A DISSERTATION ON

Optimization of pH for *Brassica juncea* Meal Protein Extraction Using Response Surface Methodology

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
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IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF TECHNOLOGY
IN FOOD TECHNOLOGY

BY
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UNDER THE SUPERVISION OF

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DECLARATION FORM

I, **Zainab Sultan**, a student of **M.Tech Food Technology** (2^{nd} year/ 4^{th} Semester), Integral University have completed my six months dissertation work entitled **"Optimization of pH for *Brassica juncea* Meal Protein Extraction Using Response Surface Methodology"** successfully from Department of Bioengineering **Integral University, Lucknow** under the guidance of **Dr. Kaiser Younis.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Zainab Sultan

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CERTIFICATE

Certificate that Ms **Zainab Sultan** (Enrollment Number 1600102573) has carried out the research work presented in this thesis entitled **"Optimization of pH for *Brassica Juncea* Meal Protein Extraction Using Response Surface Methodology"** for the award of **M.Tech Food Technology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **M.Tech Food Technology.**

I wish her good luck and bright future.

Dr. Kaiser Younis
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TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

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ZAINAB SULTAN

LIST OF ABBREVIATIONS

S.No	ABBREVIATIONS	FULL FORM
1.	DMMP	Defatted mustard meal protein
2.	SpH	Soluble pH
3.	IpH	Isoelectric pH
4.	WPI	Whey protein isolate
5.	OHC	Oil holding capacity
6.	WHC	Water holding capacity
7.	FC	Foaming capacity
8.	EC	Emulsifying capacity
9.	BBD	Box-Behnken design
10.	RSM	Response surface methodology

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ABSTRACT

Protein isolates were prepared from defatted mustard meal by alkaline solubilization at pH 11 and isoelectric precipitation at pH 4. Response surface methodology was used to determine optimum conditions for extraction of protein from defatted mustard meal. Box-Behnken design including independent variables such as SpH (9, 10 and 11), IpH (3, 4 and 5) and particle size (150, 375 and 600 µm) was used to get the optimal conditions for highest protein yield and functional properties (dependent variable). Maximum yield (11.808%) was obtained when SpH, IpH and particle size, were kept 11, 4, 375 µm, respectively. Functional properties were studied for protein isolate obtained at optimal conditions with highest yield. Results showed that alkaline extraction increased the protein yield and functional properties. The protein content (dry basis) in the protein isolate was 93%. Water absorption capacity and oil absorption capacity were 1.5 g/g and 2.50 g/g, respectively. Water absorption capacity was improve due to increased interaction of hydrophilic bonds at alkaline pH. Emulsifying activity, foaming capacity and foam stability were 69.1 % and 12.56 % respectively. Emulsifying activity and foam stability, due to protein unfolding which exposes surface hydrocarbon chains and increases surface interfacial activity.

Chapter 1

INTRODUCTION

The word mustard, which is derived from the Latin *Mustum*, originally referred to the condiment. According to Sanskrit records from around 3000 BC, mustard is one of the earliest recorded spices and was one of the first crops to be domesticated. Mustumardens, also known as "hot or burning must," was a popular Roman specialty condiment made from the expressed juice of grapes or other fruits combined with ground mustard seeds. The love of mustard that the Romans had for it spread throughout Europe, where it was widely used to season meat and fish. India accounts for the world's fourth-largest oilseed economy. Mustard is one of seven edible oilseeds cultivated in India which accounts for 28.6 percent of the country's total oilseed production. In India, 3% of the total planted land is made up of oilseeds, which make up 14.1% of the total production (Shivran et al., 2020). Around 38–42 metric tonnes mustard seeds and 12–14 metric tonnes of mustard oil are produced worldwide. The agro-climatic conditions in the mustard-growing regions of India are extremely diverse, and many varieties of mustard are grown in various regions of the nation. In 2004-2005, the production of protein meal reached about 207 million metric tonnes, while the production of oilseeds reached 380 million metric tonnes (Moure et al., 2006). The most common sources of protein meal are soybeans, mustard seed, rapeseed, cotton, sunflower seed, and peanuts, which account for 69%, 30%, 12.4%, 6.9%, 5.3%, and 2.8% of global protein meal production, respectively. Mustard seeds are mainly used to obtain mustard oil, although they are also used as a condiment. It has a high oil content of good quality, a wide range of adaptability, and good production potential. After oil extraction, mustard meal is obtained which was primarily used for animal feeding. The mustard seeds are rich source of proteins and it contains a diverse range of amino acids, including essential amino acids. Depending on the seed, the protein content of defatted meals made from de-hulled oilseeds ranges from 35% to 60% on dry basis It is high in oleic acid (20-28%), linoleic acid (10-12%), linolenic acid (9.0-9.5%), and erucic acid (30-40%) (Al-Jasass & Al-Jasser, 2012). Due to the renewable nature of the raw material and the abundance and variety of sources, the protein from vegetable origin can be used in place of animal protein in food and cosmetic applications (especially legumes, cereals and oilseeds). Mustard includes all necessary amino acids in high concentrations, including aromatic acids (phenylalanine and tyrosine), sulfur-containing amino

acids (methionine and cysteine), leucine, valine, and lysine. High amounts of water-soluble proteins or albumins (mostly the 2S napin protein) have been discovered in mustard.

Proteins are often produced from protein-rich sources by alkaline solubilization and isoelectric precipitation. The method involves solubilizing proteins in acid or alkali, followed by isoelectric precipitation of soluble proteins to produce stable protein isolates. The pH shift approach has been demonstrated to increase solubility, emulsifying, and foaming capabilities considerably (Jiang et al., 2018). The pH shift technique is a promising technology for isolating protein from plant sources since it is a simple and straightforward procedure that provides high protein yields and improves the functional characteristics of protein isolates. The pH shifting method can improve the functional properties and yield a high percentage of protein (Lafarga et al., 2018). The protein isolates are more soluble under severe alkaline conditions of the protein solution because the protein structure unfolding at high pH revealed more hydrophilic amino acids, leading to improved protein solubility. The proteins become negatively charged in the presence of a more alkaline medium due to ionization of the carboxylic groups and de-protonation of the amine group, which increases the repulsive force between negatively charged proteins. Optimization of the proteins extraction from mustard meal was performed with the aid of Design Expert V 13, (Stat-Ease Inc, USA) Software. Box Behnken design was employed for assessing the influence of soluble pH (SpH) (9,10,11), insoluble pH (IpH) (3,4,5) and particle size on the proteins extraction yield and functional properties from defatted mustard meal. RSM (Response Surface Modeling) is a technique for optimising response. In response surface method, the dependent variables are referred to as responses, while the independent variables are referred to as predictor variables.

Thus taking into consideration the good effects of pH on extraction efficiency, the current study "Optimization of pH for *Brassica juncea* meal protein extraction using Response Surface Methodology" was designed.

The objective of the study is as follows:

- Optimization of protein extraction from mustard meal.
- Investigation of physiochemical and functional properties of mustard meal protein isolate.

Chapter 2

REVIEW OF LITERATURE

2.1 Plant based proteins

The consumption of animal food has been questioned due to rising health issues among people as it increases the risk of chronic diseases like diabetes, cancer, cardiovascular, etc. Animal food is referred to as the main source of protein, however, it has high calorific value and is linked to various diseases. Plant-based protein can be a good alternative to animal protein. In recent years plant-based proteins have gained popularity as they have better health-promoting benefits than animal proteins (Qin et al., 2022). Plant-based protein isolates have recently gained popularity in food applications due to their increased sustainability and lower operating costs (Gorissen et al., 2018). Many studies have shown the effectiveness of a plant-based diet on human health. Multiple clinical studies have proven the efficiency of plant-based diets for weight loss and reducing the risk of developing diabetes (Kahleova et al., 2018). In another study decrease in body weight was positively correlated with an increased intake of plant protein (Song et al., 2016). Similarly, another study observed a positive change in body mass index by replacing animal protein with a plant source (Ortolá et al., 2020). Esmaeili et al., (2016) mentioned that rice bran oil contains 12-16% of protein. Rice bran protein possesses distinct nutritional and functional properties. It is a considerate source of hypoallergenic protein and could be used as an ingredient in baby food preparations or as a nutritional and therapeutic food ingredient. Zhang et al., (2020) stated that cold-pressed rapeseed meal contains 35-40% of protein which can be utilized in various food applications. Rapeseed meal has been used as an alternative for egg protein in mayonnaise production as it is highly nutritious and has good functional properties (Qu et al., 2018; Zhang et al., 2020). Sea buckthorn seeds are rich source of protein as it contains 83% of total amino acid of which 68% are essential amino acid (Lin et al., 2022). This protein has significant hypoglycemic, anti-inflammatory, and anti-diabetic effects (Yuan et al., 2016). Amaranth grains are another important source of plant protein as it contains all the essential amino acid with methionine and lysine being the highest. Amaranth protein has gained interest in food industries as it has shown promising health benefits and has a limited amount of nutritional factors compared to other grains (Das et al., 2021). Wang et al., (2021)

reported that pecan protein has a wide range of applications in the food and pharmaceutical industries as it is a rich source of various amino acids and has excellent antioxidant properties. Thus many studies have reported numerous plants and plant byproducts for the extraction of protein, which can be utilized in various food applications and fulfill the demand for animal-free protein.

2.2 Mustard seeds

(*Brassica nigra*) and (*Brassica juncea*) belongs to the mustard family (*Brassicaceae* or *Cruciferae)* and is known by several other names, including brown mustard, Chinese mustard, and oriental mustard. Brown mustard seeds were originated from China which later came to India and yellow mustard seeds are the origin of Mediterranean region. It is thought to have originated in the Irano-Turanian area as a member of the commercially significant genus *Brassica* (Šamec & Salopek-Sondi, 2018) Mustard seeds are primarily used for the extraction of mustard oil, but it is also used as a condiment. For ages, traditional Chinese, Indian, and Arabian medicine used Indian mustard seeds to heal ailments, but it is now used in modern medicine in Europe and North America as well (Szőllősi, 2020). Oil content in *Brassica juncea (L.)* is typically 30-38 percent. Mustard oil includes a variety of fatty acids, the most important of which being eruvic and lenoleic acid.

2.2.1 Proteins in mustard seeds

Proteins are polymers of amino acids joined by peptide bonds. Protein structure is classified into four categories: primary, secondary, tertiary, and quaternary structure. The primary structure of proteins is the precise arrangement of amino acids that compose their chains. The precise sequencing of the proteins is critical since it defines the final fold and hence the functional properties of the protein. Primary protein structures are made up of a number of polypeptide chains linked together. These chains include amino acids organized in a precise sequence that is unique to the protein. Any alteration in the sequence has an effect on the whole protein. Secondary protein structure refers to local folded structures that arise inside a polypeptide as a result of interactions between backbone atoms. They have been discovered to exist in two different sorts of structures: helix and pleated sheet. This structure results from the regular folding of the polypeptide chain's backbone caused by hydrogen bonding between the

peptide bonds. Protein tertiary structure represents overall folding of polypeptide chains, as well as additional folding of the secondary structure. It produces two primary molecular shapes: fibrous and globular. Hydrogen bonds, disulphide connections, van der Waals forces, and electrostatic forces of attraction are the primary factors that stabilize the secondary and tertiary structures of proteins. The quaternary structure is formed by the spatial arrangement of numerous tertiary structures. Some proteins are made up of two or more polypeptide chains known as sub-units. The spatial arrangement of these subunits in relation to one another is referred to as quaternary structure (Rodrigues et al., 2012).

The protein content of mustard seeds ranges from 24-30 percent. Mustard protein has a good amino acid composition including essential amino acids. It contains 20-28 percent oleic acid, 10-12 percent linoleic acid, 9.0-9.5 percent linolenic acid, and 30-40 percent erucic acid (Al-Jasass & Al-Jasser, 2012). It was also discovered that yellow mustard seed flour contained more lysine and valine than brown mustard seed flour, while having less aromatic amino acids (Phenylalanine & tyrosine), sulfur-containing amino acids (Methionine & cystine), and lysine (Abul-Fadl et al., 2011). Water-soluble proteins or albumins (mainly the 2S napin protein) have been found in high concentrations (up to 45-50 percent of total protein). 11S cruciferin is a hexamer that dissociates in acidic environment. On the other hand cruciferin, has an isoelectric point (pI) at pH 7, whereas other oilseed proteins have a pI around pH 4.5-5. This affects protein solubility, with least solubility observed for cruciferin at pH 4.0 and 8.0, compared to pH 4.5 for other oilseed proteins (Arntfield, 2018).

2.3 pH shift method for extraction of protein from plant sources

Various methods have been developed to extract protein from plant sources like salt extraction, microwave assisted extraction, ultrasound assisted extraction, micellar precipitation, etc. The pH shift method is one of the simplest methods used for protein isolation. The method involves solubilizing proteins in acid or alkali, followed by isoelectric precipitation of soluble proteins to produce stable protein isolates. Various studies have reported that the pH shifting method can improve the functional properties and yield a high percentage of protein (Lafarga et al., 2018). Globular proteins can slightly unfold when subjected to severe alkaline or acidic pH conditions, providing more flexibility to the structure. Changes in inter and intra-molecular ionic

forces have been used effectively to optimize the functionality of leguminous proteins. The pH shift method has been shown to significantly improve solubility, emulsifying, and foaming properties (Jiang et al., 2018). The pH shift method is a promising technology for isolating protein from plant sources, it is a simple and easy method with high protein yields and better functional properties of protein isolates. This article reviews the research on the extraction of protein from plant sources using the pH shift method and the effect of this method on the functional properties of protein extracts. It will assist researchers in optimizing a pH range for maximum protein extraction yield and understanding the effect of pH treatment on protein properties.

