The peptide transporters such as oligopeptide permease (Opp) and the ion-linked transporter (DtpT) will then take up these peptides and transport them into the cell (Liu *et al*., 2010). The intracellular peptidases such as endopeptidases and proline-specific peptidases will then degrade these peptides into smaller peptides and amino acids that can be used by the microbe. Previous studies on Lactic acid bacteria (LAB) on dairy fermentations have shown that LAB have several proline peptidases that degrade proline-rich peptides in casein (Adams *et al*., 2020; Kieliszek *et al*., 2021). In addition to this, the amino acids produced can also be converted into several flavor compounds including alcohol, aldehydes, and esters (Smit *et al*., 2005). This exogenous amino acid uptake aids in microbial growth and survival, thereby increasing microbial protein production. This also suggests that an additional supply of inorganic nitrogen could improve microbial growth.

2.6.6 *Aspergillus oryzae*

Aspergillus oryzae is an aerobic, multinucleated, filamentous fungus that has an optimal pH for growth of 5-6 and an optimal growth temperature of 32°C to 36°C. Like other fungi, *A. oryzae* forms hyphae or mycelium that continue to grow on apical tips and replicates by branching in liquid medium with restricted air exposure. Previous reports have shown that *A. oryzae* can grow well in corn flour-based media that has a water activity (Aw) above 0.8 (Abdullah *et al*., 2000).

Aspergillus oryzae has been used in production of shoyu (soy sauce) and miso (soybean paste) in the food manufacturing industry for centuries (Daba *et al*., 2021; Kusumoto *et al*., 2021). It's use in the production of industrial enzymes for food processing has also been widely explored. *Aspergillus oryzae* is a rich source of β-amylase, which is responsible for releasing glucose during starch hydrolysis (Wang *et al.*, 2020). Apart from β-amylase, *A. oryzae* also produces several other extracellular enzymes including nuclease, cellulase and protease, which are involved in degradation of carbohydrates, polypeptides, and nucleic acids (Tsujita & Endo, 1977). Almost 70% of bread production in the U.S.A. uses *A. oryzae* as a source of protease to break down polypeptides to release peptides and amino acids that are then used for growth of yeast and production of carbon dioxide gas (Randez-Gil *et al*., 1995; Sahnoun *et al*., 2013). The by-products of fermentation (*e.g.,* stillage) of *A. oryzae* are also used as livestock feed supplements and probiotics (Uwineza *et al*., 2021). Several substrates can be used for cultivation of *A. oryzae* including wastewater from fish processing and by-products of pea processing (Sar *et al*., 2021). Previous studies on de-oiled rice bran, which is an agro-residue waste, have analyzed the role of *A. oryzae* in the conversion of cellulose to protein (Ravinder *et al*., 2003b). They have shown that *A. oryzae* has the potential to convert agro-residue waste such as de-oiled rice bran into proteinaceous feed and food. Fermentation of de-oiled rice bran with *A. oryzae* led to an increase in protein content of 9.2 to 16.4% (Ravinder *et al*., 2003). In addition to this, the results also demonstrated that supplementation of de-oiled rice bran with an additional inorganic nitrogen source could increase the protein content of the final product.

2.7 Effect of nitrogen supplementation on SCP production

Although several reports have shown an increase in protein content of fermented substrates, some reports have also shown that fermentation can decrease protein content (Osman, 2011). The reason for this could be the utilization of amino acids by fermenting microbes for their growth and survival, causing an overall reduction in protein content. Addition of a metabolizable nitrogen source to the medium can be one way to solve this as nitrogen is one the most important limiting nutrients during production of proteins. Nitrogen sources that have been studied to aid increasing protein content during SCP production include ammonia and ammonium salts such as ammonium sulphate and ammonium phosphate, urea, nitrate, and organic nitrogen sources in different substrates such as wastes (Adoki, 2008; Taran & Bakhtiyari, 2012). Deficiency of nitrogen is one of the main causes of stuck fermentations. One way to avoid this is to add and optimize supplemental nitrogen for maximum protein production. Therefore, this thesis focused on submerged fermentation of starch-rich pulse fractions with additional nitrogen supplementation to increase overall microbial protein production.

2.8 Hypotheses

As starch fractions from legume flour has a low value, its use is very limited. Economically the protein fractions can increase the value from whole flour; meanwhile starch fractions are regarded as mostly byproducts. In order for protein fractionation to be feasible means to utilize pulse flour and to reduce the number and abundance of by-products at the same time. Therefore, it is essential to find the methods using starch-fractions as a valuable material. Considering the market of starch is dominated by wheat, rice and corn, there is little space to sell pulse starch fractions as starch. Instead, it is more practical to develop a highly efficient way to utilize starch fractions for producing other valuable materials. As single cell protein is a good way to produce proteins for feed purposes, this thesis research aims to produce proteins in the form of microbial biomass from starch fractions at a high efficiency. The hypothesis proven in this study is:

- Microbial fermentation of gelatinized starch-rich pulse flour will result in production of microbial biomass, thereby increasing overall crude microbial protein content.

2.9 Objectives

Study 1:

1. To determine efficiency of different GRAS microbes (*Lactobacillus plantarum* and *Aspergillus oryzae*) in converting starch to protein in a variety of gelatinized pulse flours during submerged state fermentation.

2. To study the effect of adding different nitrogen supplements to the starch-rich substrate to enhance protein production during fermentation.

Study 2:

3. To test for changes in proximate composition, including ash, lipid, moisture, and starch content, of protein-rich fermented substrates.

4. To assess the amino acid profile and protein digestibility of protein-rich fermented substrates.

3. MATERIALS AND METHODS

3.1 Materials

Air-classified, starch rich fraction of yellow pea (Starlite) flour was kindly donated by P&H milling group. Air-classified, starch rich fractions of yellow lentil and faba bean (V-6000) flours were kindly donated by AGT Foods and Ingredients Inc. (Saskatoon, SK, Canada) for this study. Microbial strains (*Lactobacillus plantarum* NRRL B4496 and *Aspergillus oryzae* NRRL 5590) were obtained from the Agricultural Research Service, USDA (Peoria, IL, USA). All chemicals used in this study were of reagent grade and were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada) and Fisher Science (Ottawa, ON, Canada).

3.2 Methods

3.2.1 Study 1: Submerged-state fermentation of starch-rich pulse flours with GRAS microbes

3.2.1.1 Preparation of L. plantarum *and* A. oryzae *inoculum for submerged fermentation*

Prior to fermentation, *L. plantarum* was cultured in sterile Man, Rogosa and Sharpe (MRS) broth at 37°C for 24 h. Following this, 100 µL of the culture was serially diluted and plated on MRS agar to determine the number of colony forming units (CFU) present. The culture was then used as the inoculum at a concentration of 10^7 CFU/g of flour for fermentation.

Aspergillus oryzae was cultured on Potato Dextrose Agar (PDA) in 10-cm petri dishes and incubated at 30°C for 5-7 days to obtain spores. Prior to inoculating the fermentation, the spores were suspended in 20 mL of de-ionized water from each petri dish and standardized to a concentration of 10^7 spores/g of flour by counting with a hemocytometer (Bright Line, PA, USA)

in conjunction with brightfield microscopic analysis.

3.2.1.2 Gelatinization of starch-rich pulse flours

Starch-rich pulse flours suspensions of yellow field pea, yellow lentil, and faba bean were heated to 72°C – 75°C for 20 mins under stirring conditions to convert granular starch to gelatinized starch. Following gelatinization, the flour suspensions were cooled to room temperature and dissolved in sterile distilled water to form a 10% flour suspension (w/v). The flour suspension was then inoculated with either *L. plantarum*, *A. oryzae*, or a co-culture of *L. plantarum* and *A. oryzae* (v/v), at a concentration of 10^7 CFU or spores per g of flour.

3.2.1.3 Submerged-state fermentation with **L. plantarum, A. oryzae***, or a co-culture of* **L. plantarum** ***and*** **A. oryzae**

A 20-mL suspension of 10% (w/v) gelatinized flour was added to 50-mL Erlenmeyer flasks. The suspension was inoculated with *L. plantarum*, *A. oryzae*, or a combination of both microbes, at a concentration of 10^7 CFU per g of flour or 10^7 spores per g of flour and incubated at 37°C (*L. plantarum*) or 30°C (*A. oryzae* alone or in combination with *L. plantarum*), respectively. Fermented samples were removed at 24 h intervals up to 72 h when inoculated with *L. plantarum* and up to 120 h when inoculated with *A. oryzae*, alone or in co-culture with *L. plantarum*. The increase in *A. oryzae* spores was monitored by streaking fermented samples in PDA plates. At each time point, 100 µL of *L. plantarum* inoculated samples, or a combination of *L. plantarum* and *A. oryzae*-inoculated samples, were suspended in 900 µL sterile water and then serially diluted and plated on MRS agar. Total viable count of colonies of *L. plantarum* as pure culture or in co-culture was recorded as CFU/ml to quantify increase in bacterial cell number over time. Three sets of serially diluted samples were plated to obtain triplicate results. Each fermentation condition was performed in duplicate batches. The fermented samples were then centrifuged at 10,000 rpm for 20 mins (Sorval SS-34 rotor, Thermo Fisher Scientific, Waltham, MA, U.S.A.). The cell pellet obtained was rinsed twice with sterile distilled water and then oven-dried prior to further analysis. Protein content for samples collected at discrete time points were then estimated using LECO

Analyzer (LECO Corporation, FP628 series, Michigan, U.S.A.). Non-fermented, untreated starch-rich pulse flour were used as the reference for controlled comparison.

3.2.2 Study 2: Effect of nitrogen sources on protein production

The effect of four inorganic nitrogen sources (ammonium sulphate, ammonium phosphate, a combination of ammonium sulphate and phosphate at 1:1 ratio, and urea) were assessed in this study. Starch-rich flour suspension inoculated with *L. plantarum* or *A. oryzae* was supplemented with ammonium sulphate, ammonium phosphate, ammonium sulphate/phosphate (one nitrogen atom per molecule), or urea (two nitrogen atoms per molecule) at a concentration ranging from 15 g/L – 35 g/L at the beginning of fermentation. Samples were taken at 24, 48, 72, and 120 h as described in section 2.1.3, and the protein contents were estimated using a LECO Analyzer (LECO Corporation, FP628 series, Michigan, U.S.A.). Non-fermented, untreated starch-rich pulse flour were used as the reference for controlled comparison.

3.2.2.1 Proximate analysis

Moisture content was determined according to AOAC method 925.10. Protein content was determined according to AOAC method 920.87 using a conversion factor of 6.25. Starch content was determined according to AOAC method 996.11. Ash content was determined according to AOAC method 923.03 and crude lipid content by AOAC method 920.39.

3.2.2.2 Protein quality – in vitro *protein digestibility and amino acid analysis*

The *in vitro* protein digestibility (IVPD) was determined based on a pH drop method where the change in pH of the protein solution was measured while being digested by a multienzyme solution (Tinus *et al*., 2012). The multienzyme solution was made of 31 mg of chymotrypsin, 16 mg trypsin and 13 mg protease with pH adjusted to 8.00 ± 0.05. A standardized protein solution

of each sample with 62.5 ± 0.5 mg of protein in 10 mL of MilliQ water was prepared and then stirred in a pre-heated water bath at 37°C for 1 h. The pH of the protein solution was adjusted to 8.0 ± 0.05 prior to adding 1 mL of the multienzyme solution. Following this, the drop in pH was recorded every 30 sec for 10 mins to monitor the drop in pH over time. The IVPD was calculated using the following equation (Equation 1):

$$IVPD = 65.66 + 18.10 * \triangle pH_{10\ mins} \quad \text{.....Equation 1}$$

where the $\triangle pH_{10\ min}$ is the difference in pH from the initial time and after10 mins.

The amino acid profile was determined for the fermented samples with the maximum protein concentration using Pico-Tag MT amino acid analysis system and high-performance liquid chromatography (HPLC) at University of Manitoba (Winnipeg, MB). The *in vitro* protein digestibility corrected amino acid corrected amino acid score (IVPDCAAS) was calculated as a product of amino acid scores and *in vitro* protein digestibility data of the fermented samples.

3.3 Statistical analysis

All protein measurements were made in duplicate from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours. Data represent mean ± one standard deviation (n=2). The change in number of colony forming units (CFU/mL) were measured in triplicate from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours. Data represent mean ± one standard deviation (n=3). Statistics were done using SPSS software (version 28.0, Chicago, IL, U.S.A). Test of differences between samples were performed using a one-way analysis of variance (ANOVA) along with a Tukey's test.

4. RESULTS AND DISCUSSION

4.1 Effect of submerged fermentation by GRAS microbes on microbial growth and protein content of starch-rich pulse flours.

4.1.1 Optimal gelatinization temperature for starch-rich pulse flours

Granular starch in yellow pea, yellow lentil and faba bean flours were converted to gelatinized starch by heating to 72°C – 75°C (Table 1) for 20 mins under stirring conditions. The process of gelatinization was done prior to fermentation in order to improve digestibility of starch by GRAS microbes which would thereby increase growth and biomass production. As shown in Table 1, the optimal gelatinization temperature ranges from 72°C – 75°C for faba bean, yellow lentil and yellow pea flour. The difference in optimal temperature for gelatinization can be due to the difference in amount of starch present in each flour.

Table 1: Optimal gelatinization temperatures (20 mins) for starch-rich pulse flours

Starch-rich pulse flour	Gelatinization temperature
Yellow field pea flour	75°C
Yellow lentil flour	73°C
Faba bean flour	72°C

4.1.2 Effect of fermentation time on microbial number of **L. plantarum** *with no additional nitrogen supplementation*

Bacterial SCP can be produced as a protein-rich biomass by GRAS organisms like *L. plantarum* through SmF, yielding final contents with 50% – 80% protein (Garimella *et al*., 2017). To understand the increase in protein content of starch-rich pulse flours through SCP production, the growth of *L. plantarum* and increase in bacterial cell number over the course of fermentation were quantified. SmF was carried out by inoculation of gelatinized yellow pea, yellow lentil and faba bean flour suspensions with 10^7 CFU per g of flour of *L. plantarum.* The samples were incubated for 24, 48, and 72 h post-inoculation. Bacterial cell numbers were determined over the 0 h to 72 h incubation period by counting total viable colonies in each sample recorded as CFU/mL. Preliminary studies showed that bacterial growth and protein levels reduced after 72h, therefore the *L. plantarum* fermentation was only carried out up to 72 h.

When yellow pea, yellow lentil and faba bean were fermented with *L. plantarum* alone (Figure 1) or in combination with *A. oryzae* (Figure 2), there was an overall increase in microbial numbers over time. The increase in numbers can be associated with the high amount of starch available (~70%) which acts as a good carbon source for microbial growth and replication. Cell numbers increased over the first 24 h of fermentation and then gradually entered a stationary phase, reaching the highest cell density at 48 h in yellow pea (8.6 log CFU/mL), yellow lentil (8.4 log CFU/mL) and faba bean (8.3 log CFU/mL) flours. After 48 h, the microbial numbers began to gradually decline. This can be attributed to a decrease in availability of nutrients as they were utilized by microbes.

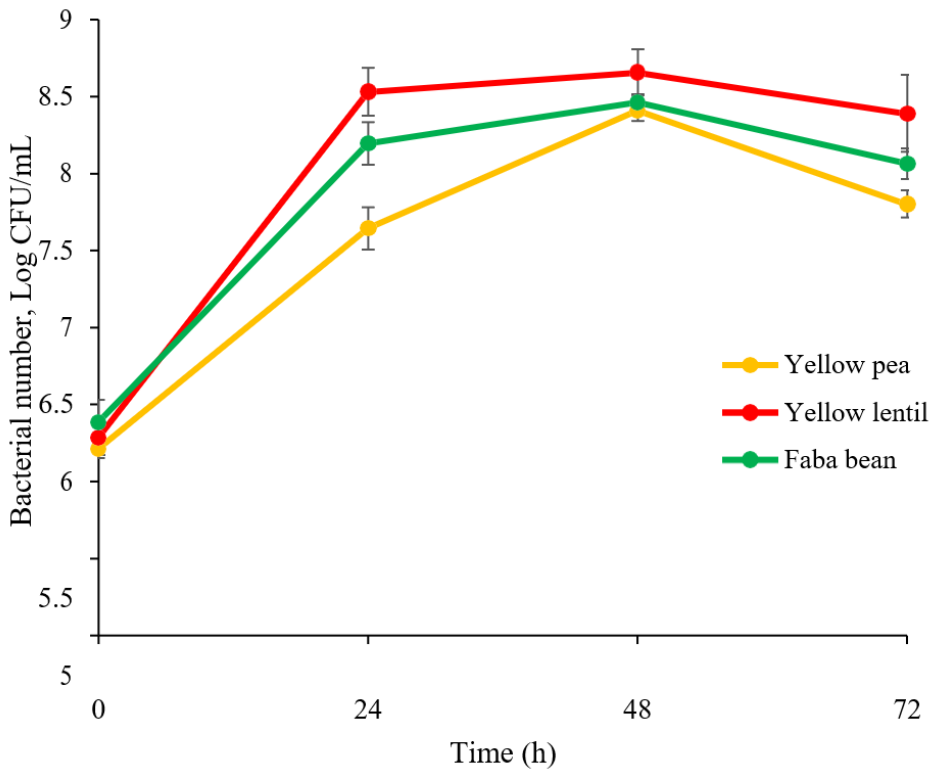


Figure 1: The change in number of colony forming units (CFU/mL) during SmF (submerged fermentation) of different starch-rich pulse flours with *L. plantarum.* Data represent the mean values from triplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=3).

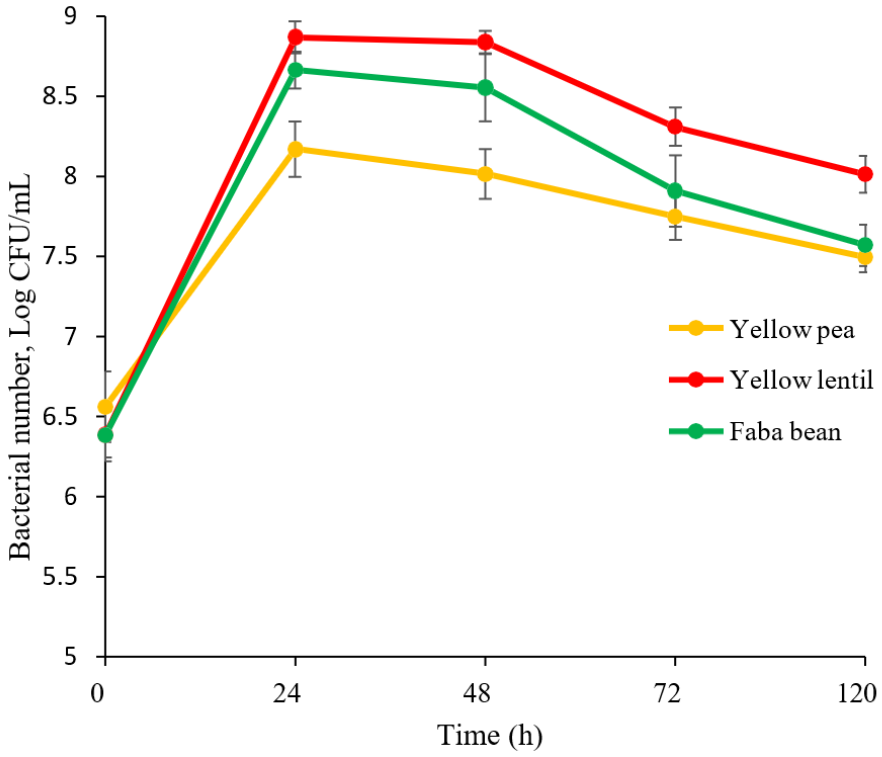


Figure 2: The change in number of colony forming units (CFU/mL) during SmF (submerged fermentation) of different starch-rich pulse flours in co-culture of *L. plantarum* and *A. oryzae*. Data represent the mean values from triplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=3).

The decrease in total bacterial counts could also be due to the accumulation of ethanol, lactic acid, antibacterial substances, and other metabolites that inhibit microbial growth (Hutkins, 2006). The increase in CFU/mL was highest in fermented yellow lentil and lowest in fermented yellow pea flour. This could be due to the difference in amylose: amylopectin ratios between yellow lentil, yellow pea and faba bean flours. Previous reports have suggested that certain microbes can utilize amylose better than amylopectin and vice versa (Wang et al., 2001). Similar patterns of change in

microbial number during fermentation have been previously reported in literature (Pranoto *et al*., 2013; Sandra Garcia, 2018; Alemneh *et al*., 2021).

As seen in Figure 2, the cell number increase of *L. plantarum* by 24 h was higher when co-fermented with *A. oryzae* than when grown as a pure culture (Figure 1). However, after 24 h, the CFU/mL numbers gradually decreased for each type of flour until the time course ended at 120 h. This could be due to the growth of *A. oryzae* in the co-culture as it would also utilize nutrients available, which could reduce availability of nutrients for *L. plantarum*. The overall CFU/mL values obtained during SmF for fermented yellow lentil was highest, followed by faba bean and yellow pea flours, regardless of the fermenting organisms used.

Although the starch content of the pulse flours used in this study was high (~70%), they could contain varying amounts of sucrose, raffinose and stachyose. For example, starch-rich yellow pea flour has 2.34% sucrose and 2% raffinose/stachyose. Previous studies have shown that most of *Lactobacillus spp.* have a starch metabolism pathway but can metabolize sucrose as a more preferred substrate (Gänzle & Follador, 2012). This could be a reason for the difference in amount of growth by *L. plantarum* in the pulse flours as the amount of sucrose in each flour may vary. In addition to this, presence of antinutritional factors such as phytic acids and tannins in the pulse flours can also negatively impact microbial growth.

4.1.3 Effect of fermentation by GRAS microbes on protein content of starch-rich pulse flours

Table 2 summarizes the change in protein content of fermented yellow pea, yellow lentil and faba bean pulse flours over time when inoculated with either *L. plantarum*, *A. oryzae* or a co-culture of *L. plantarum* and *A. oryzae*.

Overall, the protein levels increased over time in all three fermented pulse flours and the highest level of protein was achieved at the longest fermentation period: 72 h with *L. plantarum* and 120 h with *A. oryzae* and co-culture of *L. plantarum* and *A. oryzae*. Yellow pea flour had the lowest protein (7.8%) among the three flours. When fermented by *L. plantarum*, the increase in

protein content by 24 h was not significant in all three pulse flours (p>0.05). However, there was a significant increase in protein (10.2%) in fermented yellow pea flour by 72 h (p<0.05). The same applied to yellow lentil and faba bean, where the protein content increase was from 16.5% to 18.5% and 14.5% to 16.4%, respectively. This could be due to the auxotrophic nature of *Lactobacillus* species, where the microorganism is unable to synthesize certain amino acids essential for its growth and survival *(*Morishita *et al*., 1974; Makarova *et al*., 2006). Fermentation experiments of different flour substrates by others have shown an increase in protein content (El Hag *et al*., 2002; Duodu *et al*., 2003; Pranoto *et al*., 2013). Fermentation of maize flour has shown an increase in protein content from 9.0% to 12.5% by *L. plantarum* (Terefe *et al*., 2021). On the other hand, previous reports have also shown a decrease in protein content of flour substrates when fermented by *L. plantarum* (Osman, 2011). *Lactobacillus plantarum* cannot produce numerous amino acids and hence require an exogenous supply of such amino acids and peptides for their growth (Morishita *et al*., 1974; Christensen & Steele, 2003; Savijoki *et al*., 2006). To fulfill their nitrogen requirements, *L. plantarum* has a proteolytic system consisting of a complex combination of peptidases and proteinases that hydrolyze proteins in the fermentation medium to supply the amino acids required for their growth, which could lower or otherwise impact the overall protein content (Jensen & Ardö, 2010; Sun *et al*., 2015). Therefore, despite the production of SCP during fermentation by *L. plantarum,* the overall protein levels did not increase by more than ~12% as the fermenting microbes would have utilized certain amino acids for their growth and replication.

Table 2: The effect of SmF (submerged fermentation) of starch-rich pulse flours with GRAS microbes on protein content without additional nitrogen supplementation.