2.3.1 Principle of pH shift method

The pH shift method is also known as the acid and alkali solubilization or isoelectric precipitation method. In this method, alkali and acid are used to solubilize and precipitate protein at their isoelectric point. The principle of this method revolves around the solubility of protein at different pH ranges. Proteins in solution are linked together by poor protein-protein interactions and their side chains can undergo different charges as the pH of the solution changes. When the pH of the protein solution increases or decreases the side chains can accumulate high negative or positive charges, leading to enhanced opposite charges between proteins. These strong negative or positive charges will cause more interactions between protein and water, resulting in enhanced protein solubility (Yongsawatdigul & Park, 2004). The addition of acid increases the net positive charge on the side chains, while the addition of alkali increases the net negative charge on the side chains. When the negative and positive charge becomes equal on side chains a net zero charge is acquired by the protein surface, this is known as the isoelectric point of the protein. The surface net charges will decrease as the protein reaches its isoelectric point, which reduces protein–water interaction, resulting in decreased protein solubility in water. Thus extracted protein is obtained in the form of precipitate (Surasani, 2018). The method is based on increased protein solubility caused by pH changes, which allows protein separation from insoluble material, such as carbohydrates and fiber (Nisov et al., 2022). The method is explained in figure 2.1.

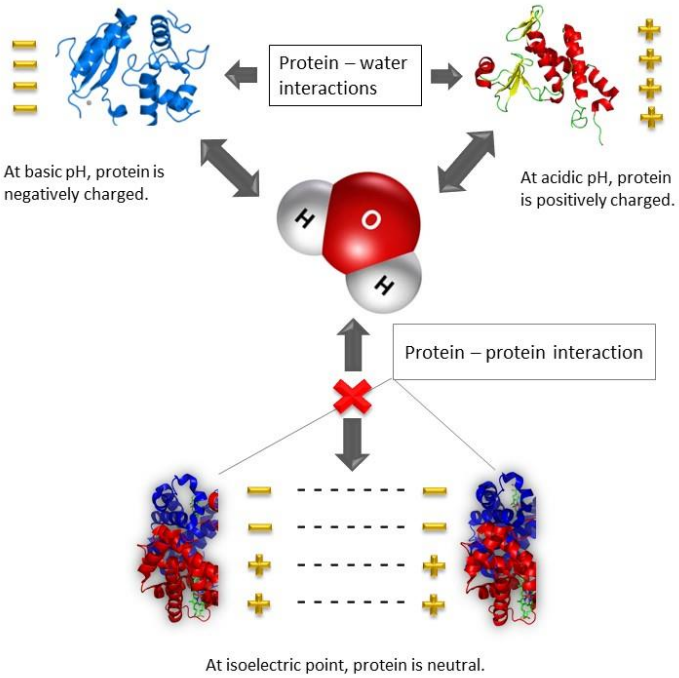

Fig 2.1 Diagrammatic representation of pH shift method

2.4 Effect of pH on the extraction yield of protein

To extract protein from defatted rapeseed meals, pH control in the extraction and precipitation process can be an efficient method. Zhang et al., (2020) revealed that raising pH values enhanced protein extraction yield from 33.58 percent to 61.25 percent, indicating that increasing the concentration of alkaline medium might improve rapeseed protein extractability. They also reported that the extraction at pH 9.0 and subsequent precipitation at pH 4.5 was the optimal process conditions for producing functionally intact rapeseed proteins with the highest yield. Jahan et al., (2022) obtained the highest yield of mustard meal protein at pH 11. They observed a higher yield of protein as the pH was raised because of the increase in protein solubility under an alkaline medium. Das et al., (2021) extracted the amaranth protein at pH 9, 10, 11, and 12. They observed the highest yield at pH 12 which shows that the yield increases more towards alkaline pH due to increased solubility. They also reported that the purity of extracted protein decreased at higher pH and the best purity of the protein was obtained at pH 9.

The initial protein content of Shiroodi and Tarom bran was 18.5 and 16.2 percent, respectively. Esmaeili et al., (2016) studied that the alkaline extraction method enhanced their

purity to 77 and 80 percent, respectively. Mir et al., (2019) isolated protein from *Chenopodium* seeds and studied the effect of pH on the characteristics of the isolated protein. They observed that at pH of 9.0-12.0 protein isolate yields ranged from 8.12-12.22 percent for QPI (*Chenopodium quinoa*) and 7.71-10.98 percent for API (*Chenopodium album),* respectively. Because protein isolates are more soluble under severe alkaline conditions of the protein solution, protein yield rose dramatically with increasing pH for both varieties of protein isolates. The proteins become negatively charged in the presence of a more alkaline medium due to ionization of the carboxylic groups and de-protonation of the amine group, which increases the repulsive force between negatively charged proteins. Further studies have been reviewed in table 2.1.

Table 2.1 Impact of the pH shift approach on protein extraction yield from various plant sources

Sample	Variables	Results	References
Hempseed cake	Alkaline pH: 8.0, 8.5, 9.0, 10.0, 10.5, 11.0, 12.0 Acidic pH:3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 Time: 1,2, 3, 4 hr Temperature: 20°, 30°, and 50°	60.6% yield with protein content- 90.3% and 57.0% yield with the protein content of 90.8% were obtained in lab-scale and pilot-scale extraction, respectively at alkaline pH of 10.5 for 1 hr at 20 °C and precipitation at pH 5.5.	(Helstad et al., 2022)
Rapeseed pressed cake	Alkaline pH: 10.5 Acidic pH: 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5	The highest protein yield of 33% was obtained at pH value 4 having a protein content of 64 ±1% on a dry basis.	(Ahlström et al., 2022)
Yellow pea	Alkaline pH: 8.5, 9.0, 9.5 Acidic pH: 4.5	The highest protein yield of 57.56% was observed at pH 9.5 having a protein content of 83.4% on a wet basis.	(Gao et al., 2020)

African mesquite bean	Alkaline pH: 9.0, 10 Acidic pH:4.5	A high yield of 30.46% having a protein content of 81.34% was observed as compared to micellization which gave a 7.95% yield with a protein content of 72.04%.	(Falade& Akeem, 2020)
Sour cherry kernel	Alkaline pH: 8.0, 9.5, 11.0 Acidic pH: 4.2 Solid-solvent (g/mL): 1/10, 1/20, 1/30 Incubation time (h): 1, 2, 3	A maximum yield of 79.6% was observed at pH 9, solid to solvent ratio 1/30, and incubation time 3 h.	(Kasapoğlu et al., 2021)
Pumpkin seed oil cake	Alkaline pH: 10 Acidic pH: 5	The protein yield obtained was 63.5% having 94.3% protein content.	(Bučko et al., 2015)
Defatted conophor nut flour	Alkaline pH: 10 Acidic pH: 4.5	The obtained extract contained protein content ranging from 86.86% to 87.74%.	(Iyenagbe et al., 2017)
Moringa leaves	Alkaline pH: 11.5, 12 Acidic pH: 1, 4, 4.5, 5	The highest yield of 6.50% having a protein content of 75.77% was obtained at the combination of pH 11.5 and 4.5	(Bocarando-Guzmán et al., 2022)
Chia seeds	Alkaline pH: 8, 10, 12 Acidic pH: 3.0, 4.5	The highest recovery yield of protein (17%) was observed at the combination of pH 12 and 4.5	(López et al., 2018)

Durum wheat bran	Alkaline pH: 9.5 Acidic pH: 4.2	The yield ranged from 20.5% to 24.8% having a protein content of 61%	(Alzuwaid et al., 2020)
Defatted tomato seed meal	Alkaline pH: 7.5 Acidic pH: 3.9 Extraction time: o, 18, 36, 54, and 72 h Mixing time: 10, 15, 20, 25, and 30 min	The highest yield of 80.37% was obtained at the optimized conditions of 82.81/1 (v/w) solvent-solid ratio. The extraction time was 49.76 hours, while the mixing time was 24.6 minutes.	(Mechmeche et al., 2017)
Jatropha seed cake	Alkaline pH: 8, 9, 10, 11, 12 Acidic pH: 3.5, 4.0, 4.5, 5.0, 5.5 Temperature (°C): 30, 40, 50, 60, 70 Extraction time: 0.5, 0.75, 1, 1.25, and 1.75 h	Under the optimal condition of 60 °C temperature, alkaline pH 11.0, acidic pH 4.41, and extraction duration of 0.78 h, an extraction yield of 38.66 percent with a protein content of 41.98 was produced.	(Ahluwalia et al., 2020)

2.5 Effect of pH on the functional properties of protein

2.5.1 Solubility

Protein solubility is an important parameter in the food industry because it affects other functional qualities like emulsifying and foaming capability (Çelik et al., 2019). The solubility of rapeseed protein isolates increased significantly with increasing pH. Rapeseed protein structure unfolding at high pH revealed more hydrophilic amino acids, leading to improved protein solubility. Protein buildup at lower precipitation pH values was most likely due

to the decreased rapeseed protein solubility (Zhang et al., 2020). Ge et al., (2021) isolated protein from eight traditional Chinese beans. Protein solubility was found to be lowest at the isoelectric point (pH 5.0) and rose considerably when the pH varied from 5.0. Solubility was much higher at basic pH values than at acidic pH values, indicating that the surface net charge of legume proteins has a considerable impact on their solubility. Chen et al., (2019) observed that the solubility of whey protein isolate (WPI) increased considerably at pH ranges of 2-4 and 6-10. Moreover, the solubility of WPI at pH 5 decreases significantly from 35.16 to 31.30%. They concluded that the lowest solubility was observed at the isoelectric point of WPI. Yuliana et al., (2014) isolated protein from cashew nut shells (defatted) and studied the influence of pH on solubility. They noticed the lowest solubility of 48.44 percent at pH 3, and the solubility improved with variation in pH. Protein solubility of 82.31 percent and 84.75 percent was observed at pH 2 and 11, respectively. At high or low pH, proteins get unfolded due to net positive and negative charges, leading to more exposed groups and causing increased solubility. While at the isoelectric point, protein forms clusters due to intermolecular forces, thus decreasing solubility. Cui et al., (2013) studied the influence of pH on the functional characteristics of soybean protein hydrolysates. The result showed a U-shape trend at various pH levels, they observed the lowest solubility near the isoelectric point, and the highest solubility was observed at pH 9.0. Çelik et al., (2019) demonstrated the impact of pH on protein extracts of sour cherry kernels. The highest solubility was observed at (92.96 ± 1.66 %) at pH 12.0 and the lowest solubility (12.41 ± 1.23%) was observed at pH 5.0. Greater protein solubility at alkaline pH was most likely correlated with higher positive or negative charges, resulting in ionic interactions.

To investigate the influence of pH on solubility, the pH of the casein solution was varied to 3.0, 4.0, 5.0, 6.0, and 8.0. Casein solubility was lowest around the isoelectric point at pH 3.0 to 5.0 and rose considerably to pH 7.0, where the solubility was maximum. This shows that protein exposes more hydrophilic groups at alkaline pH due to structural changes, thus increasing solubility (Biasutti et al., 2007). Ahmed et al., (2011) observed the effect of pH on functional characteristics of selected legume flours. The lowest solubility of pigeon pea, phaseolus, cowpea, and hyacinth bean flours at pH 4.0 was determined to be 15.22%, 30.06% , 25.32% and 15.75 %, respectively, indicating that the flour protein's isoelectric point is at pH 4.0. As the pH was moved away from the isoelectric point on either side, the solubility rose. The increased net charge obtained at both acid and alkaline pH levels resulted in solubility due to

wheat protein unfolding. It was also discovered that at alkaline pH, the degree of unfolding increased. In a study done by (Majzoobi & Abedi, 2014), the lowest water solubility of the gluten protein (18%) was reported at pH 6 and the water solubility enhanced to 23.3 percent after the pH was reduced from 6 to 3. This could be due to a slight deterioration of the gluten in the acidic state. Further increased water solubility (21.5%) was observed at pH 9. Pham et al., (2017) investigated the effects of pH on the functional properties of pumpkin seed protein fractions. At pH 3–4, 4–5, and 4–5, the lowest solubility of albumin, glutelin, and globulin concentrate were observed. The protein concentrates were more soluble when the pH decreased from 3 to 2 and increased from 5 to 12 and the maximum albumin concentrate solubility (92.4 percent) was observed at pH 9–10. Peng et al., (2020) studied the influence of pH on the functional properties of mildly fractionated soy protein. According to the study, the lowest value of protein solubility was obtained at processing pH levels between 4.5 and 5.5, which is close to the isoelectric point (pI) of soy protein. Soy protein fraction (SPF) solubility improved dramatically when the processing pH was adjusted apart from an isoelectric point on either side. The maximum solubility was reported at pH 7.5. Mir et al., (2019) studied the effect of pH on the characteristics of isolated protein from *Chenopodium* seeds. At pH 3, the solubility of QPI (*Chenopodium quinoa*) (28.34 %) and API (*Chenopodium album)* (21.41 %) was the lowest and the solubility improved with increasing pH. At pH 11, QPI (78.46 %) and API (76.40 %) had the highest solubility. This could be due to less bonding between proteins and water, which increases intermolecular interactions of proteins and causes agglomeration and sedimentation, lowering solubility. Das et al., (2021) observed that with increasing or decreasing pH on either side of the isoelectric point of protein the solubility of amaranth protein isolates improved exponentially. The results also showed that pH 4 to 6 recorded the lowest solubility. This can be due to the presence of globular proteins which are less soluble at this pH range.