		Protein content (%, d.b.)		
GRAS Microbe	**Time**	**Yellow pea**	**Yellow lentil**	**Faba bean**
Untreated		7.8 ± 0.1^{c}	16.5 ± 0.2^{b}	14.5 ± 0.1^{c}
L. plantarum	0h	7.9 ± 0.1^{cA}	16.8 ± 0.1^{bA}	14.8 ± 0.1^{cA}
	24h	8.4 ± 0.1^{cB}	16.1 ± 0.1^{bB}	15.2 ± 0.1^{abA}
	48h	9.6 ± 0.3^{bA}	17.9 ± 0.3^{aB}	15.5 ± 0.1^{bB}
	72h	10.2 ± 0.1^{aB}	18.5 ± 0.2^{aB}	16.4 ± 0.3^{aB}
A. oryzae	0h	7.9 ± 0.1^{eA}	17.0 ± 0.2^{dA}	14.7 ± 0.1^{dA}
	24h	9.1 ± 0.2^{dA}	17.5 ± 0.3^{dA}	15.5 ± 0.3^{dA}
	48h	10.5 ± 0.2^{cA}	18.8 ± 0.1^{cAB}	19.8 ± 0.6^{cA}
	72h	15.6 ± 0.2^{bA}	20.6 ± 0.4^{bA}	24.2 ± 0.5^{bA}
	120h	16.7 ± 0.2^{aB}	22.8 ± 0.3^{aA}	26.3 ± 0.3^{aB}
***L. plantarum* +**	0h	7.9 ± 0.1^{dA}	16.7 ± 0.1^{dA}	14.7 ± 0.1^{dA}
A. oryzae	24h	8.7 ± 0.1^{dAB}	16.9 ± 0.2^{dA}	14.8 ± 0.0^{dA}
	48h	11.0 ± 0.5^{cA}	19.4 ± 0.3^{cA}	19.6 ± 0.5^{cA}
	72h	16.5 ± 0.4^{bA}	21.7 ± 0.5^{bA}	23.7 ± 0.1^{bA}
	120h	18.9 ± 0.2^{aA}	23.3 ± 0.4^{aA}	27.5 ± 0.2^{aA}

Data represents mean values from duplicate batches of each fermented starch-rich pulse flours (yellow pea, yellow lentil and faba bean flour) ± one standard (n=2). Abbreviations: SmF (submerged fermentation); GRAS (generally recognized as safe); *L. plantarum* (*Lactobacillus plantarum*); *A. oryzae* (*Aspergillus oryzae*); *L. plantarum + A. oryzae* (*Lactobacillus plantarum + Aspergillus oryzae*); d.b. (dry weight basis). Data with the same superscript letter are not significantly different ($p>0.05$). Lower case letters denote significant differences in protein values over time when fermented by same microbe ($p<0.05$). Upper case letters denote significant differences in protein values when fermented by different microbes ($p<0.05$).

When fermented by *A. oryzae,* the protein levels of yellow pea, yellow lentil and faba bean increased significantly and almost doubled by 120 h after fermentation ($p<0.05$). This could be because fungi, such as *A. oryzae,* can produce microbial biomass with a high protein concentration, containing up to 63% protein (Nasseri *et al*., 2011, Mahboubi *et al*., 2017). They are also relatively chemosynthetic, meaning they can synthesize biomolecules, like amino acids, required for their metabolism and growth from less complex organic compounds, or even use inorganic building blocks like ammonium (Zhao *et al*., 2015). Our findings also correlate well with previous results where production of SCP production from de-oiled rice bran by *A. oryzae* increased protein content from 9.2% to 28.1% after 72 h of fermentation (Rudravaram *et al.,* 2006). Previous reports have shown that fermentation with *A. oryzae* similarly yielded a high level of protein increase in soybean meals (Hong *et al*., 2004). Studies by others on soybean meals have also established the highest accumulation of protein when fermented with *A. oryzae* from 50.7% to 82.0% (Serba *et al*., 2020). A report on dry biomass production also showed a 36.6% increase in protein level following cultivation of *A. oryzae* in an effluent obtained from starch production from wheat and corn (Jin *et al*., 1998; Souza Filho *et al*., 2019).

Co-culture fermentation by *L. plantarum* and *A. oryzae* also showed significant increase in protein content of pulse flours ($p<0.05$). The co-culture fermentation yielded the highest protein levels in comparison to single strain culture fermentation at 120 h, with yellow pea at 18.9%, yellow lentil at 23.3%, and faba bean at 27.5%. Co-culture fermentation can increase rate of conversion of substrates, and overall microbial fitness, especially when the two microbes positively-interact with each other (Canon *et al.,* 2021). Some of the most effective ways of interaction would be exchange of nitrogen compounds or cross-feeding, where one microorganism takes in a primary substrate and converts it into a product that benefits the other microbe (*e.g.*, using nitrogen to make amino acids which is then used by less-chemosynthetic organisms) (Morris *et al*., 2013). Previous reports have shown that mixed culture fermentation could enhance overall bioconversion of carbon in substrates and microbial yield (Zhu *et al*., 2020). One limiting factor of *A. oryzae* is that it does not exhibit strong cellulase, pectinase or β-glycosidase producing abilities. These enzymes are necessary for the breakdown of plant cell wall polysaccharides. On the other hand, *L. plantarum* produces such carbohydrases, including pectinases and β-glycosidase

(Karam & Belarbi, 1995; Anand *et al*., 2016), that can help degrade plant cell walls to improve starch hydrolysis by both *L. plantarum* and *A. oryzae*, and thereby increase protein content.

4.2 Effect of additional nitrogen supplementation on protein production by GRAS microbes during submerged fermentation of starch-rich pulse flours

Although fermentation by *L. plantarum*, *A. oryzae* and a co-culture of both increased the overall protein levels in starch-rich yellow pea, yellow lentil and faba bean flours, the maximum amount of protein achieved was not sufficient to make the fermented substrates reach the targeted level of >45% protein. One way to improve efficiency of protein production by microbes during SmF is to supply additional nitrogen to the medium (Reihani & Khosravi-Darani, 2019; Bratosin *et al*., 2021). Deficiency of nitrogen is one of the main causes of "slowed" fermentations. By supplying additional nitrogen, we can ensure that nitrogen is not a limiting factor, and therefore protein production should improve. In this study, supplemental nitrogen was added at the beginning of fermentation since the initial nitrogen levels were low in the three pulse flours as shown in Table 2. The effect of four different nitrogen sources (ammonium sulphate, ammonium phosphate, a 1:1 ratio of ammonium sulphate and ammonium phosphate, and urea) at a concentration ranging from 5 g/L – 35 g/L were assessed in this study. Preliminary tests with 5 g/L and 10 g/L of each nitrogen source and their effect on protein production showed that there was an increase in protein levels after fermentation. However, it was not sufficient to produce protein-rich biomass. This could have been since the nitrogen concentration was not sufficiently high. Therefore, higher concentrations ranging from 15 g/L to 35 g/L were analyzed in detail. Any excess nitrogen supplements that remained in the fermented samples were removed by centrifugation at 4000 rpm for 20 minutes and the pellet was rinsed with distilled water prior to analysis by LECO analyzer to ensure that the nitrogen measured by LECO analyzer was only due to proteins.

4.2.1 Effect of different nitrogen sources on microbial growth during submerged fermentation

Each fermentation batch was supplemented with ammonium sulphate, ammonium phosphate, a 1:1 ratio of ammonium sulphate and ammonium phosphate, or urea at a concentration ranging from 15 g/L – 35 g/L followed by inoculation with either *L. plantarum*, *A. oryzae* or a co-culture of *L. plantarum* and *A. oryzae*. Samples collected at each time point (0, 24, 48, 72, and 120 h) were then analyzed for change in CFU/mL by counting total viable colonies in each sample after serial dilution and incubation. The CFU/mL were analyzed in triplicate from each of the duplicated runs of fermented yellow pea, yellow lentil and faba bean.

Figures 3 and 4 show the effect of supplementation of the highest concentration (35 g/L) of each nitrogen source on the change in microbial number over time during fermentation by *L. plantarum* and co-culture of *L. plantarum* and *A. oryzae* (Figure 4). There was an overall increase in CFU/mL in fermented yellow pea, yellow lentil and faba bean flours when supplemented with nitrogen compared to change in bacterial number when no additional nitrogen was supplied.

Figures 3 and 4 show that both urea and ammonium sulphate improved microbial growth than ammonium phosphate or a combination of ammonium sulphate and ammonium phosphate. Similar to when no additional nitrogen supplemented samples were provided, the cell number increased from 0 h to 24 h and then gradually slowed down over time for all treatments. These results were supported by previous studies that have shown addition of nitrogen supplements increased microbial growth during fermentation (Rajoka *et al*., 2012; Taran & Bakhtiyari, 2012; Nicolas *et al*., 2017). It has also been noted in the literature that among various nitrogen sources, ammonia is the preferred nitrogen source that supports a faster growth rate in bacteria (Wang *et al*., 2016). This is due to the upregulation of both expression and activity of glutamine synthetase, which acts as a precursor and of the ammonium transporter AmtB by the nitrogen regulatory system. This nitrogen dietary preference for ammonium sulphate and urea was likely to reflect on the metabolism and utilization of nutrients by *L. plantarum* and protein production.

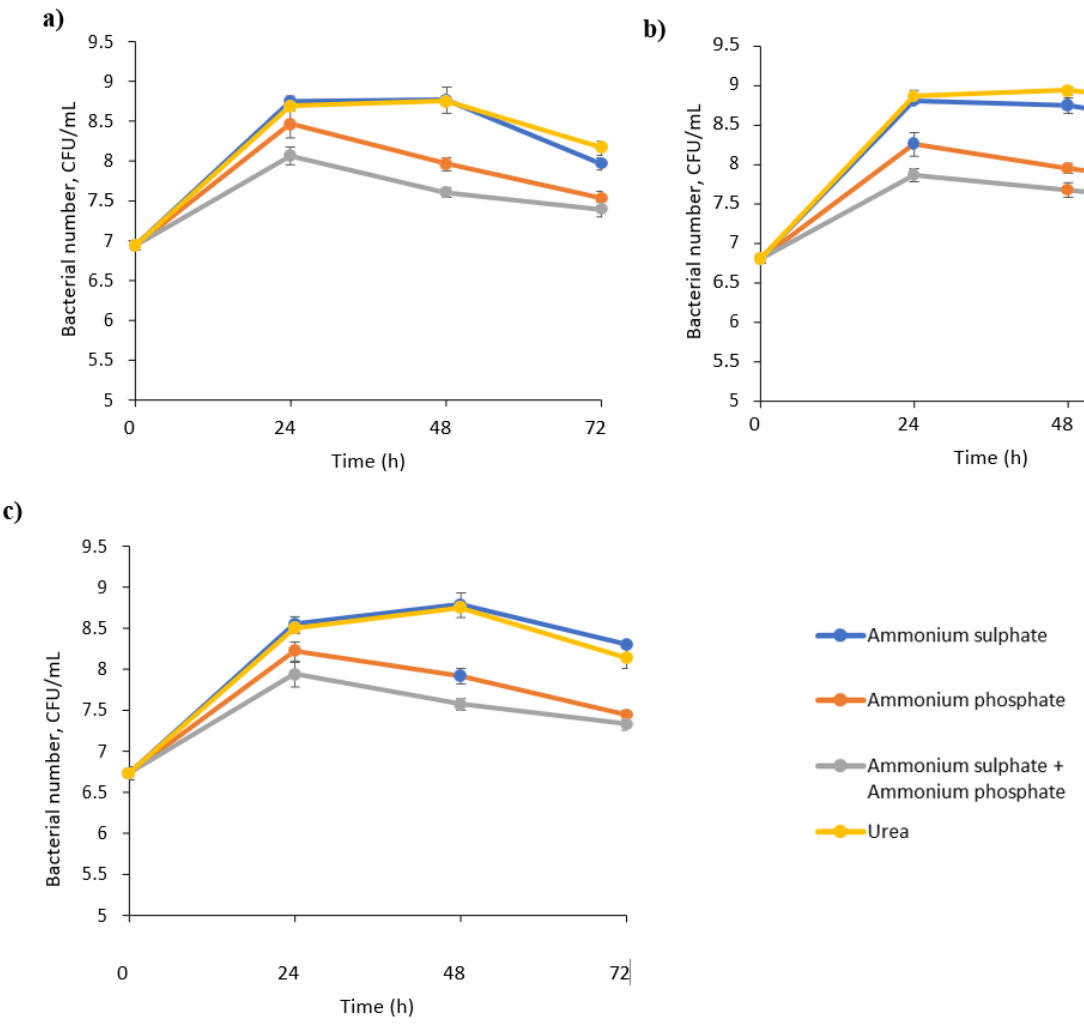


Figure 3: The change in number of CFU/mL during SmF with *L. plantarum* using **a)** yellow pea flour, **b)** yellow lentil flour, and **c)** faba bean flour supplemented with 35 g/L nitrogen source.

Data represent the mean values from triplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=3).

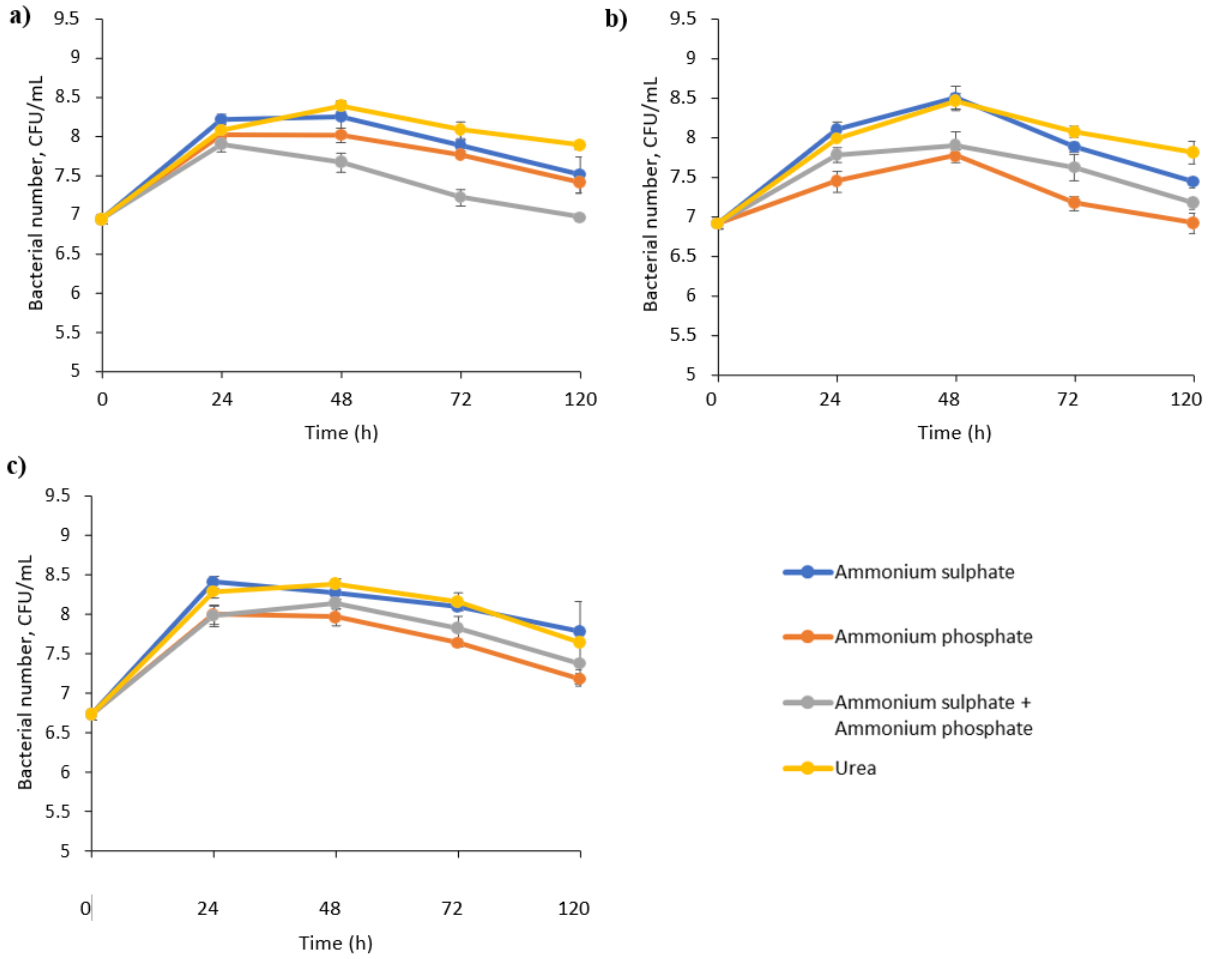


Figure 4: The change in number of CFU/mL during SmF with co-culture of *L. plantarum* and *A. oryzae* using **a)** yellow pea flour, **b)** yellow lentil flour, and **c)** faba bean flour supplemented with 35 g/L nitrogen source.

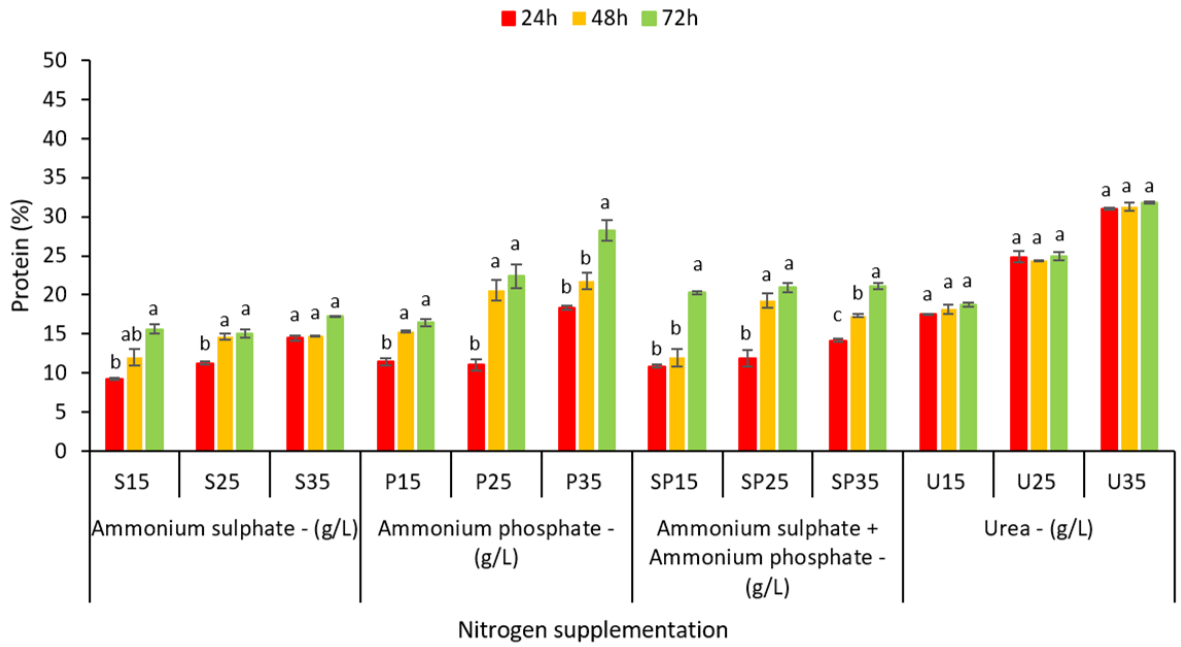
Data represent the mean values from triplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=3).

4.2.2 Effect of nitrogen supplementation during submerged fermentation on protein content

The effect of different nitrogen sources and their concentration over time on the protein yield of fermented starch-rich flours when fermented by *L. plantarum* is summarized in Figure 5. Preliminary tests showed that bacterial growth and protein levels reduced after 72h, therefore the *L. plantarum* fermentation was only carried out up to 72 h. Overall, a maximum protein yield was achieved for 72 h fermented yellow pea, yellow lentil and faba bean flours when supplemented with 35 g/L of urea at the beginning of fermentation. They reached 31.8%, 49.8% and 38.3% (w/w), respectively. The highest difference in protein level was observed for fermented yellow lentil flour where the protein content increased from 16.5% to 49.8%.

Figure 5 shows an increase in protein content with increasing concentration of nitrogen source in fermented yellow pea (Figure 5A), yellow lentil (Figure 5B) and faba bean (Figure 5C) flours. The highest protein yield achieved in the three flours supplemented with ammonium sulphate was 17.2% (yellow pea), 26.6% (yellow lentil) and 21.8% (faba bean) with 35 g/L concentration at 72 h. The increase in protein yield with increasing fermentation time when supplemented with ammonium sulphate was not significant in all three pulse flours ($p>0.05$). The highest protein yield achieved with 35 g/L of ammonium phosphate supplemented flour samples (yellow pea, yellow lentil and faba bean) were 28.3%, 32.3% and 23.9%, respectively. The increase in protein yield with increasing fermentation time when supplemented with ammonium phosphate was significant in all three pulse flours ($p<0.05$). The highest protein yield achieved with 35 g/L of ammonium sulphate and ammonium phosphate (1:1 ratio) supplemented flour samples (yellow pea, yellow lentil and faba bean) were 21.2%, 25.5% and 19.1%, respectively. The increase in protein yield with increasing fermentation time when supplemented with ammonium sulphate and ammonium phosphate was significant in yellow pea and faba bean ($p<0.05$) but not significant in yellow lentil ($p>0.05$). However, these values were still lower than the yield achieved with 35 g/L of urea supplemented flour samples which were 31.8%, 49.8% and 38.3% for yellow pea, yellow lentil and faba bean, respectively.

a)



b)

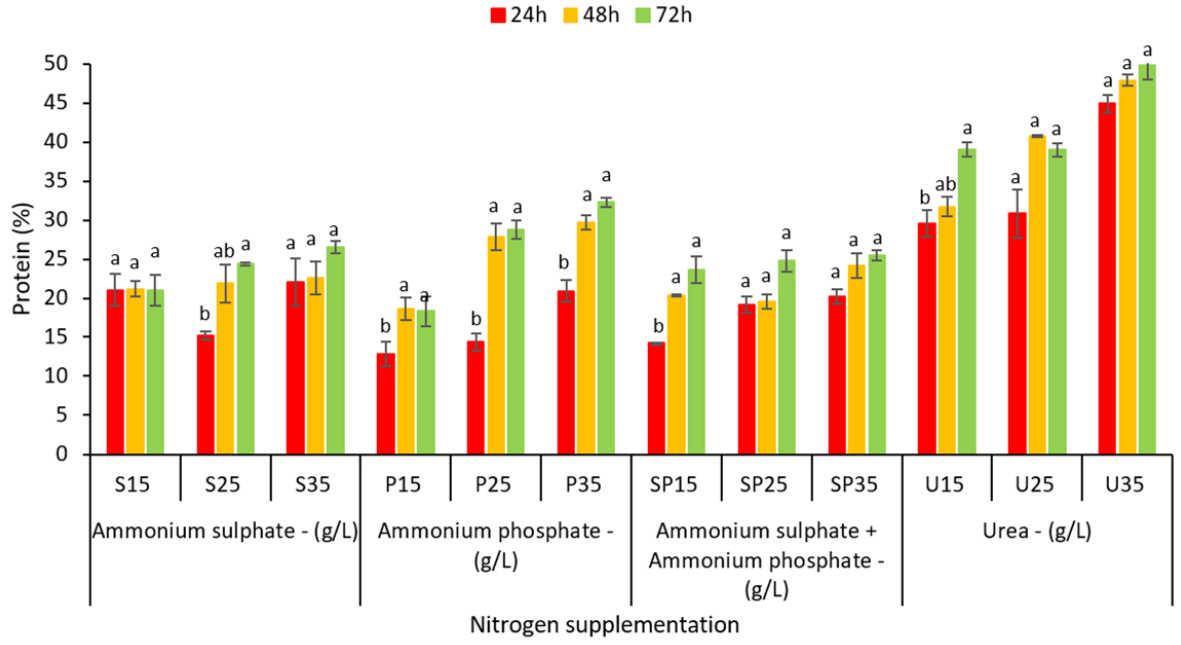


Figure 5 (continued)