Thus it was reviewed that solubility is highly influenced by the pH of the extraction solvent. Many researchers have observed that the solubility is recorded as the lowest near isoelectric point and increased considerably with increasing pH.

2.5.2 Emulsifying capacity

Emulsion activity is the tendency of a protein to produce an emulsion by absorbing oil at the oil-water interaction (Mir et al., 2019). The most significant factors influencing a protein's emulsifying ability are surface hydrophobicity and concentration. Furthermore, a larger proportion of hydrophobic amino acids in the protein molecule increases emulsification (Çelik et al., 2019). Zhang et al., (2020) showed that rapeseed proteins synthesized at low precipitation pH had improved emulsification capabilities, which might be due to protein unfolding, which exposes surface hydrocarbon chains and increases surface interfacial activity. They also discovered that emulsifying activity and protein isolate stability were much greater at pH 3.0 and steadily reduced as precipitation pH increased. The significant emulsifying value of rapeseed proteins at low precipitation pH levels may be related to their high interfacial charge and surface zeta potential. Protein isolates from Chinese beans were found to have emulsifying characteristics at pH 3.0, 7.0, and 9.0. All isolates had considerably higher emulsifying ability index (EAI) values at pH 7.0 and 9.0 than at pH 3.0. All isolates showed better solubility at basic pH than at acidic pH values, which might be a reason for better EAI (Ge et al., 2021). Chen et al., (2019) observed that the emulsifying ability and emulsifying stability of pH shift treated whey protein isolate (HWPI) was higher than the control whey protein isolate (WPI). They further mentioned that when compared with untreated WPI, the HWPI was more effective in absorbing the oil/water interface and formed a more stable emulsion. Yuliana et al., (2014) recorded the highest emulsifying activity of 70% at pH 10 while the lowest EA of 40% was observed at pH 3. The pH influences emulsifying capacity (EC) by distributing charge on protein molecules. When the pH was raised to alkaline levels, dipole-dipole repulsion between adjacent droplets increased due to which the polarised protein molecules' hydration also increased. Because of these conditions, interfacial energy was lowered, leading to emulsion droplet coalescence. Ahmed et al., (2011) observed the influence of pH on the functional properties of selected legume flours. At pH 4.0, the studied samples had minimum EA values of 24.23, 30.0, 33.33, and 30.0 percent for pigeon pea, phaseolus, cowpea, and hyacinth bean flours, respectively. This could be due to increased binding proteins, which reduces hydrophobicity of the surface and decreases protein net charge and solubility. They reported the highest emulsification ability (EA) value at pH 8.0. The impact of pH on the functional characteristics of protein fractions of pumpkin seeds was studied. Protein concentrates exhibited the lowest

emulsifying capacity (EC) at pH 4 due to their poor protein solubility. Protein diffusion to the water-oil interface was delayed, and film formation around suspended particles in an o/w emulsion was inhibited, lowering the EC of protein. Pumpkin seed glutelin had the lowest emulsifying stability (ES) in the isoelectric zone, whereas high or low pH levels considerably improved emulsifying ability (ES) (Pham et al., 2017). According to (Lawal et al., 2005) the partial dissociation of protein molecules generated the high emulsifying stability (ES) at very high acidic or alkaline pH values. This event may result in mutual cohesion of oil droplets and protein molecules, as well as the stabilization of the protein film that surrounds the oil droplets in the emulsion. Esmaeili et al., (2016) reported that emulsifying activities and emulsion stabilities of rice bran protein were enhanced by increasing the pH from 5.0 to 8.0. The maximum emulsifying stability was found at pH 7.

Mir et al., (2019) studied the effect of pH on the characteristics of protein isolates of *Chenopodium* seeds. They observed that the emulsifying ability for QPI (*Chenopodium quinoa*) ranged from 55.09 to 64.07 %, while for API (*Chenopodium album)* ranged from 47.41 to 57.34 % and emulsion activity increased with increasing pH. The greatest emulsion activity for QPI and API was 64.70 percent and 57.34 percent at pH 11 while the lowest values were 55.09 percent and 47.41 percent at pH 3. The particle size, surface charge, electrostatic hindrance, and particle flexibility all have a significant impact on the emulsification property. Aside from that, non-polar regions on the surface of protein molecules have been revealed to be significant for protein adsorption at the water-oil interface during emulsification.

2.5.3 Foaming capacity

Foaming capacity is determined by the ability of soluble proteins to diffuse toward the air-water interface, as well as fast molecular modification and rearrangement at the interface. As a result, flexible protein molecules can foam well while globular proteins have a low foaming capacity due to the difficulty of surface dissociation (Çelik et al., 2019). Zhang et al., (2020) observed that the foaming capacity (FC) of rapeseed protein decreased with increasing pH, most likely as a result of reduced flexibility and capacity to construct effective surface membranes. At pH 3, rapeseed protein isolates had the maximum foaming capability and foaming stability. As pH falls, net charges on the protein reduce links between protein side-chains, lowering hydrophobic interactions while increasing protein flexibility and therefore foaming ability. Ge et

al., (2021) studied the foaming capacity (FC) and protein stability (FS) of Chinese beans at pH 3.0, 5.0, 7.0, and 9.0. Because of their decreased solubility at isoelectric pH, all proteins showed the lowest foaming characteristics at pH 5.0. However, the foaming stability of protein increased at pH 7 and pH 9 as greater protein solubility improved foaming stability. A protein with high foaming stability represents a greater interfacial film of adsorbed proteins. As a result, the protein has higher visco-elasticity in general, which may result in more stable foam. Yuliana et al., (2014) recorded a minimum foaming capacity (FC) of 28.65% at pH 3 due to the protein structure at the isoelectric point. FC ranging from 32.34% to 90.01% was observed at pH above the isoelectric point.

Ahmed et al., (2011) investigated the effect of pH on the functional characteristics of different bean flours. At pH 4.0, the chosen legume flours exhibited minimum FC values of 26%, 25%, 20%, and 24% for cowpea, phaseolus, pigeon pea, and hyacinth bean flours, respectively. At pH 12.0, the maximum FC was observed for phaseolus (84%), pigeon pea (70%), cowpea (94%), and hyacinth bean (90%) flours. Some protein isolates' higher foaming capacity (FC) might be due to greater solubility, quick unfolding at the air-water interface, low interfacial cohesion, and the extensibility of protein colloidal particles. Majzoobi & Abedi, (2014) discovered that increasing the pH from 3 to 9 increased the foam volume of gluten protein from 46 to 92 percent. Pham et al., (2017) studied the impact of pH on the functional characteristics of pumpkin seed protein extracts. The lowest foaming capacity (FC) was recorded at pH 3, 4, and 5 for albumin, globulin, and glutelin respectively. However, the FC for protein concentrates significantly improved when the pH was reduced from 3 to 2 or increased from 5 to 10. The maximum foaming capacity (FC) of albumin, globulin and glutelin was obtained at pH 10. High pH raises the net charge of proteins, limiting hydrophobic interactions and increasing protein flexibility. This effect may increase protein dispersion and modification at the air-water interface, allowing for the encapsulation of air bubbles. As a result, foaming capacity (FC) improves. Pham et al., (2017) also studied the effect of pH on foaming stability (FS) of protein concentrates. The protein extract has high foaming stability (FS) in the isoelectric zone (pH 3-5). At pH 4, globulin, glutelin and albumin had the highest FS. As the pH declines from 3 to 2 or raises from 5 to 10, the FS reduced significantly. The proteins' FS was maximized near the isoelectric point due to the lack of attraction and repulsion interactions. Esmaeili et al., (2016) observed the functionality of rice bran protein at various pH values. The foaming capacity of

Shiroodi and Tarom rice bran protein (RBP) improved gradually as the pH was raised from 5.0 to 8.0. At pH 5, the minimum foaming capacity of Shiroodi and Tarom RBP was observed to be 70% and 78.3% respectively and the highest foaming capacity of Shiroodi and Tarom RBP was observed at pH 8.

As per the review, it was observed that the foaming capacity increases in highly acidic and alkaline pH due to the high net charge on protein molecule which reduces hydrophobic groups and increases the solubility of protein with water and air. However, foaming stability was observed higher near the isoelectric point because the charge on the protein surface becomes neutral causing minimum activity and disturbance to the foam.

2.5.4 Water holding capacity (WHC) and oil holding capacity (OHC)

The protein's water holding capacity is considered to be a measure of its ability to effectively retain water. Many foods' sensory qualities are influenced by the interactions of protein and lipids. Proteins with poor solubility and high hydrophobic groups can absorb a lot of oil (Çelik et al., 2019). Ge et al., (2021) reported that oil holding capacity (OHC) and water holding capacity (WHC) of protein fractions of Chinese beans were found to be pH-dependent. At pH 5, protein fractions showed the lowest water holding and oil holding capacity values. Acid and neutral pH values had a substantially stronger influence on oil holding capacity than water holding capacity. The results indicated that the OHC values at pH 3.0 and 7.0 were substantially greater than the WHC values for all protein fractions. Under the changing pH conditions, the protein's surface exposes more hydrophobic groups, which may combine with oil molecules to boost the oil absorption ability of protein isolates. Yuliana et al., (2014) studied the influence of pH on the water-holding ability of protein isolate of defatted cashew nut. Since proteins accumulate at and near the isoelectric point which decreases protein interaction with water, the water-absorbing capacity of protein isolates was minimum at pH 3, while WAC increased with an increase in pH from 3 to 8. In a study done by (Sodini et al., 2006), water holding capacity was substantially higher (44%)in yogurts supplemented with whey protein concentrate (WPC) obtained from whey at pH 6.4 than in yogurts supplemented with WPC derived from whey at pH value 5.8 (39%). Before heat treatment, a higher acidic pH contributes to a more heterogeneous covering of the caseins by the deactivated whey protein. A more unequal covering of casein micelles may allow for an even more open structure of the gel matrix, allowing for greater water

loss during centrifugation. Majzoobi & Abedi, (2014) observed that at pH 6 the water-holding capability of gluten protein was 1.2 (g H2 O/g sample). These values considerably increased to 2.8 and 3.2 (g H2 O/g sample) at pH 9 and 3, respectively.

Pham et al., (2017) studied the effects of pH on the functional properties of pumpkin seed protein fractions. At pH values 4 and 5, globulin, albumin, and glutelin had the lowest WAC. The best WAC was demonstrated for globulin and glutelin at pH 2, while the albumin had the highest WAC at pH 10. When the pH of the protein concentrates was elevated from 5 to 10 or dropped from 4 to 2, the WAC rose. This is because changes in pH influence both the net charge and the distribution of oppositely charged ions on the protein surface. Peng et al., (2020) studied the influence of pH on the functional properties of mildly fractionated soy protein. At pH 4.5 the WHC was recorded lowest and the WHC increased when the pH was changed from more basic and acidic. This is because the total charge on the soy protein is near zero when the pH is in the isoelectric zone. Protein-protein interactions are at their peak at this point, and fewer water-binding structures are exposed. At pH 7.5 highest WHC was recorded because the polarity of protein increases as its net charge increases, increasing bound water. Mir et al., (2019) studied the effect of pH on the characteristics of protein extracted from *Chenopodium* seeds. They demonstrated that the water binding ability of QPI ((*Chenopodiumquinoa)* and API (*Chenopodium album)* protein extract significantly enhanced with increasing pH. The highest water-binding capacity of API (205.27 %) and QPI (174.90 %) was reported at pH value 11, while the poorest values of QPI (124.27%) and API (110.40%) were found at pH 3.

It was discussed that WHC and OHC are solubility dependant, and their solubility changes with pH. Because of the high solubility, the WHC is high and the OHC is low. As a result, a rise in alkaline pH results in improved WHC because hydrophilic linkages are exposed and store more water. While OHC is greater towards the isoelectric zone because solubility is lowest and there are more hydrophobic groups present.

2.5.5 Gelling capacity

The gelling properties are essential in various food processing industries for the production of foods like yogurt, ice cream, soups, etc. It also implies that during the thermal process, the stabilized structure of proteins undergoes intermolecular interactions and forms a new bond which induces gel formation. Das et al., (2021) studied the effect of pH treatment on

the gelling capacity of amaranth (*Amaranthus hypochondriacus*) seed protein isolates. The gelation behavior was observed higher at pH 9, 10, and 11. Because of the presence of several non-protein molecules, pH 12 exhibited a decreased gel-forming ability. The presence of excellent protein molecules, which improve gelation by mixing a high number of protein structures, may be related to increased gelation behavior at pH 9, 10, and 11. Naik et al., (2022) extracted bitter melon seed protein at alkaline pH 9 and precipitated the extract at pH 4. They evaluated the least gelling capacity and discovered that gel was formed at a concentration of 7.5 (w/v) and above.

Pham et al., (2017) observed that the least gelling capacity was recorded between pH 4-6 while higher gelling capacity was recorded at pH 3 and from pH 7-10 because of increased repulsive forces among protein molecules and higher net charge of the protein molecules. It was thus observed that the gelation is concerned with the solubility of protein which is higher at alkaline pH. The high gelling capacity was obtained at high alkaline pH.