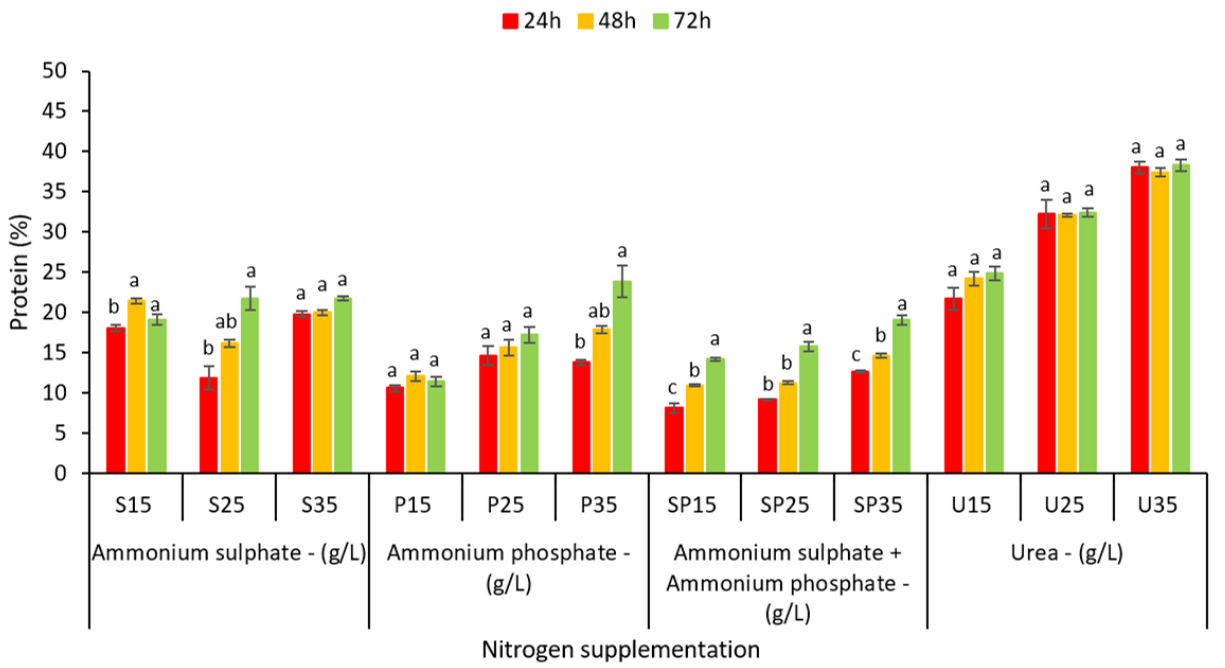
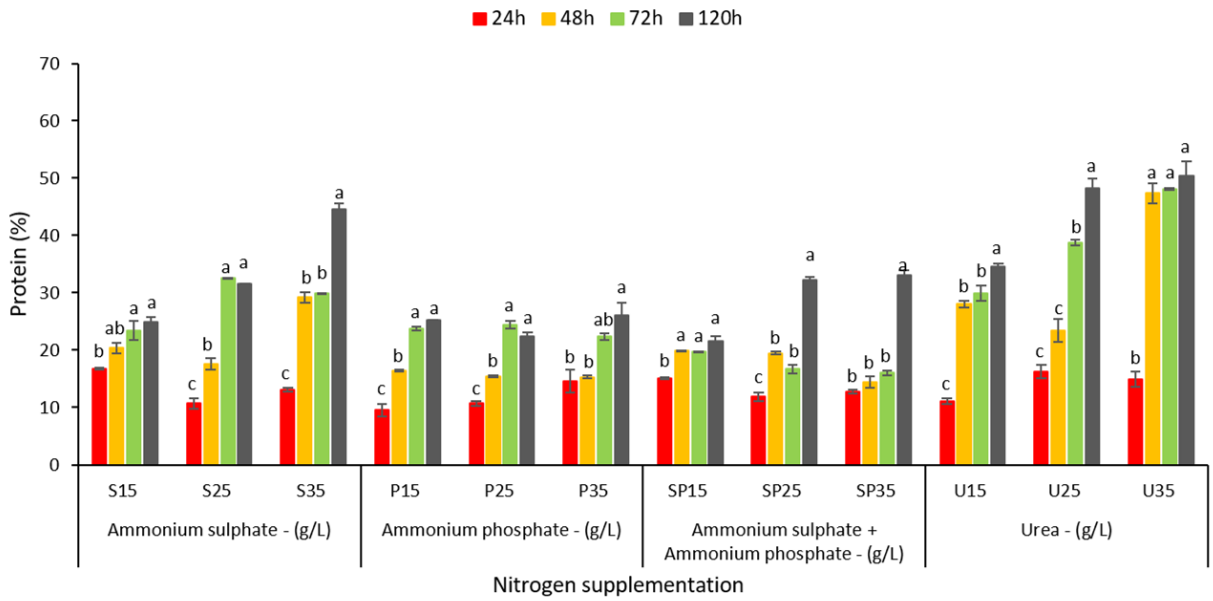


Figure 5: The change in protein content (%) during SmF with *L. plantarum* for **a)** yellow pea flour, **b)** yellow lentil flour, and **c)** faba bean flour when supplemented with nitrogen sources at varying concentration.

Data represent the mean values from duplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=2). Data with the same superscript letter are not significantly different (p>0.05). Lower case letters denote significant differences in protein values over time (p<0.05).

Figure 6 summarizes the effect of different nitrogen sources, concentration and fermentation time on protein content when fermented by *A. oryzae*. A similar pattern of change in protein levels was observed between *A. oryzae*-fermented samples and *L. plantarum*-fermented samples (Figure 5) where an overall significant increase in protein levels was noted over time with increasing concentration of nitrogen ($p<0.05$). However, the increase in protein at any given time point was much higher when fermented with *A. oryzae* than *L. plantarum*. Supplementation with ammonium phosphate, or a combination of ammonium sulphate and ammonium phosphate, in all three flours resulted in similar protein levels. The optimal fermentation time for *L. plantarum* samples was observed to be 72 h as the maximum amount of protein was produced at 72 h. However, the optimal fermentation time for *A. oryzae* was observed to be 120 h. This can be attributed to the fact that fungi such as *A. oryzae* have a relatively slower growth rate compared to bacteria such as *L. plantarum*.

a)



b)

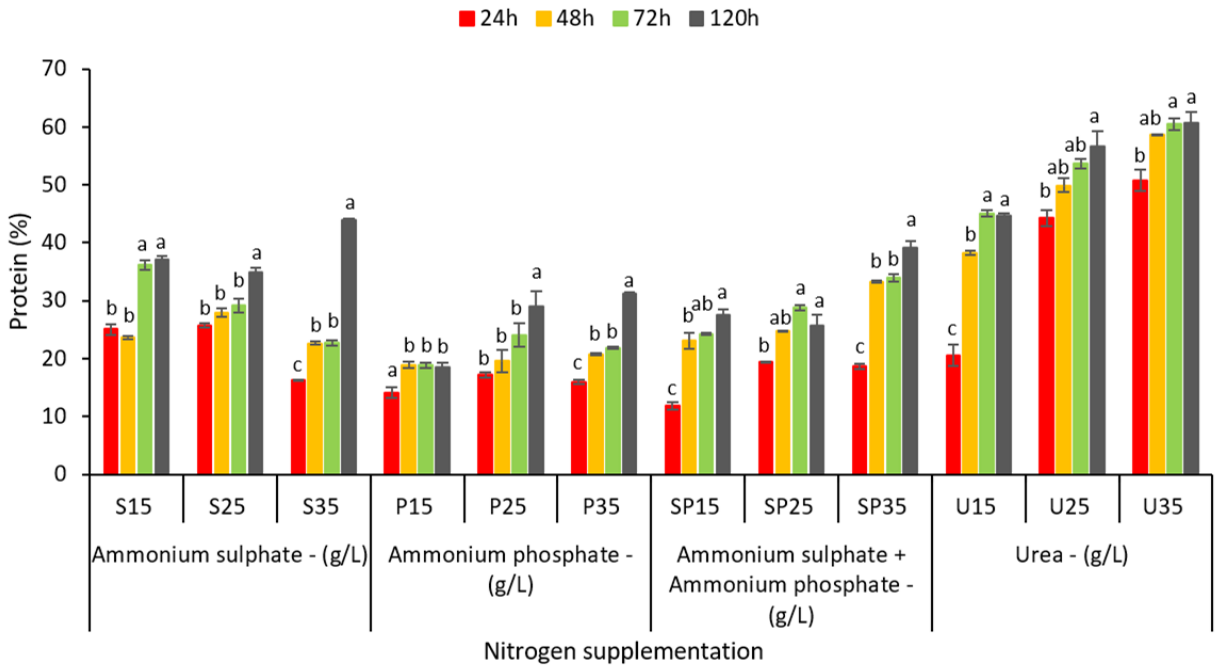


Figure 6 (continued)

c)

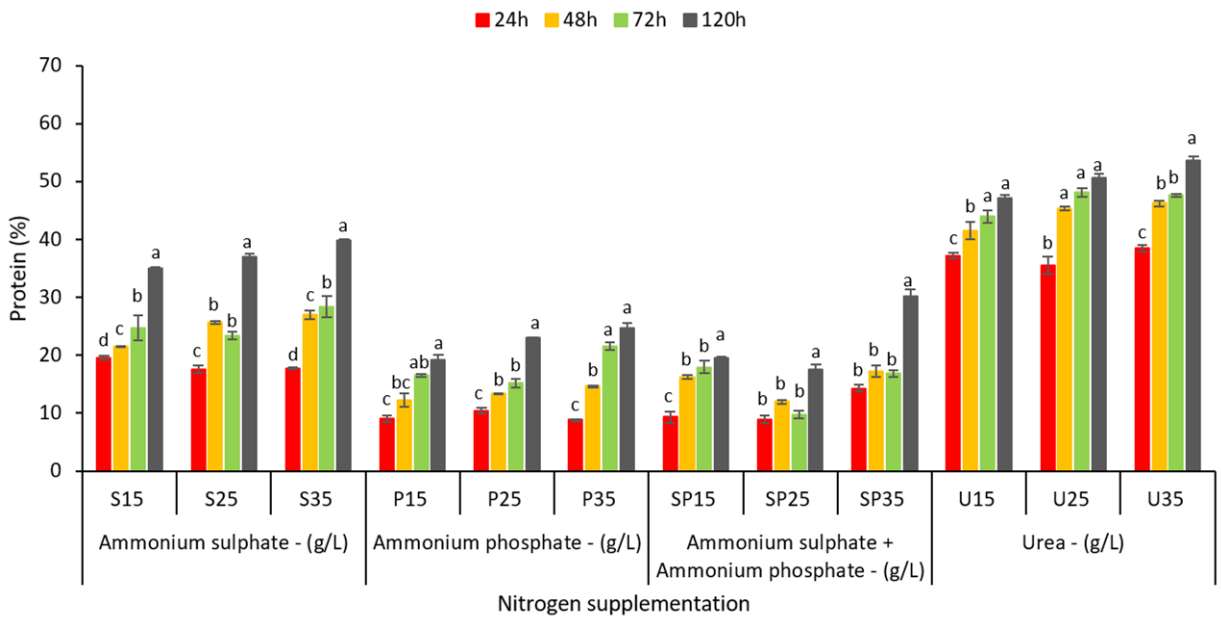


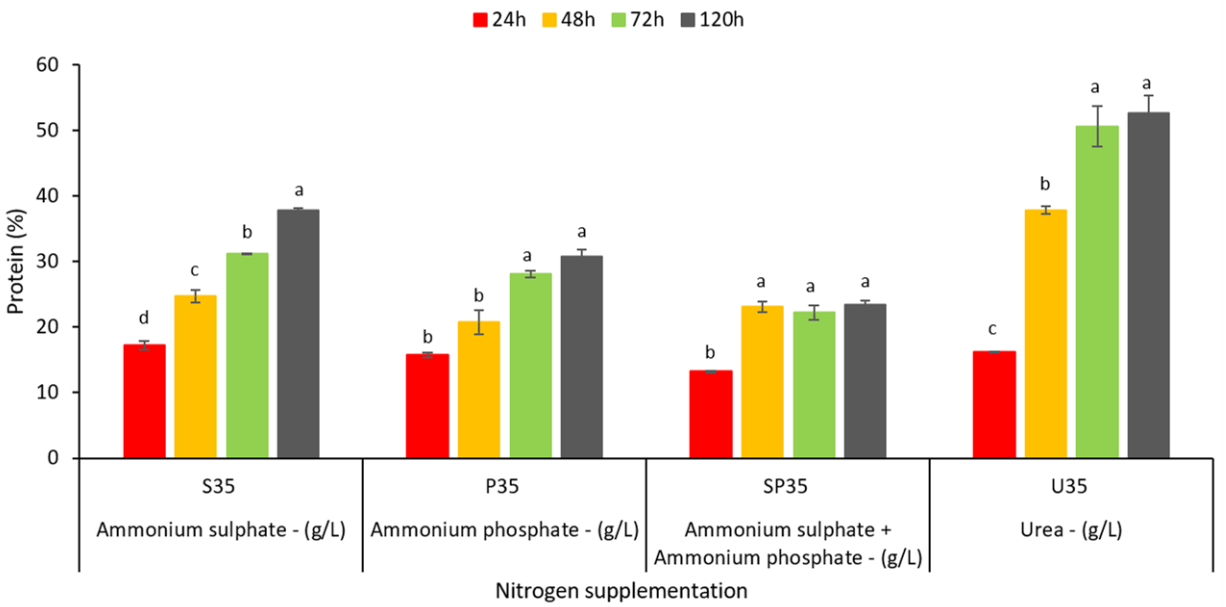
Figure 6: The change in protein content (%) during SmF with *A. oryzae* for **a)** yellow pea flour, **b)** yellow lentil flour, and **c)** faba bean flour.

Data represent the mean values from duplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=2). Data with the same superscript letter are not significantly different (p>0.05). Lower case letters denote significant differences in protein values over time (p<0.05).

Yellow lentil and yellow pea flours supplemented with ammonium sulphate showed a higher yield in protein levels than faba bean flour. However, like *L. plantarum* fermented samples, supplementation with urea at 35 g/L yielded the highest protein content in yellow pea, yellow lentil and faba bean flours, attaining concentrations of 50.4%, 60.6%, and 53.6%, respectively.

Since 35 g/L concentration of nitrogen sources gave the highest yield of protein during fermentation by *L. plantarum* or *A. oryzae*, this concentration was chosen to be assessed with fermentation by a co-culture of *L. plantarum* and *A. oryzae*. The effect of additional nitrogen supplementation of pulse flours during fermentation by a co-culture of *L. plantarum* and *A. oryzae* is summarized in Figure 7. Ammonium sulphate supplemented samples show a higher protein yield in all three flours than ammonium phosphate supplemented samples. As in Figures 5 and 6, this condition showed that the highest protein yield was achieved with the supplementation of urea compared to other nitrogen sources. The highest protein contents achieved overall were 52.7% for yellow pea, 60.3% for yellow lentil and 58.1% for faba bean flours with supplementation of urea at 35 g/L.

a)



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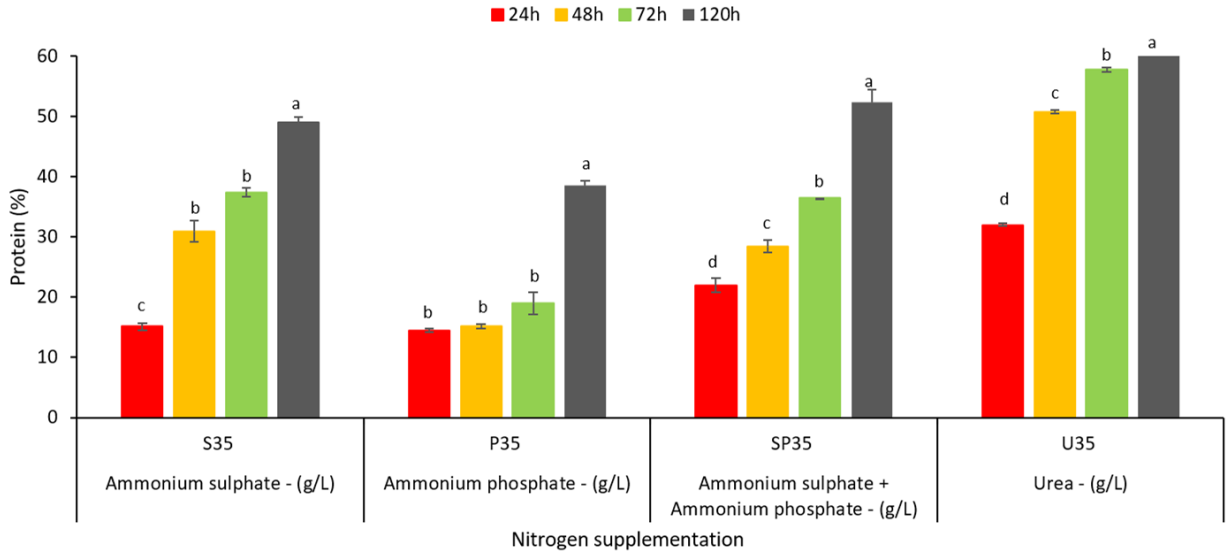

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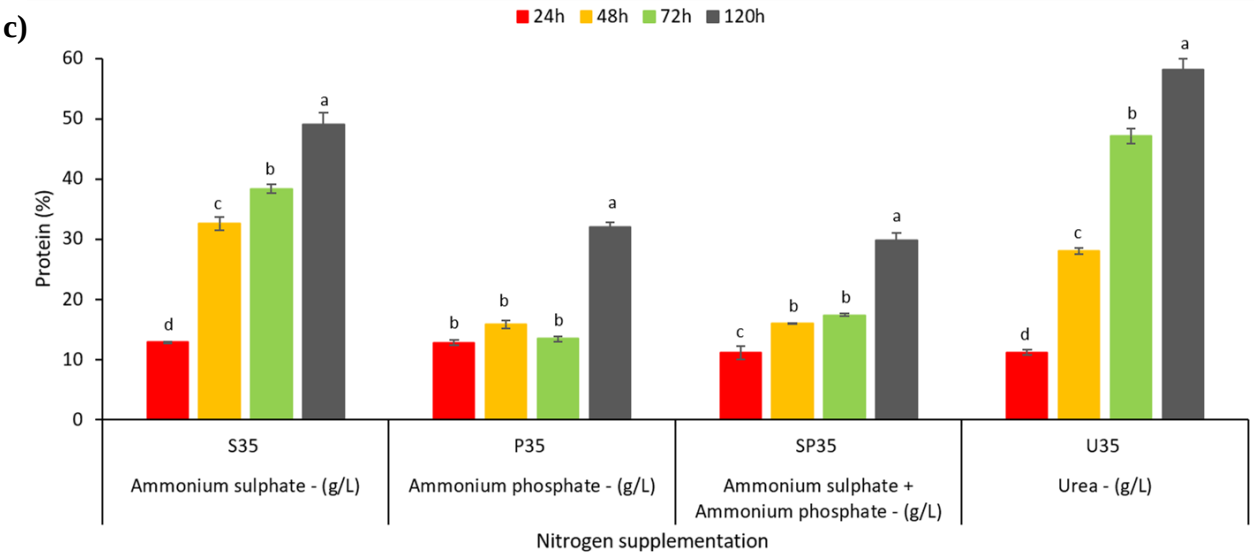

Figure 7: The change in protein content (%) during SmF with co- culture of *L. plantarum* and *A. oryzae* for **a)** yellow pea flour, **b)** yellow lentil flour, and **c)** faba bean flour.

Data represent the mean values from duplicate analyses from duplicate fermented batches of yellow pea, yellow lentil and faba bean flours ± one standard deviation (n=2). Data with the same superscript letter are not significantly different (p>0.05). Lower case letters denote significant differences in protein values over time (p<0.05).

Previous studies have investigated different N:C ratios for SCP production from GRAS microbes (Kwatra *et al*., 2021). It has been evaluated that a ratio of N:C as high as 1:6 and 1:8 resulted in highest yield of SCP from microbes in oil-rich salad oil manufacturing wastewater (Zheng *et al*., 2005). Several nitrogen sources have been tested in literature for their effect on SCP production including sodium nitrate, ammonium chloride, ammonium nitrate, corn steep liquor, peptone and tryptone (Nigam, 1998; Schultz *et al*., 2006; Pradeep & Pradeep, 2013). However, several reports have shown that ammonium sulphate, ammonium phosphate and urea have resulted in higher protein yields compared to supplementation with other nitrogen sources (Rajoka *et al*., 2006; Zhao *et al*., 2010).

Generally, a typical microbial cell is composed of 12% nitrogen by dry weight, which is the major constituent of protein and nucleic acids (Kim & Gadd, 1986). Due to such structural requirements, the nitrogen source is one of the most vital factors required for protein synthesis and cell proliferation during fermentation by microbes. In addition to nitrogen, sulfur and phosphorus are also important for SCP production (Goldman & Dennett, 1991). Ammonium sulphate consists of about 21.2% of nitrogen and 24.3% of sulfur, ammonium phosphate consists of 12.2% of nitrogen and 26.9% of phosphorus, and urea consists of 46.6% of nitrogen. The price of ammonium sulphate is $14.99/lb and ammonium phosphate are $8.99/lb but urea is $1.10/lb which is much cheaper and is food and feed grade. The generally acceptable level of urea used in animal feed is 1.5-2%. This typically makes these inorganic compounds an excellent supplementation for SCP production. Based on the results obtained from this study, supplementation with urea resulted in maximum SCP production. This can be attributed to the high nitrogen content of urea that is much higher than either ammonium sulphate or ammonium phosphate. This led to the understanding that nitrogen was more of a limiting factor than either sulfur or phosphorus and in the presence of more nitrogen, protein yield would increase without additional supplement of sulphur and phosphorus.

4.3 Proximate composition

The most suitable conditions (fermentation time, nitrogen source, and concentration of nitrogen supplementation) that resulted in highest yield of protein when fermented with GRAS microbes in the starch-rich pulse flours were selected for further proximate analysis (Table 3).

Overall, protein content in samples fermented by co-culture of *L. plantarum* and *A. oryzae* was significantly higher compared to single strain cultures; 50.5% in yellow pea, 58.6% in yellow lentil, and 58.1% in faba bean ($p<0.05$). Protein content of yellow pea and yellow lentil fermented by a co-culture of *L. plantarum* and *A. oryzae* was only slightly higher (1% - 2%) than samples fermented by *A. oryzae* alone. However, co-culture fermentation produced ~10% higher protein than *A. oryzae* alone in faba bean samples. This can be attributed to the varied rates of starch digestion by *L. plantarum* and *A. oryzae*. Although *A. oryzae* produces a higher content of amylase that is used for starch hydrolysis into fermentable sugars, the production of amylase by *L. plantarum* can further enhance overall rate of starch utilization, thereby improving the protein production.

As the protein levels increased, the starch content was observed to have a significant decrease ($p<0.05$). This can be attributed to the utilization of carbohydrates by the microbes as an energy source (Osman, 2011). Consumption of starch in yellow pea flour was the highest where untreated flour initially had 70.3% starch and co-culture fermented flour only had 30.6% starch remaining. Previous reports on fermentation of maize flour by *L. plantarum* show a similar decrease in carbohydrate content after 24 h (Fidelis *et al*., 2014; Chibuike *et al*., 2018). Despite fermentation times of 72 h in case of *L. plantarum* and 120 h in case of *A. oryzae*, there was still starch remaining in all of the fermented substrates. Most of the remaining starch could be resistant starch, as the presence of resistant starch in pulses is estimated to be approximately 15% to 20%. Break down of resistant starch by microbial enzymes takes longer, therefore longer fermentation periods could increase further utilization of starch.

Ash content in fermented samples were lower than untreated flours. The lowest was observed for samples fermented by *L. plantarum* followed by *A. oryzae* and co-culture. Lipid content was also found to be lower in fermented samples. The decrease in lipids and ash possibly

occurred due to the utilization of lipids and minerals by fungi. Reduction in lipid and ash contents due to microbe utilization have been reported in literature (Terefe *et al*., 2021). The high amounts of moisture in *A. oryzae* and co-culture fermented samples could be due to the fungal hyphae that retain higher amounts of moisture than bacteria to support growth and metabolism during fermentation and therefore may require longer drying time to remove moisture.

Table 3. Proximate composition of fermented samples with highest protein yield

Sample	Nutrient composition (%, d.b.)				Moisture (%)
	Protein	Starch	Ash	Lipid	
a)Yellow pea					
Untreated	7.8 ± 0.1^{c}	70.3 ± 0.8^{a}	1.4 ± 0.1^{a}	1.0 ± 0.2^{a}	4.0 ± 0.1^{c}
LPYP	27.4 ± 0.2^{b}	68.2 ± 0.9^{a}	0.6 ± 0.1^{c}	0.2 ± 0.1^{c}	4.0 ± 0.0^{c}
AOYP	49.5 ± 0.7^{a}	48.6 ± 1.1^{b}	0.9 ± 0.1^{b}	0.9 ± 0.1^{ab}	10.0 ± 0.2^{b}
LPAOYP	50.5 ± 0.9^{a}	30.6 ± 0.9^{c}	1.2 ± 0.0^{a}	0.7 ± 0.1^{b}	15.1 ± 0.1^{a}
b) Yellow lentil					
Untreated	16.5 ± 0.2^{d}	68.6 ± 1.0^{a}	2.2 ± 0.1^{a}	1.4 ± 0.1^{a}	3.8 ± 0.1^{c}
LPLE	37.2 ± 0.6^{c}	60.6 ± 0.8^{b}	0.8 ± 0.1^{d}	0.7 ± 0.1^{b}	3.6 ± 0.1^{c}
AOLE	56.3 ± 0.4^{b}	40.3 ± 1.0^{c}	1.1 ± 0.0^{c}	1.2 ± 0.1^{a}	8.9 ± 0.0^{b}
LPAOLE	58.6 ± 0.5^{a}	33.4 ± 0.3^{d}	1.4 ± 0.1^{b}	1.5 ± 0.1^{a}	13.5 ± 0.1^{a}
c)Faba bean					
Untreated	14.5 ± 0.1^{d}	63.8 ± 1.6^{a}	2.0 ± 0.2^{a}	0.8 ± 0.2^{a}	3.8 ± 0.1^{d}
LPFB	38.5 ± 0.8^{c}	58.9 ± 0.7^{b}	0.8 ± 0.1^{c}	0.4 ± 0.1^{a}	4.7 ± 0.2^{c}
AOFB	48.3 ± 0.7^{b}	49.9 ± 1.0^{c}	0.9 ± 0.1^{c}	0.7 ± 0.2^{a}	9.7 ± 0.2^{b}
LPAOFB	58.1 ± 0.7^{a}	31.5 ± 0.5^{d}	1.6 ± 0.1^{b}	0.7 ± 0.2^{a}	12.3 ± 0.2^{a}

Data represents mean values from triplicate analysis of each fermented starch-rich pulse flours (yellow pea, yellow lentil and faba bean flour) ± one standard (n=3).