2.6 Nutritional profile of protein extracted through pH shift method

According to certain studies, the amino acid content of food proteins, especially the number of essential amino acids, has a strong relationship with their nutritive value.

pH shift treatment is a non-thermal extraction protein thus it protects the amino acid profile which can be degraded at high temperatures. However, the highly alkaline condition is found to have adverse effects on the nutritional properties of the extracted protein, such as degraded amino acids and low digestibility. Also, extremely alkaline conditions reduce the purity of the protein extract as phenol oxidizes and binds with the proteins under alkaline conditions (Fetzer et al., 2019; Vahedifar & Wu, 2022). When protein is precipitated at low pH phytate-protein electro-static complexes are formed. Since the isoelectric point differs among the extracted proteins, fewer fractions of protein are recovered at a specific isoelectric point (Vahedifar & Wu, 2022). Many studies have reported that high alkaline and acidic conditions can damage the amino acid profile and can cause the production of toxic compounds. Protein changes are the most important chemical reactions that take place during alkaline treatment. Under alkali conditions, free or protein-bound L-amino acids are converted into their mirror images (enantiomers), known as D-amino acids and other reactions (e.g., protein cross-linking) may occur, causing the amino acids to racemize (Yang et al., 2012). This phenomenon is explained in

figure 2.2. At high pH, L-amino acid residues racemize to D-isomers, resulting in the generation of cross-linked organic molecules like lysinoalanine. These protein reactions can lower the amount of essential L-amino acids in foods, thus lowering their nutritional quality and reliability. Protein digestibility and biocompatibility is also reduced, and toxic byproducts such as lysinoalanine are produced (Souza et al., 2016).When exposed to pH 12 for 5 minutes at room temperature, the amount of lysinoalanine in native hemp protein was 0.81 mg/100 g protein; the lysinoalanine concentration remained stable when subjected to pH 12 for 5 minutes at room temperature. However, when it was kept for 60 minutes at pH 12 and the temperature was maintained at 50ºC, the concentration increased to 57 mg/100 g protein. Thus, temperature and incubation time proves to be an important variable in the amount of lysinoalanine that can be produced during pH treatment (Jiang et al., 2018).

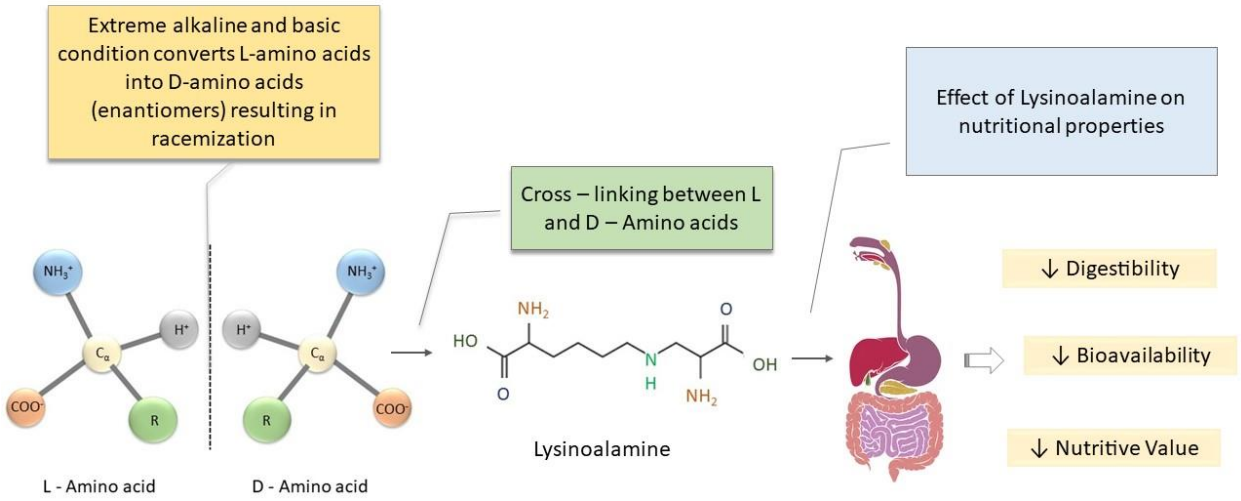

Fig 2.2 Racemization of amino acids in high alkaline and acidic conditions

Rice protein extracted with 0.3 percent NaOH had lower cysteine and arginine content than rice protein isolated with 0.1 percent and 0.2 percent NaOH at ambient temperature. Thus, it was discovered that the extent of protein denaturation is concerned with the alkaline solutions used in the extraction process, and the use of cold temperatures should be suggested when minimizing the negative impacts of the alkaline medium on food proteins (Yang et al., 2012).

Mir et al., (2019) studied the effect of pH on the characteristics of protein extracts from two varieties of *Chenopodium* seeds. They discovered that *Chenopodium* is abundant in essential amino acids, whereas methionine and cysteine are deficient in both varieties of *Chenopodium.* Because albumins are hydrophilic and high in sulfur-containing amino acids, the bulk of them eliminated during flour washing before alkali treatment. QPI (*Chenopodium quinoa)* and API (*Chenopodium album)* had a significant amount of aspartic acid and glutamic acid, indicating that these protein extracts have acidic properties. The current study also found higher levels of leucine (67.0, 67.2 percent), valine (41.9, 41.0 percent), and threonine (40.9, 40 percent) for QPI and API, respectively. The effect of different pH values on the amino acid profile of extracted protein is shown in table 2.2.

Table 2.2: Impact of pH on amino acid profile

Sample	Variables	Amino acid profile (g/100g)									References
		His	Leu	Ile	Lys	Met	Phe	Thr	Trp	Val	
Bitter melon seed	Alkaline pH-9 Acidic pH – 4	2.30	0.90	2.0	0.16	0.20	0.0	0.80	-	0.30	(Naik et al., 2022)
Pea protein	Alkaline pH-9.6 Acidic pH-4.5	2.1	6.4	4.0	5.9	0.75	4.0	2.80		4.36	(Wang et al., 2020)
Sea buckthorn seed	Alkaline pH-11 Acidic pH- 5	2.31	6.92	3.48	2.97	0.8	3.38	2.61		3.83	(Lin et al., 2022)
Sesame seed	Alkaline pH-8.68 Acidic pH-5.32	3.46	9.04	5.92	3.59	-	-	5.04	1.23	6.91	(Sá et al., 2022)

Tunisian black cumin seeds	Alkaline pH-9,10,11 Acidic pH-4.5	1.28	3.10	3.64	1.90	0.87	-	2.09	-	2.26	(Trigui et al., 2021)
Turkish black cumin seeds	Alkaline pH-9,10,11 Acidic pH-4.5	1.22	3.34	2.09	1.96	0.77	-	1.91	-	2.73	(Trigui et al., 2021)
Unprocessed alfalfa protein isolates	Alkaline pH-10 Acidic pH- 4	5.62	7.86	6.72	6.58	5.03	3.07	4.81	5.66	4.99	(Sahni et al., 2020)
Processed alfalfa protein isolates	Alkaline pH-10 Acidic pH- 4	5.76	9.08	6.86	6.38	5.04	3.47	4.48	4.49	5.28	(Sahni et al., 2020)

Chapter 3

MATERIAL AND METHODS

This chapter comprises of all the details related to the materials and methods which were used for the isolation of protein from defatted mustard meal (DFF). The present study entitled "Optimization of pH for *Brassica juncea* meal protein extraction using Response Surface methodology" was conducted in food processing lab of Department of Bioengineering, Integral University, Lucknow.

3.1 Procurement of raw material

Defatted mustard *(Brassica juncea L*) meal was obtained from local oil pressing machinery of Lucknow. The composition of DMM is given in table.

3.2 Chemicals and equipments used

1. Sodium hydroxide, 35% Hydrochloric acid and distilled water was procured from the department of Bioengineering.

2. Sieves (standard test sieve, as per ISS: 460–1962) of different mesh sizes range of 150, 375, and 600 µm.

3. pH meter

4. Homogenizer

5. Centrifuge

3.3 Preparation of sample

Defatted mustard meal was obtained after oil extraction from mustard seeds. Mustard meal was cleaned and grounded to obtain the flour of particle size under the range of 150 µm, 375 µm, 600 µm as shown in figure 3.1. The grounded meal was packed in HDPE polybags and stored at -18 degree Celsius.

a.  b. 

Fig 3.1 a. Mustard meal b. Grounded mustard meal

3.4 Proximate analysis

3.4.1 Moisture content

Moisture content of defatted mustard meal was determined by the (AOAC, 2000) method. 1g of mustard meal was uniformly grounded and kept in hot air oven at 105 ºC for 3 hours. The dried meal was cooled in desiccator and weighed for the final reading. The moisture content (wet basis) in percentage was determined gravimetrically, where the difference of sample weight before and after the drying was divided by the sample weight given in equation below

$$Moisture\ content\ (\%) = \frac{W1 - W2}{W1} \times 100$$

Where,

W1= Weight (g) of sample before drying

W2= Weight (g) of sample after drying

3.4.2 Fat content

The fat content of mustard meal was determined using Soxhlet apparatus by (AOAC, 2000) method. 3g of mustard meal was placed in thimble. The hexane (200ml/sample) was used as an extraction solvent and the sample was heated for around 14 hours. Following the extraction of fat, the round bottom flask comprising of extracted fat was placed in a 70 °C oven for 1 hour so that solvent should evaporate. The fat content was determined by the difference in weight of the round bottom flask before and after the extraction of fat as shown below

$$Fat\ content\ (\%) = \frac{a-b}{c} \times 100$$

Where,

a= weight of flask after oil extraction (g)

b= weight of empty flask (g)

c= weight of sample (g)

3.4.3 Ash content

The ash content of mustard meal was determined by the (AOAC, 2000) method. 2 g of mustard meal was weighed in a pre-weighed crucible and placed in the muffle furnace at 550°C for 5 hours. The sample was cooled in desiccator and the final weight was recorded. The calculation is given below

$$Ash\ content(\%) = \frac{weigt\ of\ ash(g)}{weight\ of\ sample(g)} \times 100$$

3.4.4 Protein content

1 g of sample was digested in a digestion flask using 10ml H_2SO_4, Cu2SO4 and K2SO4 catalyst at the ratio of 1:5. When the solution is completely digested and clear green color appears, sodium hydroxide solution (40 %) is added to neutralize the solution. Then distillation is performed in which ammonia gas releases and is trapped in sulfuric acid solution. The nitrogen content which can be converted into protein content by multiplying it with Kjeldahl factor 6.25 for protein content determination in mustard meal,

3.5 Isolation of mustard meal protein

The extraction of mustard meal protein was done using the method of (Jahan et al., 2022) with a few changes. Ground mustard meal was mixed with deionized water in a ratio of 1:10 and left for one hour, allowing cellular components to be dissolved in aqueous solution. The pH of the solution was adjusted to 9, 10 and 11 using 1N NaOH. After that solution is centrifuged at 5000 rpm for 15 min. the supernatant was collected carefully and the PH of supernatant is adjusted to 3, 4 and 5 using 1M HCl. The supernatant is centrifuged again at 5000 rpm for 15 min and the protein extract was collected in the form of precipitate. The precipitate was washed with deionized water and the pH was readjusted to neutral using 1N NaOH. The precipitate is

dried at room temperature and dried protein extract was stored at -20 ºC for further analysis. The schematic representation of isolation process is given in figure 3.2.

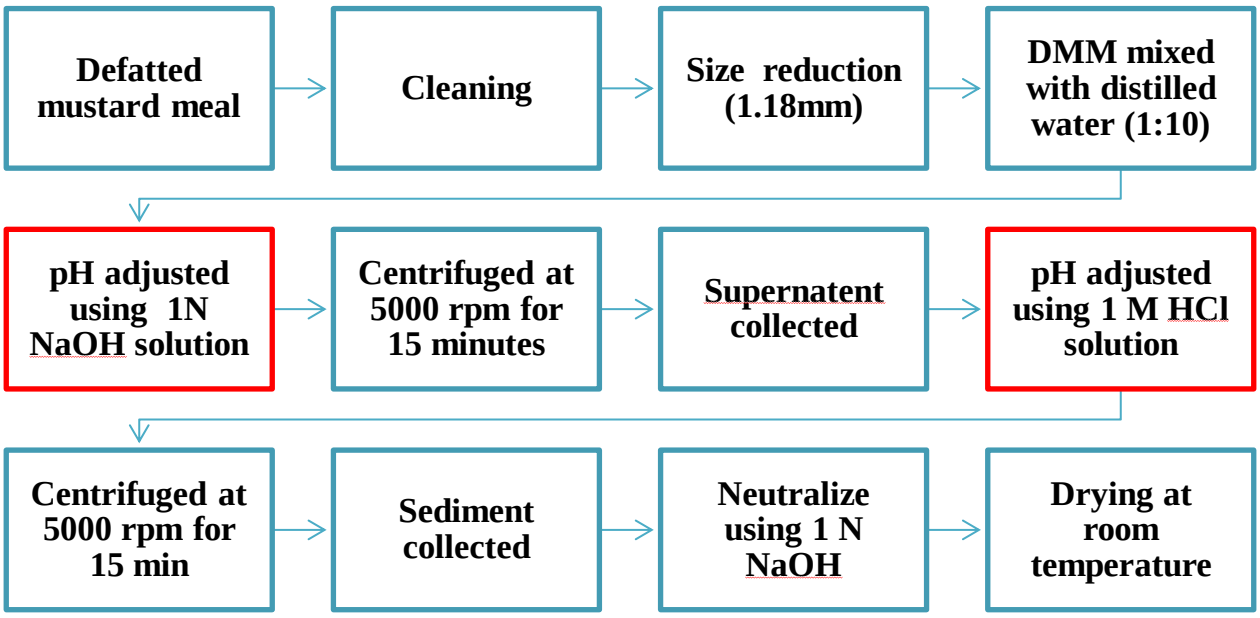

Fig 3.2 Schematic representation of protein isolation from defatted mustard meal

3.6 Determination of extraction yield

The extraction yield of protein was determined using the method of (Jahan et al., 2022) . The protein yield was measured by dividing the weight of the protein isolate produced by the amount of protein originally present in the mustard meal as shown in following equation

$$\text{Protein yield } \% = \frac{\text{Extracted protein (g)}}{\text{Total protein in sample(g)}} \times 100$$

3.7 Functional properties of Defatted mustard meal protein

3.7.1 Emulsification property

Emulsification property of defatted mustard meal was determined using the method of (Malik et al., 2017). 5 mL of 2% protein solution was added in 5 mL of soybean oil. The solution's pH was adjusted to alkaline and then it was homogenized. The emulsion was

centrifuged for 5 minutes at 3500 rpm. The height of the emulsified layer and the total contents of the tube were measured. The emulsifying activity was calculated using the following formula

$$\text{EA\%} = \frac{\text{Height of emulsified layer (ml)}}{\text{Height of total content in the tube (ml)}} \times 100$$

3.7.2 Foaming ability

The foaming ability of defatted mustard meal was determined using the method of (Malik et al., 2017). 50 ml of 3% protein solution was whipped by shaking the solution vigorously for 1 minute. The height of froth formed was noted immediately. The percentage of foaming ability was calculated using formula given below

$$\text{FA\%} = \frac{\text{Volume after whipping – volume before whipping (ml)}}{\text{Volume before whipping (ml)}} \times 100$$

3.7.3 Water holding capacity

The water holding capacity of defatted mustard meal was determined using the method of (Malik et al., 2017). One gram of protein isolate was combined with 10 ml of distilled water. The mixture was allowed to stand for 30 minutes before being centrifuged (4500 rpm for 30 min). After discarding the supernatant, the tubes were inverted at 45° for 25 minutes to drain the remaining liquid from the protein sediment. The water holding capacity was calculated as shown in equation given below

$$\text{WHC \%} = \frac{\text{a}-\text{b}}{\text{c}} \times 100$$

Where

a = mass of the tube with the protein isolate and absorbed water

b = mass of the tube and protein isolate.