Abbreviations: *LP* (*Lactobacillus plantarum*); *AO* (*Aspergillus oryzae*); *LPAO (Lactobacillus plantarum + Aspergillus oryzae*); YP (yellow pea); LE (yellow lentil); FB (faba bean). Data with the same superscript letter are not significantly different (p>0.05).

4.4 Analysis of protein quality and *in vitro* protein digestibility (IVPD)

4.4.1 Amino acid profile analysis

The overall quality of microbial proteins enriched in fermented pulse flours under the best conditions determined in this study was studied by analyzing changes to the amino acid profiles after fermentation. The amino acid composition (g per 100 g of sample on an as-is basis), essential amino acid (EAA) concentration (mg/g protein) and amino acid scores of fermented flours are summarized in Tables 4, 5 and 6. The limiting amino acid in all the fermented flours were found to be methionine and cysteine, except for untreated yellow pea flour where tryptophan was the lowest. However, the amino acid score obtained for tryptophan was 1.05 which was still much higher compared to amino acid scores obtained for methionine and cysteine in other fermented flours. Methionine and cysteine scores were lower in *L. plantarum* fermented samples than *A. oryzae* or co-culture fermented samples. Overall, the amino acid scores show that fermentation has reduced amino acid levels. This can be attributed to the utilization of EAA by fermenting microbes for their own growth and metabolism. Although the individual amino acid concentrations achieved in this study were lower when compared to pulse proteins, they were still comparable to the amino acid concentrations of feedstuffs such as soybean meal and poultry by-product meal (Sriperm *et al.*, 2011). The concentration of lysine and methionine is 32.3 mg/g and 7.7 mg/g in soybean meal and 33.5 mg/g and 9.9 mg/g in poultry by-product meal. This is similar to the lysine and methionine concentrations obtained in this study for fermented pulse flours, with lysine content ranging from 24 mg/g – 32 mg/g and methionine content ranging from 8 mg/g - 11 mg/g in *A. oryzae* or co-culture fermented samples. This indicates that *A. oryzae* or co-culture fermented samples could be suitable for use as feedstuff in terms of amino acid concentrations.

Table 4. Amino acid composition (g per 100 g of sample on an as is basis) of protein enriched fermented flours

Amino acid	Sample											
	YP	**LE**	**FB**	**LPYP**	**LPLE**	**LPFB**	**AOYP**	**AOLE**	**AOFB**	**LPAOYP**	**LPAOLE**	**LPAOFB**
Aspartic acid	1.05	1.93	1.76	1.05	1.71	1.81	2.02	3.32	2.65	2.10	3.39	3.10
Glutamic acid	1.56	2.77	2.55	1.13	2.14	2.51	2.62	4.45	3.78	2.61	4.43	4.40
Serine	0.41	0.83	0.72	0.35	0.71	0.79	0.81	1.38	1.15	0.88	1.47	1.36
Glycine	0.41	0.65	0.61	0.29	0.51	0.60	0.77	1.15	1.00	0.83	1.22	1.26
Histidine	0.20	0.51	0.41	0.17	0.32	0.32	0.36	0.68	0.52	0.42	0.73	0.58
Arginine	0.59	1.21	1.21	0.52	1.04	1.22	1.08	1.92	1.72	1.22	2.16	2.16
Threonine	0.35	0.59	0.53	0.29	0.51	0.56	0.72	1.11	0.91	0.84	1.21	1.15
Alanine	0.43	0.70	0.64	0.38	0.61	0.68	1.08	1.70	1.36	1.33	1.65	1.68
Proline	0.33	0.65	0.59	0.29	0.56	0.62	0.67	1.15	1.02	0.77	1.23	1.23
Tyrosine	0.27	0.47	0.44	0.19	0.39	0.49	0.63	0.99	0.85	0.66	1.05	1.02
Valine	0.40	0.78	0.66	0.36	0.66	0.71	0.96	1.53	1.28	1.04	1.58	1.53
Methionine	0.08	0.16	0.11	0.07	0.12	0.09	0.24	0.38	0.24	0.20	0.39	0.27
Cysteine	0.15	0.15	0.17	0.06	0.07	0.10	0.15	0.19	0.19	0.18	0.25	0.34
Isoleucine	0.35	0.71	0.60	0.32	0.63	0.65	0.89	1.46	1.16	0.93	1.44	1.35
Leucine	0.58	1.19	1.03	0.54	1.04	1.14	1.40	2.35	2.03	1.46	2.38	2.31
Phenylalanine	0.36	0.81	0.59	0.33	0.68	0.61	0.86	1.54	1.12	0.89	1.52	1.28
Lysine	0.63	1.11	0.94	0.53	0.94	1.02	1.20	1.94	1.47	1.33	1.99	1.85
Tryptophan	0.09	0.14	0.14	0.07	0.12	0.12	0.18	0.29	0.24	0.22	0.31	0.31

Abbreviations: THR (threonine); VAL (valine); CYS (); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); TRP (tryptophan); *LP* (*Lactobacillus plantarum*); *AO* (*Aspergillus oryzae*); *LPAO (Lactobacillus plantarum + Aspergillus oryzae*); YP (yellow pea); LE (yellow lentil); FB (faba bean). Measurements were performed once on each sample.

Table 5. Essential amino acid concentration (mg/g protein) of protein enriched fermented flours

Sample	Amino acid								
	THR	VAL	MET+CYS	ILE	LEU	PHE+TYR	HIS	LYS	TRP
YP	45	52	30	45	75	81	26	81	12
LE	36	47	19	43	72	77	31	67	9
FB	36	46	19	41	71	71	28	65	9
LPYP	11	13	5	12	20	19	6	19	3
LPLE	14	18	5	17	28	29	8	25	3
LPFB	15	18	5	17	30	29	8	26	3
A0YP	15	19	8	18	28	30	7	24	4
A0LE	20	27	10	26	42	45	12	34	5
A0FB	19	27	9	24	42	41	11	30	5
LPA0YP	17	21	8	18	29	31	8	26	4
LPA0LE	21	27	11	25	41	44	13	34	5
LPA0FB	20	26	11	23	40	39	10	32	5
FAO Score	34	35	25	28	66	63	19	58	11

Abbreviations: THR (threonine); VAL (valine); CYS (); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); TRP (tryptophan); *LP* (*Lactobacillus plantarum*); *AO* (*Aspergillus oryzae*); *LPAO (Lactobacillus plantarum + Aspergillus oryzae*); YP (yellow pea); LE (yellow lentil); FB (faba bean). Measurements were performed once on each sample.

Table 6. Amino acid scores of protein-enriched fermented flours

Sample	Amino acid score								
	THR	**VAL**	**MET+CYS**	**ILE**	**LEU**	**PHE+TYR**	**HIS**	**LYS**	**TRP**
YP	1.32	1.48	1.18	1.60	1.13	1.28	1.37	1.39	1.05
LE	1.07	1.35	0.75	1.54	1.09	1.23	1.64	1.16	0.79
FB	1.07	1.31	0.77	1.48	1.08	1.13	1.49	1.12	0.86
LPYP	0.31	0.37	0.19	0.41	0.30	0.30	0.32	0.33	0.23
LPLE	0.40	0.50	0.21	0.60	0.42	0.46	0.45	0.43	0.29
LPFB	0.43	0.52	0.21	0.61	0.45	0.45	0.44	0.45	0.27
A0YP	0.43	0.55	0.31	0.64	0.43	0.48	0.39	0.42	0.33
A0LE	0.58	0.78	0.40	0.93	0.63	0.71	0.64	0.59	0.46
A0FB	0.55	0.76	0.35	0.86	0.64	0.65	0.56	0.52	0.45
LPA0YP	0.49	0.59	0.30	0.66	0.44	0.49	0.43	0.45	0.39
LPA0LE	0.61	0.77	0.44	0.88	0.62	0.70	0.66	0.59	0.47
LPA0FB	0.58	0.75	0.42	0.83	0.60	0.63	0.53	0.55	0.49

(*) Indicates the first limiting amino acid.

Abbreviations: THR (threonine); VAL (valine); CYS (cysteine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); TRP (tryptophan); *LP* (*Lactobacillus plantarum*); *AO* (*Aspergillus oryzae*); *LPAO (Lactobacillus plantarum + Aspergillus oryzae*); YP (yellow pea); LE (yellow lentil); FB (faba bean). Measurements were performed once on each sample.

4.4.2 **In vitro** ***protein digestibility (IVPD) and corrected amino acid score (IVPDCAAS)***

Protein quality of untreated and fermented flours was also examined by measuring change in *in vitro* digestibility (Table 4). The IVPD of yellow pea, yellow lentil, and faba bean fermented by *L. plantarum* was found to significantly increase from 68.6% to 71.6%, 59.2% to 80.2% and 56.9% to 81.6%, respectively ($p<0.05$). Samples fermented by *A. oryzae* also showed a slight increase in IVPD but not as high as for *L. plantarum*. Fermentation has been used in previous studies to improve IVPD in legume samples (Pranoto *et al*., 2013). Increase in digestibility could be due to an increase in protease activity and reduction of antinutritional factors (Gilani *et al*., 2005). However, samples fermented by co-culture showed a decrease in digestibility. This can be attributed to the presence of fungal cell wall composed of glucans, chitin, and glycoproteins in *A. oryzae*, or co-culture fermented samples that act as a barrier between intracellular microbial proteins and digestives enzymes used in the IVPD assay which reduces the exposure of proteins and thereby reduces protein digestibility values.

Protein digestibility was also determined taking the amino acid scores into account as IVPDCAAS. The IVPDCAAS values were found to decrease from 71.9% in untreated yellow pea flour to 13.4%, 21.7% and 15.2% in *L. plantarum*, *A. oryzae* or co-culture fermented samples, respectively. Similarly, IVPDCAAS values were also found to decrease in yellow lentil and faba bean flours after fermentation from 44.2% to 26.9% and 43.6% to 22.8%, respectively. The reduction in IVPDCAAS values can be attributed to the change in amino acid profiles after fermentation. The limiting amino acid (methionine and cysteine) scores of yellow pea flour became reduced from 1.18 to 0.19 when fermented by *L. plantarum* and 0.3 when fermented by *A. oryzae* or co-culture. Similarly, the limiting amino acid scores of yellow lentil and faba bean flours were also found to become reduced after fermentation, with the largest decrease observed when fermented by *L. plantarum*. This could be attributed to the auxotrophic nature of *L. plantarum* that utilizes EAA such as methionine and cysteine for the microbial growth. Overall, despite the increase in protein content after fermentation, EAA content does not meet FAO recommended scores at this point. One possible reason for this could be the inaccessibility of intracellular proteins to proteinases due to the presence of microbial cell wall. However, previous

studies on the effect of fermentation or hydrolysis of protein enriched flours have shown better IVPDCAAS values than obtained in this study. Previous studies on effect of fermentation by *A. oryzae* on protein enriched pea flour show that IVPDCAAS values decrease over fermentation time from 66.7% to 63.6%, which is still higher compared to the value obtained from starch-rich pea flour. Similarly, a study compared the IVPDCAAS values of faba bean flours and protein isolates and showed that the IVPDCAAS values for flours ranged from 55.8% to 80.7%, whereas the faba bean protein isolates had lower IVPDCAAS values that ranged from 47.3% to 67.2% (Kumitch *et al*., 2020). These IVPDCAAS values are still much higher compared to starch-rich faba bean flour used in this study (Shi *et al*., 2022).

Table 7. Protein digestibility analysis of fermented samples with highest protein content

Sample	Limiting amino acid[1]	Limiting amino acid score[1]	IVPD (%)[2]	IVPDCAAS (%)[1]
a) Yellow pea				
Untreated	TRP	1.05	68.6 ± 1.0^{b}	71.9^{a}
LPYP	MET+CYS	0.19	71.6 ± 0.4^{a}	13.4^{d}
AOYP	MET+CYS	0.31	69.3 ± 0.3^{c}	21.7^{b}
LPAOYP	MET+CYS	0.30	50.1 ± 0.8^{d}	15.2^{c}
b) Yellow lentil				
Untreated	MET+CYS	0.75	59.2 ± 1.0^{d}	44.2^{a}
LPLE	MET+CYS	0.21	80.2 ± 0.6^{a}	16.5^{d}
AOLE	MET+CYS	0.40	64.8 ± 0.4^{b}	25.9^{c}
LPAOLE	MET+CYS	0.44	61.4 ± 0.7^{c}	26.9^{b}
b) Faba bean				
Untreated	MET+CYS	0.77	56.9 ± 0.8^{c}	43.6^{a}
LPFB	MET+CYS	0.21	81.6 ± 0.7^{a}	16.9^{c}
AOFB	MET+CYS	0.35	64.4 ± 0.4^{b}	22.6^{b}
LPAOFB	MET+CYS	0.42	53.8 ± 0.4^{d}	22.8^{b}

[1]Measurements were performed once on each fermented starch-rich pulse flours. Measurements were calculated from amino acid content in 1 g of sample protein divided by amino acid content of 1g of reference protein determined by FAO.

[2]Data represents mean values from duplicate analysis of each fermented starch-rich pulse flours (yellow pea, yellow lentil and faba bean flour) ± one standard (n=2).

Abbreviations: *LP* (*Lactobacillus plantarum*); *AO* (*Aspergillus oryzae*); *LPAO* (*Lactobacillus plantarum + Aspergillus oryzae*); YP (yellow pea); LE (yellow lentil); FB (faba bean); MET (methionine); CYS (cysteine); TRP (Tryptophan); IVPD (*In vitro* protein digestibility); IVPDCAAS (*In vitro* protein digestibility corrected amino acid score). Data with the same superscript letter are not significantly different (p>0.05).

5. OVERALL CONCLUSIONS

Protein concentrates/isolates from pulses have desirable nutritional and functional properties that make them useful for the food industry (Shevkani *et al*., 2019). Pulse flour fractionation yields high amounts of protein concentrate that are highly desirable; however, this process also generates large amounts of starch-rich fractions as a co-product (Schutyser & van der Goot, 2011). The starch-rich co-products are of low value due to their beany flavor and low protein content. The main aim of this research was to develop an efficient way of utilizing the under-utilized starch fractions by using them as substrates for SCP production.

The highest yield of protein was achieved at the longest fermentation period which was 72 h when fermented by *L. plantarum* and 120 h when fermented by *A. oryzae* or a co-culture of the two strains. This can be due to the replication rate, substrate utilization rate and growth rate of each microbe during fermentation. The overall protein yield achieved with co-culture fermentation was higher than fermentation runs using single strain cultures. This suggests that a positive interaction between *L. plantarum* and *A. oryzae* resulted in better microbial growth and protein production. The primary hypothesis that submerged fermentation of starch-rich pulse flours with GRAS microbes will convert starch into microbial proteins was proven to be true. The highest protein yield achieved in starch-rich yellow pea, yellow lentil and faba bean flours after fermentation was 10.2%, 18.5% and 16.4%, respectively. However, the main goal of this research was to obtain protein-rich fermented substrates which was not produced solely by fermentation of starch-rich fractions. Therefore, the effect of nitrogen supplementation from different sources (ammonium sulphate, ammonium phosphate and urea) at varying concentrations (15 g/L – 35 g/L) were analyzed. Nitrogen supplementation was found to aid in microbial growth and protein production increasing protein content of fermented yellow pea, yellow lentil and faba bean flours above ~50%. It was found that urea supplemented at 35 g/L resulted in yield of highest protein production when fermented by either single or multi strain cultures. The protein-rich fermented

substrates were then further analyzed for proximate compositions changes including starch, lipid, ash, and moisture contents. Supporting the research model, as the protein levels increased, the starch levels were found to decrease. The lipid and ash content were also found to decrease possibly due to microbial utilization for growth. Following this, the protein-rich fermented substrates were analyzed for IVPD, and it was found that fermentation by *L. plantarum* improved the protein digestibility greatly whereas fermentation by *A. oryzae* or the co-culture did not do so significantly. This could be due to the presence of a microbial cell wall that prevents enzymes' access to intracellular proteins for digestion during the digestibility assay. The IVPDCAAS values were also found to become decreased to < 25%. This could be due to the reduction in sulphur containing amino acids, methionine, and cysteine. In order to improve IVPDCAAS values, the fermentation process could be further optimized to increase production of sulphur containing amino acids. Overall, with further improvements, protein-rich fermented substrates produced through this method will have great potential as protein ingredients, especially in the animal feed industry. SCP produced in this study has greatly improved protein content by greater than 45% and with similar individual amino acid concentrations to current feedstocks in the animal feed market such as soybean meal and poultry by-product meals. With further improvements, protein-rich fermented substrates produced through this method will have improved potential as protein ingredients, especially in the animal feed industry. Therefore, utilization of low economical value pulse starch as raw materials to produce SCP may serve as a promising strategy to better utilize starch-rich co products.

6. FUTURE DIRECTIONS

Based on the findings of this research, future work could focus on the following:

- SCP production yield and efficiency varies based on several factors, especially the choice of microorganisms. In this study, *L. plantarum* and *A. oryzae* have shown promising levels of SCP production, however, several other GRAS microbes have also been of great interest for SCP production from agro-wastes based on previous literature. The efficiency of protein production by such microbes, including *Candida sp.*, *Saccharomyces sp.*, *Pluteus oreatus* and *Corynebacterium glutamicum* using starch-rich pulse fractions could be explored.
- Urea supplementation resulted in highest SCP production in this study. However, several other inexpensive nitrogen sources, including corn steep liquor and ammonium nitrate have shown promising effects on SCP production in literature. Such nitrogen sources could also be studied to measure their effect on protein production by microbes during submerged fermentation.
- A major limitation of using SCP for food and feed applications is the presence of high nucleic acid content about 6% - 10%. To meet WHO/FAO regulatory guidelines, the amount of nucleic acid must be reduced to less than 2%. Therefore, nucleic acid reduction by chemical treatments with sodium hydroxide or sodium chloride, addition of exogenous nucleases or thermal shock could be a promising future approach.
- Apart from high nucleic acid content, the presence of fungal cell wall is also a limitation that reduces protein digestibility (but would increase fiber). Therefore, cell wall destruction using mechanical (sonication, high pressure homogenization, wet milling), chemical (acid/base, detergent) and enzymatic (lytic enzymes or autolysis) methods can be carried out to improve overall protein digestibility.

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8. APPENDICES

Appendix 1A:

The change in protein content (%) during submerged fermentation with *L. plantarum* for yellow pea flour when supplemented with nitrogen sources at varying concentration

Time (h)	Ammonium sulphate - (g/L)			Ammonium phosphate - (g/L)			Ammonium sulphate + Ammonium phosphate - (g/L)			Urea - (g/L)		
	S15	**S25**	**S35**	**P15**	**P25**	**P35**	**SP15**	**SP25**	**SP35**	**U15**	**U25**	**U35**
24h	9.3	11.3	14.5	11.4	11.0	18.4	10.9	11.9	14.1	17.5	24.8	31.0
48h	12.0	14.7	14.8	15.3	20.6	21.8	12.0	19.3	17.4	18.2	24.4	31.3
72h	15.6	15.1	17.2	16.5	22.4	28.3	20.3	20.9	21.2	18.8	25.0	31.8

Appendix 1B:

The change in protein content (%) during submerged fermentation with *L. plantarum* for lentil flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)			Ammonium phosphate - (g/L)			Ammonium sulphate + Ammonium phosphate - (g/L)			Urea - (g/L)		
	S15	**S25**	**S35**	**P15**	**P25**	**P35**	**SP15**	**SP25**	**SP35**	**U15**	**U25**	**U35**
24h	21.1	15.2	22.1	12.8	14.4	20.9	14.2	19.2	20.3	29.6	30.9	44.9
48h	21.2	21.9	22.6	18.6	27.9	29.7	20.3	19.6	24.2	31.7	40.7	47.9
72h	21.0	24.4	26.6	18.3	28.7	32.3	23.6	24.8	25.5	39.0	39.0	49.8

Appendix 1C:

The change in protein content (%) during submerged fermentation with *L. plantarum* for faba bean flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)			Ammonium phosphate - (g/L)			Ammonium sulphate + Ammonium phosphate - (g/L)			Urea - (g/L)		
	S15	**S25**	**S35**	**P15**	**P25**	**P35**	**SP15**	**SP25**	**SP35**	**U15**	**U25**	**U35**
24h	18.1	11.9	19.8	10.6	14.7	13.8	8.2	9.3	12.7	21.7	32.2	38.0
48h	21.4	16.2	20.0	12.1	15.7	17.9	11.0	11.3	14.7	24.2	32.1	37.4
72h	19.1	21.8	21.8	11.4	17.2	23.9	14.2	15.8	19.1	24.9	32.5	38.3

Appendix 2A:

The change in protein content (%) during submerged fermentation with *A. oryzae* for yellow pea flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)			Ammonium phosphate - (g/L)			Ammonium sulphate + Ammonium phosphate - (g/L)			Urea - (g/L)		
	S15	**S25**	**S35**	**P15**	**P25**	**P35**	**SP15**	**SP25**	**SP35**	**U15**	**U25**	**U35**
24h	16.8	10.7	13.1	9.5	10.7	14.5	15.1	11.8	12.7	11.1	16.2	14.9
48h	20.4	17.6	29.2	16.4	15.4	15.3	20.0	19.5	14.5	28.0	23.4	47.3
72h	23.4	32.6	29.9	23.7	24.4	22.4	19.8	16.7	16.0	30.0	38.8	48.1
120h	24.8	31.6	44.7	25.2	22.4	26.0	21.5	32.3	33.1	34.6	48.2	50.4

Appendix 2B:

The change in protein content (%) during submerged fermentation with *A. oryzae* for lentil flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)			Ammonium phosphate - (g/L)			Ammonium sulphate + Ammonium phosphate - (g/L)			Urea - (g/L)		
	S15	**S25**	**S35**	**P15**	**P25**	**P35**	**SP15**	**SP25**	**SP35**	**U15**	**U25**	**U35**
24h	25.1	25.7	16.3	14.2	17.3	15.9	11.9	19.4	18.7	20.6	44.3	50.9
48h	23.7	28.0	22.7	19.0	19.6	20.8	23.1	24.8	33.3	38.2	49.9	58.7
72h	36.2	29.2	22.8	18.9	24.1	21.9	24.2	28.8	33.9	45.1	53.7	60.5
120h	37.1	35.0	44.0	18.6	29.0	31.3	27.5	25.7	39.2	44.6	56.6	60.6

Appendix 2C:

The change in protein content (%) during submerged fermentation with *A. oryzae* for faba bean flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)			Ammonium phosphate - (g/L)			Ammonium sulphate + Ammonium phosphate - (g/L)			Urea - (g/L)		
	S15	**S25**	**S35**	**P15**	**P25**	**P35**	**SP15**	**SP25**	**SP35**	**U15**	**U25**	**U35**
24h	19.6	17.5	17.7	9.0	10.4	8.8	9.3	8.9	14.2	37.2	35.5	38.5
48h	21.5	25.6	27.0	12.2	13.4	14.6	16.2	12.0	17.3	41.5	45.4	46.2
72h	24.7	23.4	28.3	16.5	15.2	21.5	17.9	9.7	16.9	44.0	48.1	47.6
120h	35.0	36.9	39.8	19.2	23.0	24.7	19.5	17.5	30.1	47.2	50.6	53.6

Appendix 3A:

The change in protein content (%) during submerged fermentation with *L. plantarum + A. oryzae* for yellow pea flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)	Ammonium phosphate - (g/L)	Ammonium sulphate + Ammonium phosphate - (g/L)	Urea - (g/L)
	S35	**P35**	**SP35**	**U35**
24h	17.3	15.7	13.2	16.2
48h	24.8	20.8	23.1	37.8
72h	31.2	28.0	22.2	50.6
120h	37.9	30.7	23.5	52.7

Appendix 3B:

The change in protein content (%) during submerged fermentation with *L. plantarum + A. oryzae* for lentil flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)	Ammonium phosphate - (g/L)	Ammonium sulphate + Ammonium phosphate - (g/L)	Urea - (g/L)
	S35	**P35**	**SP35**	**U35**
24h	15.1	14.5	22.0	32.0
48h	30.9	15.2	28.4	50.8
72h	37.4	19.0	36.4	57.8
120h	49.0	38.6	52.4	60.3

Appendix 3C:

The change in protein content (%) during submerged fermentation with *L. plantarum + A. oryzae* for faba bean flour when supplemented with nitrogen sources at varying concentration.

Time (h)	Ammonium sulphate - (g/L)	Ammonium phosphate - (g/L)	Ammonium sulphate + Ammonium phosphate - (g/L)	Urea - (g/L)
	S35	**P35**	**SP35**	**U35**
24h	12.9	12.8	11.2	11.2
48h	32.6	15.9	16.0	28.0
72h	38.3	13.4	17.4	47.1
120h	49.0	32.0	29.8	58.1