C= mass of the protein isolate

3.7.4 Oil holding capacity

The oil holding capacity of defatted mustard meal was determined using the method of (Malik et al., 2017). One gram of protein isolate was combined with 10 ml of soybean oil. The

mixture was allowed to stand for 30 minutes before being centrifuged (4500 rpm for 30 min). After discarding the supernatant, the tubes were inverted at 45° for 25 minutes to drain the remaining liquid from the protein sediment. The oil holding capacity was calculated as given below

$$\text{OHC } \% = \frac{a - b}{c} \times 100$$

Where

a = mass of the tube with the protein isolate and absorbed oil

b = mass of the tube and protein isolate.

C= mass of the protein isolate

3.8 Box-Behnken design

Optimization of the proteins extraction from mustard meal was performed with the aid of Design Expert V 13, (Stat-Ease Inc, USA) Software. Box Behnken design was employed for assessing the influence of soluble pH (SpH) (9,10,11), insoluble pH (IpH) (3,4,5) and particle size on the proteins extraction yield and functional properties from defatted mustard meal. Protein yield was estimated as the response variables. Table 3.1 represents the values of independent variables, and table 3.2 represents their levels. The whole experimental design consisted of 17 runs that included five replicates at the central point conducted in a randomized manner to decrease chances of unpredicted variations. The data of protein yield depend on A, B and C (independent variables) was evaluated by using quadratic model.

Table 3.1 Independent variables and their levels

S.No.	Factors	Symbols	Levels		
			-1	0	+1
1.	SpH	A	9	10	11
2.	IpH	B	3	4	5
3.	Particle size	C	150	375	600

Table 3.2 Box-Behnken design for pH and particle size

Std	Run	Factors		
		A:SpH	B:IpH	C:Particle size
3	1	9	5	375
7	2	9	4	600
15	3	10	4	375
2	4	11	3	375
14	5	10	4	375
4	6	11	5	375
10	7	10	5	150
8	8	11	4	600
9	9	10	3	150
5	10	9	4	150
6	11	11	4	150
11	12	10	3	600
1	13	9	3	375
12	14	10	5	600
17	15	10	4	375
16	16	10	4	375
13	17	10	4	375

3.9 Physical and structural properties of defatted mustard meal

3.9.1 Viscosity

The viscosity of protein isolates were determined using Ostwald capillary viscometer. First distilled water (DW) is filled in bulb 1 of viscometer and the DW is sucked up to bulb 2 using a rubber tube. The DW is allowed to free flow and the time taken by the DW to reach from upper mark to the lower mark is noted for calculation as shown below. Same procedure is followed by using 1% protein solution and time is noted for calculation. The density of protein

solution ($\rho 2$) is determined using specific gravity bottle as given below and the standard value of density of water ($\rho 1$) is 0.997g/ml.

$$\rho 2 = \frac{W3-W1}{W2-W1}$$

Where,

W1= Weight of empty specific gravity bottle

W2= Weight of specific gravity bottle + DW

W3= Weight of specific gravity bottle + sample liquid (protein solution)

$$\mu 2 = \frac{\rho 2\ t2}{\rho 1\ t1} \times \mu 1$$

Where,

$\rho 1$ = Density of water (g/ml)

$\rho 2$ = Density of protein solution (g/ml)

$\mu 1$ = viscosity of water (cp)

$\mu 2$ = viscosity of protein solution (cp)

t1 = mean time of flow of water from mark A to B on bulb 2

t2 = mean time of flow of protein solution from mark A to B on bulb 2

3.9.2 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE mustard protein isolates were determined using the method of [19] using EPS301 electrophoresis apparatus (GE Healthcare Laboratories, Richmond, CA, USA) and Mini VE electrophoresis tank (Amersham Biosciences Company, USA). Polyacrylamide at 12 % was used as resolving gel and acrylamide at 5 % was used as stacking gel. 10 mg of samples were mixed with 100 µl of sample buffer (0.0625 M Tris154 HCl, 10% glycerin, 2% SDS, and 5% 2-β-mercaptoethanol, 0.0025% bromophenol blue) and were heated in a boiling water bath for 5 min to denature all the proteins. The samples were then promptly chilled using ice. Wells were

loaded with 10 l of sample and ran at an 80 V voltage. When the samples reached the resolving gel, the voltage was raised to 120 V and the experiment was repeated until the tracking dye reached the bottom of the gel. Gels were removed and stained for about 2 hours with Coomassie Brilliant Blue R-250 stain solution (GE Healthcare Laboratories), then destained with a solution of 20% methanol and 10% acetic acid until a clean background was attained. For determining the molecular weights of each band, a 3B prestained protein ladder containing proteins ranging in size from 3.5-245 kDa was utilised as a benchmark. The gel was scanned using the Alpha Ease FC gel imaging equipment (Alpha 164 Inc., USA).

3.9.3 Fourier transforms infrared spectrometer (FT-IR)

The Fourier Transform Infrared Spectrophotometer (FT-IR) is used for detecting the functional groups present in protein extract. 10 mg of dried extract was encapsulated in 100 mg of KBr pellet to make transparent sample discs. The spectra were collected using a Perkin Elmer Fourier transform infrared spectrometer (FTIR) in the wave number range of 400– 4000 cm^{-1} (Jahan et al., 2022).

Chapter 4

RESULT AND DISCUSSION

4.1 Proximate analysis of mustard meal

The proximate composition of defatted mustard meal on dry basis is shown in the table 5. The mustard meal had a protein content of 23.15 g/100 g which is considered adequate for protein extraction.

4.2 Model Fitting

RSM was used to optimize protein yield and functional properties of mustard meal. The influence of SpH, IpH and particle size, were investigated with Box-Behnken design. The result of protein yield (%), WHC (g/g), OHC (g/g), FC (%), EC (%) and viscosity (cp) obtained after 17 runs is presented in table 4.1 . From table 4.1 it can be observed that the protein yield ranged from 3.1 % to 15.4%, WHC ranged from 0.62 to 2.044 (g/g), OHC ranged from 1.99 to 3.13(g/g), FC ranged from 3% to 14.5%, EC ranged from 27% to 63.9% and viscosity ranged from 0.72 to 0.95 (cp).

Table 4.1 Box-Behnken design with experimental value from pH shift method

		Factors			Responses					
Std	Run	A:SpH	B:IpH	C:Particle size	Yield %	WHC g/g	OHC g/g	FC %	EC %	Viscosity cP
3	1	9	5	375	5.15	0.59	2.98	6.87	48.1	0.94
7	2	9	4	600	6.5	0.62	3.2	3.75	37.3	0.813
15	3	10	4	375	7.2	0.87	3	10	56.1	0.85
2	4	11	3	375	11.35	2.24	2.79	14.54	57.9	0.86
14	5	10	4	375	7.2	0.952	3	10	56.8	0.85
4	6	11	5	375	11.6	1.38	2.02	15.36	71.4	0.85
10	7	10	5	150	6.2	0.99	2.65	13.33	45.8	0.91

8	8	11	4	600	10	1.49	2.64	12.34	62.1	0.85
9	9	10	3	150	4.95	1.83	3.1	12.2	33.2	0.81
5	10	9	4	150	7	0.65	3.14	4.1	38.1	0.82
6	11	11	4	150	15.4	1.59	2.74	12.66	63.9	0.88
11	12	10	3	600	3.1	1.88	3.01	11.8	34	0.76
1	13	9	3	375	4.2	1.25	2.92	5.84	37.6	0.71
12	14	10	5	600	4.45	0.958	2.69	13	45	0.88
17	15	10	4	375	7.2	0.952	3.3	10.4	56.3	0.88
16	16	10	4	375	7.3	0.898	3.1	10	56.5	0.87
13	17	10	4	375	7.4	0.995	3	10.2	55.9	0.81

4.3 Statistical Analysis of the responses

4.3.1Extraction yield:

The feasibility of the model for the extraction yield was tested using partial sum of squares techniques on the response surface quadratic model. The yield value of extracted protein ranged from 15.4% to 3.1%. The maximum yield value (15.4 %) was obtained for 150 µm particle size via the combination of 11 SpH and 4 IpH, from pH shift treatment. While the minimum yield value (3.1%) was obtained for 600 µm particle size via the combination of 10 SpH and 3 IpH. Protein yield at this combination was similar to the extraction of protein isolates at pH combination of 9-12 from *Chenopodium* seeds in which the yield ranged from 8.2-12.22 % for QPI (*Chenopodium quinoa*) and 7.71-10.98 % for API (*Chenopodium album*) (Mir et al., 2019).

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on protein yield by ANOVA is shown in Table 4.2. The Model F-value of 92.01 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AC, A^2, B^2 are significant model terms.

Table 4.2 ANOVA showing the variables as a linear, quadratic and interaction terms on protein yield of defatted mustard meal

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	153.73	9	17.08	92.01	< 0.0001	significant
A-SpH	81.28	1	81.28	437.84	< 0.0001	
B-IpH	1.81	1	1.81	9.72	0.0169	
C-Particle size	11.28	1	11.28	60.77	0.0001	
AB	0.1225	1	0.1225	0.6599	0.4434	
AC	6.00	1	6.00	32.33	0.0007	
BC	0.0025	1	0.0025	0.0135	0.9109	
A^2	36.21	1	36.21	195.04	< 0.0001	
B^2	18.88	1	18.88	101.70	< 0.0001	
C^2	0.9202	1	0.9202	4.96	0.0613	
Residual	1.30	7	0.1856			
Lack of Fit	1.27	3	0.4225	52.81	0.0011	significant
Pure Error	0.0320	4	0.0080			
Cor Total	155.03	16				

Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 52.81 implies the Lack of Fit is significant. There is only a 0.11% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R^2 of 0.8689 is in reasonable agreement with the Adjusted R^2 of 0.9808. This demonstrated that the generated models could accurately depict the actual relationship between the parameters.

A second order polynomial equation was developed representing an empirical relationship between the response (Extract Yield) and the independent variables viz. SpH (A), IpH(B) and particle size(C). The equation for extract yield is given below:

Yield (%)= 7.26 + 3.1875 * A + 0.475 * B + -1.1875 * C + -0.175 * AB + -1.225 * AC + 0.025 * BC + 2.9325 * A^2 + -2.1175 * B^2 + -0.4675 * C^2

Figure 4.1 shows the combined effect of factors on the mustard meal protein extraction at the central point of the experimental design. The extraction yield was drawn by changing only one factor while others were kept constant. It was observed that all the factors had a significant effect on the extraction yield of protein. The relatively flat line of factor C, as per the plot, indicated lower effect on the extraction yield. Factor A (SpH) has the most prominent curve, indicating its significance in the extraction. Then came factor B (IpH), which also had a good influence on protein yield. In comparison to A and B, factor C (particle size) showed a relatively flat line but had a considerable influence on protein yield.

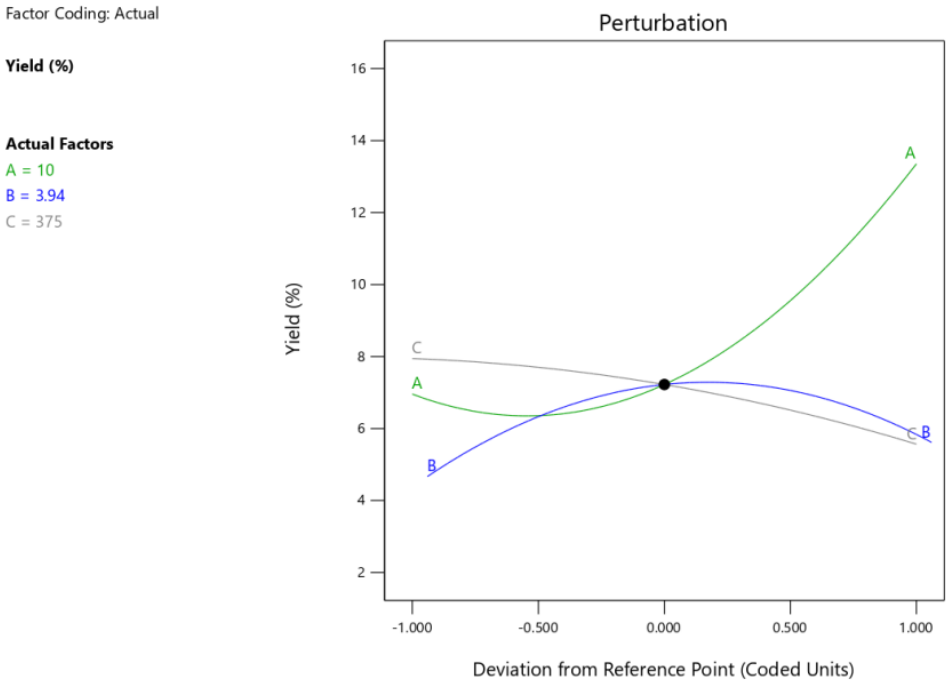

Fig 4.1 Perturbation plot for yield% of mustard meal protein isolates (A: SpH, B: IpH, C: particle size)

4.3.2 Water holding capacity (WHC)

The effectiveness of the model for the WHC was tested using partial sum of squares techniques on the response surface quadratic model. The WHC value of extracted protein ranged from 2.24 (g/g) to 0.59 (g/g). The maximum WHC value (2.24 g/g) was obtained for 375 µm particle size via the combination of 11 SpH and 3 IpH, from pH shift treatment which was higher

than the WHC of whey protein concentrate obtained at pH 6.4 (Majzoobi & Abedi, 2014). While the minimum WHC value (0.59 g/g) was obtained for 375 µm particle size via the combination of 9 SpH and 5 IpH. This can be due to the reason that protein-protein interactions are high at lower pH values, and fewer water-binding structures are exposed while an increase in alkaline pH improves WHC because hydrophilic bonds are exposed and can store more water (Peng et al., 2020).

Table 4.3 ANOVA showing the variables as a linear, quadratic and interaction terms on WHC of defatted mustard meal

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.67	9	0.4074	143.09	< 0.0001	significant
A-SpH	1.61	1	1.61	565.83	< 0.0001	
B-IpH	1.35	1	1.35	472.90	< 0.0001	
C-Particle size	0.0016	1	0.0016	0.5507	0.4822	
AB	0.0100	1	0.0100	3.51	0.1031	
AC	0.0012	1	0.0012	0.4303	0.5328	
BC	0.0017	1	0.0017	0.5904	0.4674	
A^2	0.0115	1	0.0115	4.05	0.0842	
B^2	0.6058	1	0.6058	212.76	< 0.0001	
C^2	0.0436	1	0.0436	15.33	0.0058	
Residual	0.0199	7	0.0028			
Lack of Fit	0.0102	3	0.0034	1.39	0.3677	not significant
Pure Error	0.0098	4	0.0024			
Cor Total	3.69	16				

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on WHC by ANOVA is shown in Table 4.3. The Model F-value of 143.09 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The Lack of Fit F-value of 1.39 implies the Lack of Fit is not significant relative to the pure error. There is a 36.77% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R^2 of 0.9517 is in reasonable agreement with the Adjusted R^2 of 0.9876; i.e. the difference is less than 0.2. This demonstrated that the generated models could accurately depict the actual relationship between the parameters.

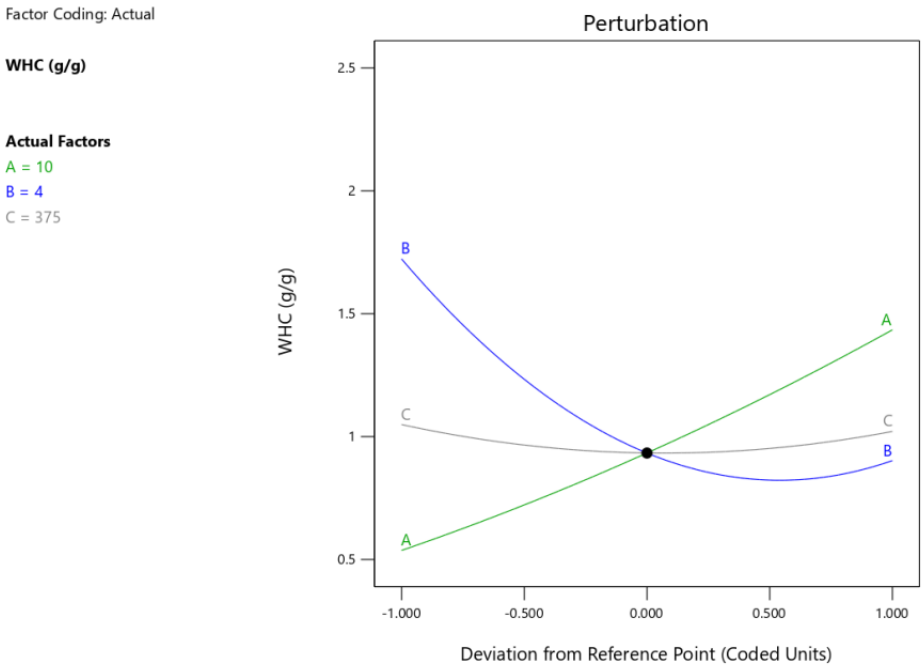

Fig 4.2 Perturbation plot for WHC (g/g) of mustard meal protein isolates (A: SpH, B: IpH, C: particle size)

A second order polynomial equation was developed representing an empirical relationship between the response (WHC) and the independent variables viz. SpH (A), IpH(B) and particle size(C). The equation for WHC is given below:

WHC (g/g) = 0.9334 + 0.44875 * A + -0.41025 * B + -0.014 * C + -0.05 * AB + -0.0175 * AC + -0.0205 * BC + 0.0523 * A^2 + 0.3793 * B^2 + 0.1018 * C^2

Figure 4.2 shows the combined effect of factors on the WHC of mustard meal protein isolates at the central point of the experimental design. The water holding capacity was drawn by changing only one factor while others were kept constant. It was observed that all the factors had a significant effect on the WHC of protein. The line of factor A, as per the plot, indicated that WHC increased with the increasing IpH. It was also observed that WHC decreased drastically

when IpH was increased. Then came factor C (particle size), which showed minimum influence on WHC.

4.3.3 Oil holding capacity (OHC)

The feasibility of the model for the OHC was tested using partial sum of squares techniques on the response surface quadratic model. The OHC value of extracted protein ranged from 3.14 (g/g) to 2.02 (g/g). The maximum OHC value (3.14 g/g) was obtained for 150 µm particle size via the combination of 9 SpH and 4 IpH, from pH shift treatment which were similar to the results of (Ge et al., 2021). This can be due to the reason that the protein's surface exposes more hydrophobic groups at low alkaline pH values, which may combine with oil molecules to boost the oil absorption ability of protein isolates (Çelik et al., 2019; Ge et al., 2021). While the minimum OHC value (2.02 g/g) was obtained for 375 µm particle size via the combination of 11 SpH and 5 IpH because solubility is highest and there are more hydrophilic groups present at high alkaline pH values.

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on OHC by ANOVA are shown in Table 4.4. The Model F-value of 14.90 implies the model is significant. There is only a 0.09% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.The Lack of Fit F-value of 0.05 implies the Lack of Fit is not significant relative to the pure error. There is a 98.30% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R^2 of 0.8964 is in reasonable agreement with the Adjusted R^2 of 0.8866; i.e. the difference is less than 0.2.

A second order polynomial equation was developed representing an empirical relationship between the response (OHC) and the independent variables viz. SpH (A), IpH(B) and particle size(C). The equation for OHC is given below:

OHC (g/g) = 3.08 + -0.25625 * A + -0.185 * B + -0.01125 * C + -0.2075 * AB + -0.04 * AC + 0.0325 * BC + -0.1675 * A^2 + -0.235 * B^2 + 0.0175 * C^2

Table 4.4 ANOVA showing the variables as a linear, quadratic and interaction terms on OHC of defatted mustard meal

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.35	9	0.1502	14.90	0.0009	Significant
A-SpH	0.5253	1	0.5253	52.10	0.0002	
B-IpH	0.2738	1	0.2738	27.16	0.0012	
C-Particle size	0.0010	1	0.0010	0.1004	0.7606	
AB	0.1722	1	0.1722	17.08	0.0044	
AC	0.0064	1	0.0064	0.6348	0.4518	
BC	0.0042	1	0.0042	0.4191	0.5381	
A^2	0.1181	1	0.1181	11.72	0.0111	
B^2	0.2325	1	0.2325	23.06	0.0020	
C^2	0.0013	1	0.0013	0.1279	0.7312	
Residual	0.0706	7	0.0101			
Lack of Fit	0.0026	3	0.0009	0.0505	0.9830	not significant
Pure Error	0.0680	4	0.0170			
Cor Total	1.42	16				

Figure 4.3 shows the combined effect of factors on the OHC of mustard meal protein isolates at the central point of the experimental design. The extraction yield was drawn by changing only one factor while others were kept constant. It was studied that factor A (SpH) and factor B (IpH) was increase OHC started declining. The relatively flat line of factor C, as per the plot, indicated lower effect on the OHC. In comparison to factor A and B, factor C (particle size) showed a relatively flat line but had a considerable influence on protein's OHC.

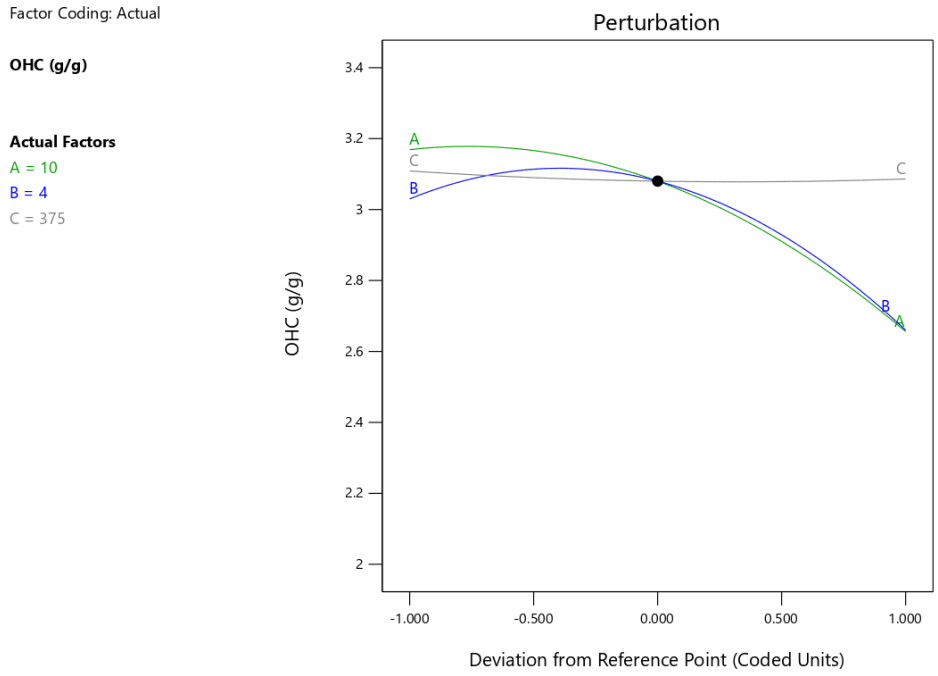

Fig 4.3 Perturbation plot for OHC (g/g) of mustard meal protein isolates (A: SpH, B: IpH, C: particle size)

4.3.4 Foaming Capacity

The feasibility of the model for the FC was tested using partial sum of squares techniques on the response surface quadratic model. The FC value of extracted protein ranged from 15.36 (%) to 4.1 (%). The maximum FC value (15.36 %) was obtained for 375 µm particle size via the combination of 11 SpH and 5 IpH, from pH shift treatment. While the minimum FC value (4.1 %) was obtained for 150 µm particle size via the combination of 9 SpH and 4 IpH. Similar results were observed by Ahmed et al., (2011) where minimum FC of bean flours were observed at pH 4 and and maximum FC was observed as the pH was increased till pH 12. Majzoobi & Abedi, (2014) also discovered that increasing the pH from 3 to 9 increased the foam volume of gluten protein because high pH raises the net charge of proteins, limiting hydrophobic interactions and increasing protein flexibility (Pham et al., 2017)

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on FC by ANOVA are shown in Table 4.5. The Model F-value of 932.03 implies the model is

significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 0.31 implies the Lack of Fit is not significant relative to the pure error. There is 82.05% chance that a Lack of Fit F-value this large could occur due to noise.

Table 4.5 ANOVA showing the variables as a linear, quadratic and interaction terms on FC of defatted mustard meal

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	188.68	9	20.96	932.03	< 0.0001	Significant
A-SpH	147.40	1	147.40	6553.39	< 0.0001	
B-IpH	2.18	1	2.18	97.10	< 0.0001	
C-Particle size	0.2450	1	0.2450	10.89	0.0131	
AB	0.0110	1	0.0110	0.4902	0.5065	
AC	0.0002	1	0.0002	0.0100	0.9231	
BC	0.0012	1	0.0012	0.0545	0.8222	
A^2	15.50	1	15.50	689.17	< 0.0001	
B^2	25.30	1	25.30	1124.78	< 0.0001	
C^2	0.0005	1	0.0005	0.0237	0.8820	
Residual	0.1574	7	0.0225			
Lack of Fit	0.0294	3	0.0098	0.3068	0.8205	not significant
Pure Error	0.1280	4	0.0320			
Cor Total	188.83	16				

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on FC by ANOVA are shown in Table 4.5. The Model F-value of 932.03 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2 are

significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 0.31 implies the Lack of Fit is not significant relative to the pure error. There is 82.05% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R^2 of 0.9964 is in reasonable agreement with the Adjusted R^2 of 0.9981; i.e. the difference is less than 0.2.

A second order polynomial equation was developed representing an empirical relationship between the response (FC) and the independent variables viz. SpH (A), IpH(B) and particle size(C). The equation for FC is given below:

FC (%) = 10.12 + 4.2925 * A + 0.5225 * B + -0.175 * C + -0.0525 * AB + 0.0075 * AC + 0.0175 * BC + -1.91875 * A^2 + 2.45125 * B^2 + 0.01125 * C^2

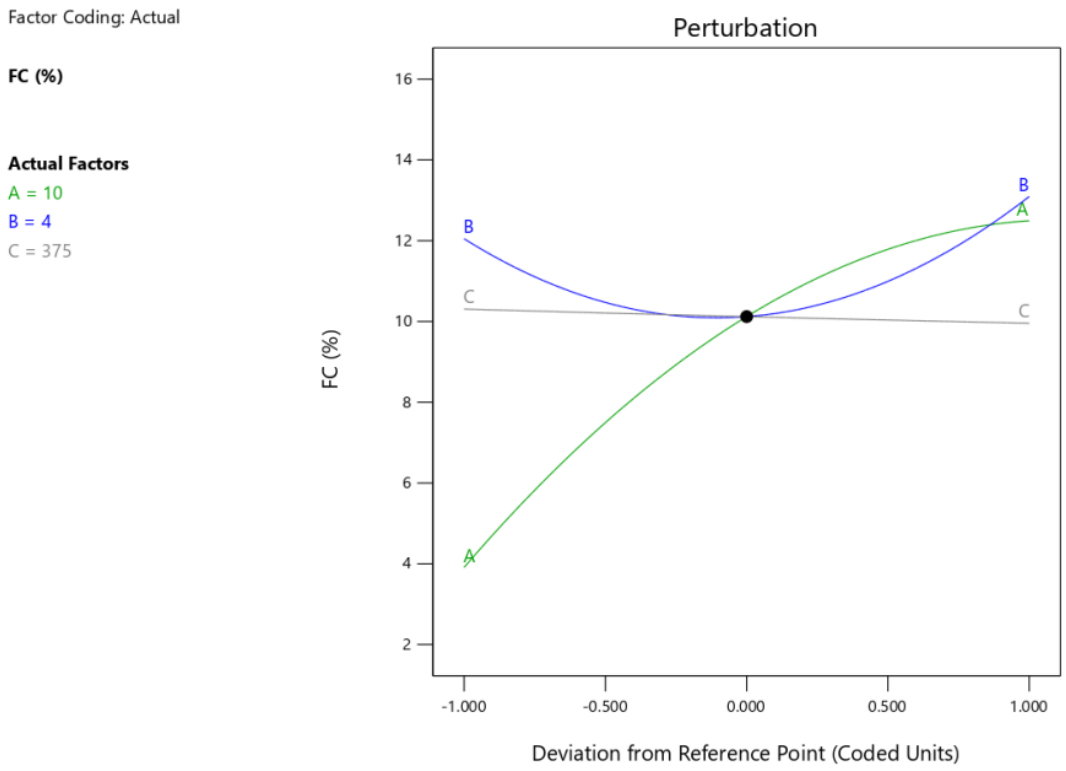

Fig 4.4 Perturbation plot for FC% of mustard meal protein isolates (A: SpH, B: IpH, C: particle size)

Figure 4.4 shows the combined effect of factors on the FC of mustard meal protein isolates at the central point of the experimental design. The extraction yield was drawn by changing only one factor while others were kept constant. It was observed that factor A and factor B had a significant effect on the FC of protein. Factor A (SpH) has the most prominent curve, indicating its significant effect on FC. Then came factor B (IpH), which also had a good influence on FC. The relatively flat line of factor C, as per the plot, indicated lower effect on the FC. In comparison to factor A and B, factor C (particle size) exhibited a reasonably flat line but had a significant impact on the FC of protein.

4.3.5 Emulsifying capacity (EC)

The feasibility of the model for the EC was tested using partial sum of squares techniques on the response surface quadratic model. The EC value of extracted protein ranged from 71.4 (%) to 33.2 (%). The maximum FC value (71.4 %) was obtained for 375 µm particle size via the combination of 11 SpH and 5 IpH, from pH shift treatment. While the minimum EC value (33.2 %) was obtained for 150 µm particle size via the combination of 10 SpH and 3 IpH. It was discovered that increasing alkaline pH has a positive impact on EC of mustard meal protein isolates. Similar trend was observed by Yuliana et al., (2014) where the highest EC of 70% was recorded at pH 10 while the lowest EC of 40% was observed at pH 3. When the pH was raised to alkaline levels, dipole-dipole repulsion between adjacent droplets increased due to which the polarised protein molecules' hydration also increased. Because of these conditions, interfacial energy was lowered, leading to emulsion droplet coalescence.

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on EC by ANOVA is shown in Table 4.6. The Model F-value of 217.56 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 19.10 implies the Lack of Fit is

significant. There is only a 0.78% chance that a Lack of Fit F-value this large could occur due to noise.

Table 4.6 ANOVA showing the variables as a linear, quadratic and interaction terms on EC of defatted mustard meal

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2091.74	9	232.42	217.56	< 0.0001	significant
A-SpH	1109.21	1	1109.21	1038.30	< 0.0001	
B-IpH	283.22	1	283.22	265.12	< 0.0001	
C-Particle size	0.8450	1	0.8450	0.7910	0.4033	
AB	2.25	1	2.25	2.11	0.1900	
AC	0.2500	1	0.2500	0.2340	0.6433	
BC	0.6400	1	0.6400	0.5991	0.4643	
A^2	72.17	1	72.17	67.55	< 0.0001	
B^2	189.58	1	189.58	177.46	< 0.0001	
C^2	430.37	1	430.37	402.86	< 0.0001	
Residual	7.48	7	1.07			
Lack of Fit	6.99	3	2.33	19.10	0.0078	significant
Pure Error	0.4880	4	0.1220			
Cor Total	2099.22	16				

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on EC by ANOVA is shown in Table 4.6. The Model F-value of 217.56 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 19.10 implies the Lack of Fit is significant. There is only a 0.78% chance that a Lack of Fit F-value this large could occur

due to noise. The Predicted R^2 of 0.9464 is in reasonable agreement with the Adjusted R^2 of 0.9919; i.e. the difference is less than 0.2.

A second order polynomial equation was developed representing an empirical relationship between the response (EC) and the independent variables viz. SpH (A), IpH(B) and particle size(C). The equation for FC is given below:

EC (%) = 56.32 + 11.775 * A + 5.95 * B + -0.325 * C + 0.75 * AB + -0.25 * AC + -0.4 * BC + 4.14 * A^2 + -6.71 * B^2 + -10.11 * C^2

Figure 4.5 shows the combined effect of factors on the EC of mustard meal protein isolates at the central point of the experimental design. The extraction yield was drawn by changing only one factor while others were kept constant. It was observed that all the factors had a significant effect on the EC of protein. The downward curved line of factor C, as per the plot, indicated lower effect on the EC. Factor A (SpH) has the most prominent curve, indicating its significant effect on EC. Then came factor B (IpH), which also had a good influence on FC. In comparison to factor A and B, factor C (particle size) exhibited decline in curved line but had a notable impact on the EC of protein

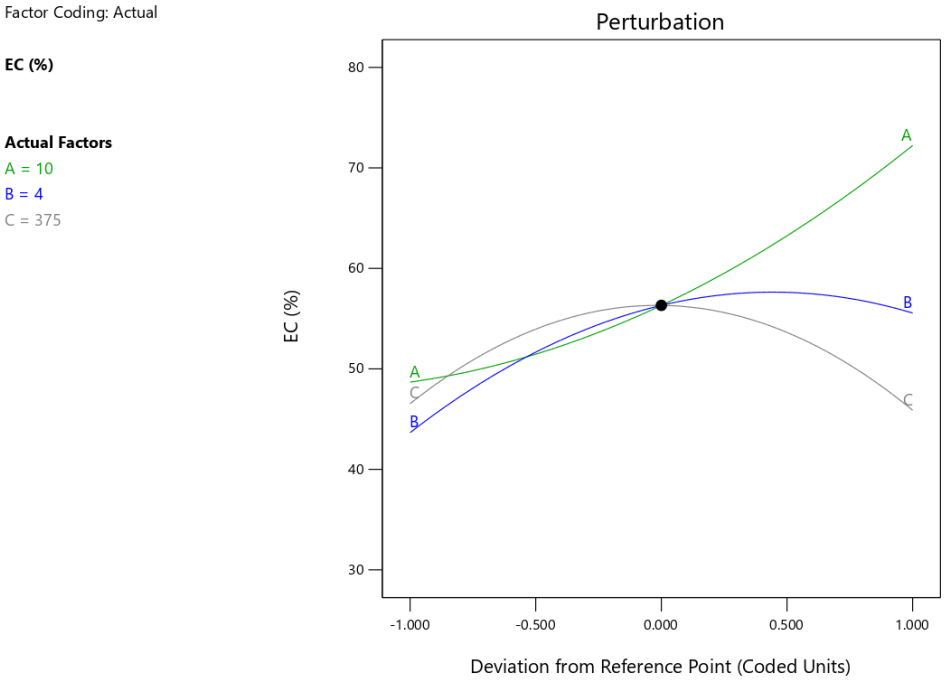

Fig 4.5 Perturbation plot for EC% of mustard protein isolates (A: SpH, B: IpH, C: particle size)

4.3.6 Viscosity

The feasibility of the model for the viscosity was tested using partial sum of squares techniques on the response surface quadratic model. The viscosity value of extracted protein ranged from 0.94 (cp) to 0.71 (cp). The maximum FC value (0.94 cp) was obtained for 375 µm particle size via the combination of 9 SpH and 5 IpH, from pH shift treatment. While the minimum viscosity value (0.71 cp) was obtained for 375 µm particle size via the combination of 9 SpH and 3 IpH. It was discovered that increasing alkaline pH has a positive impact on viscosity of mustard meal protein isolates.

Table 4.7 ANOVA showing the variables as a linear, quadratic and interaction terms on visocity of defatted mustard meal

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0436	6	0.0073	19.28	< 0.0001	Significant
A-SpH	0.0031	1	0.0031	8.17	0.0170	
B-IpH	0.0242	1	0.0242	64.17	< 0.0001	
C-Particle size	0.0017	1	0.0017	4.54	0.0590	
AB	0.0144	1	0.0144	38.19	0.0001	
AC	0.0001	1	0.0001	0.3507	0.5669	
BC	0.0001	1	0.0001	0.2652	0.6178	
Residual	0.0038	10	0.0004			
Lack of Fit	0.0009	6	0.0001	0.2063	0.9566	not significant
Pure Error	0.0029	4	0.0007			
Cor Total	0.0474	16				

The statistical analysis of the model equations and the significance of the coefficients were estimated by the P-value and F-test. The significance of each term through P-values on viscosity by ANOVA is shown in Table 4.7. The Model F-value of 19.28 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model

reduction may improve your model. The Lack of Fit F-value of 0.21 implies the Lack of Fit is not significant relative to the pure error. There is a 95.66% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R^2 of 0.8639 is in reasonable agreement with the Adjusted R^2 of 0.8727; i.e. the difference is less than 0.2.

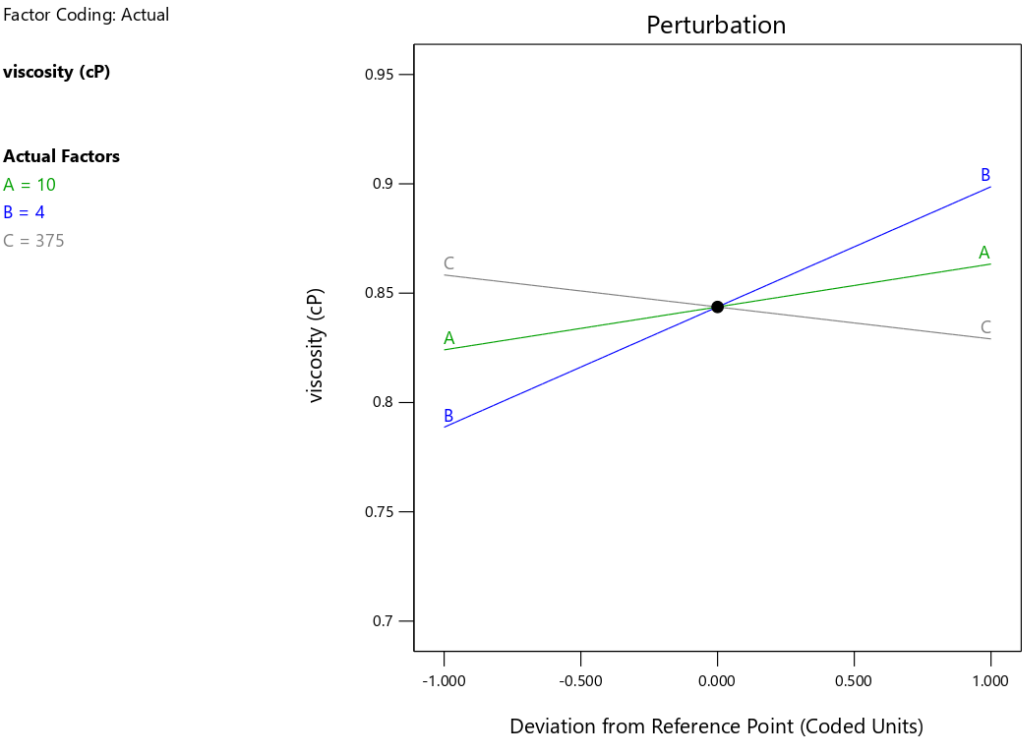

Fig 4.6 Perturbation plot for viscosity (cp) of mustard meal protein isolates (A: SpH, B: IpH, C: particle size)

A second order polynomial equation was developed representing an empirical relationship between the response (viscosity) and the independent variables viz. SpH (A), IpH(B) and particle size(C). The equation for FC is given below:

Viscosity (cp) = 0.843706 + 0.019625 * A + 0.055 * B + -0.014625 * C + -0.06 * AB + -0.00575 * AC + 0.005 * BC

Figure 4.6 shows the combined effect of factors on the viscosity of mustard meal protein isolates at the central point of the experimental design. The viscosity was drawn by changing only one factor while others were kept constant. It was observed that all the factors had a

significant effect on the viscosity of protein. Factor B (IpH) has the most significant effect on viscosity of protein isolates. Then came factor A (SpH), which also had a good influence on viscosity. In comparison to factor A and B, factor C (particle size) exhibited least impact on the viscosity of protein isolates.

4.4 Influence of independent variables on yield and functional properties of protein

To understand the interactions between several factors, we have generated the 3D response surface graph. Each response surface shows a function of any two factors, and the third factor is kept fixed. If the response surface graph was a curvature, this means that quadratic term was efficient on the plot.

4.4.1 Extraction yield

Fig. 4.7a illustrates the values of protein yield by varying SpH and IpH while fixing the particle size. It was observed that on increasing the SpH the protein yield increases while on raising the IpH, the protein yield first increases then starts to decline after pH 4.5. The highest yield was observed at pH 11, which depicts that increasing alkaline pH enhances the solubility and increases the yield of defatted mustard meal protein.

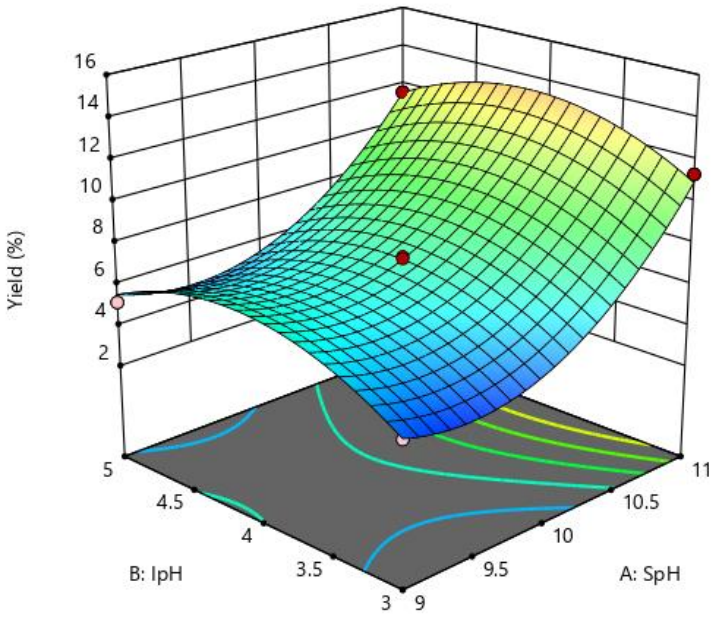

Fig 4.7a Response surface plot for the effect of SpH and IpH on protein yield

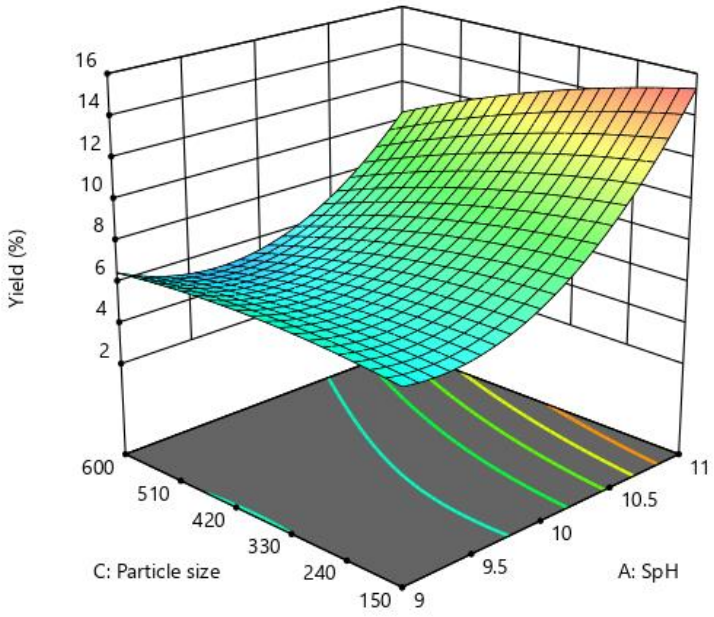

Fig 4.7b Response surface plot for the effect of SpH and particle size on protein yield

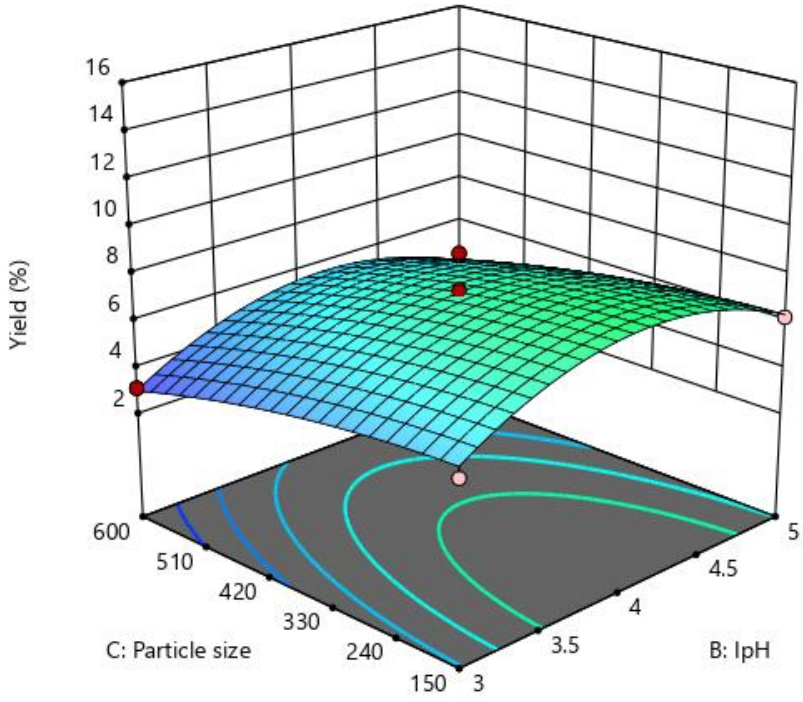

Fig 4.7c Response surface plot for the effect of IpH and particle size on protein yield

Fig. 4.7b demonstrates the values of protein yield by varying SpH and particle size while fixing the IpH. Particle size seems to have little influence on protein yield, however raising the pH enhances the yield of protein. It was observed that the yield is pH dependent and it increases with increasing alkaline pH upto pH 11. Fig. 4.7c shows the values of protein yield by varying IpH and particle size while fixing the SpH. It was observed that on increasing the isoelectric pH yield increases upto pH 4.5 then it declines,while particle size has no significant effect on protein yield.

4.4.2 Water Holding Capacity (WHC)

Fig. 4.8a shows the values of WHC of protein isolates by varying SpH and IpH while fixing the particle size. A drastic increase in WHC was observed on increasing the alkaline pH value upto pH 11. It was observed that WHC was minimum between pH 4 and 4.5 but increased on either side of this pH range because the solubility of protein is lowest at isoelectric point leading to least holding of water.

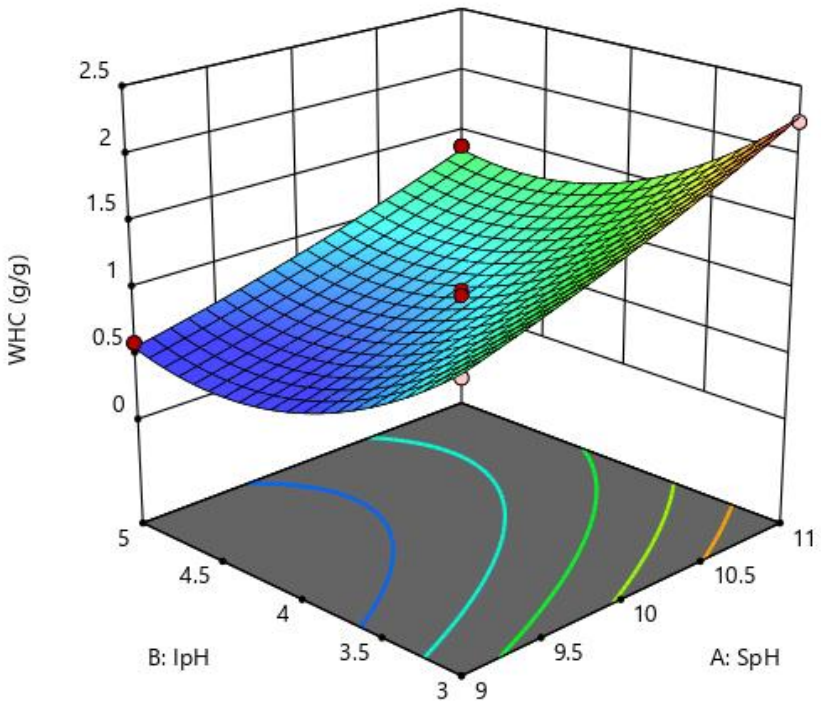

Fig 4.8a Response surface plot for the effect of IpH and SpH on WHC of protein

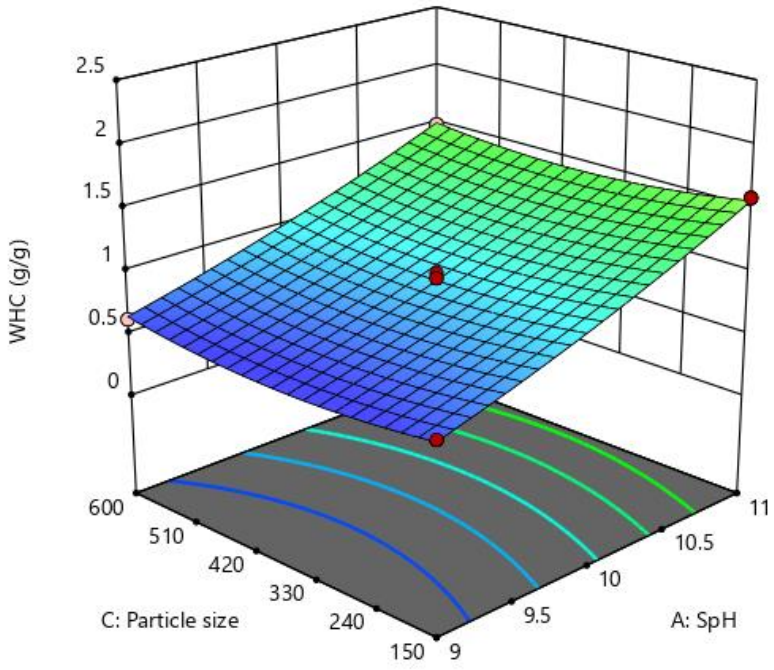

Fig 4.8b Response surface plot for the effect of SpH and particle size on WHC of protein

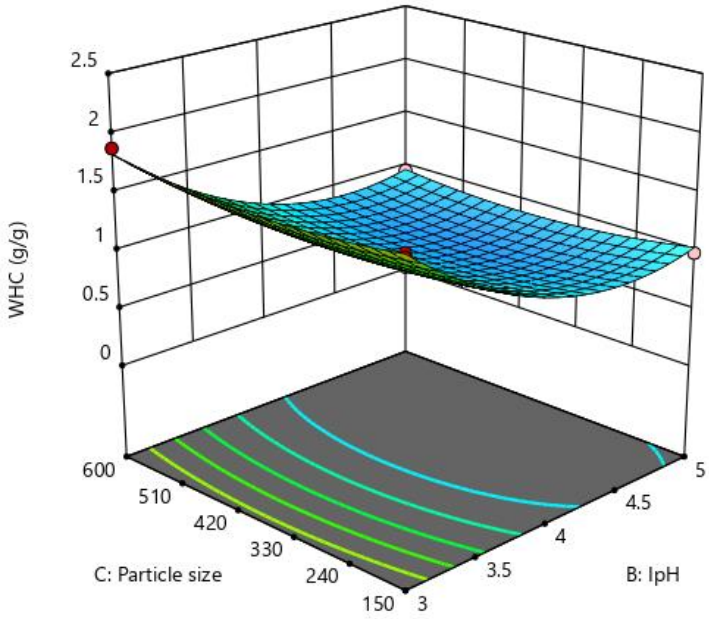

Fig 4.8c Response surface plot for the effect of IpH and particle size on WHC of protein

Fig 4.8b depicts the values of WHC of protein isolates by varying SpH and particle size while fixing the IpH. WHC levels increased significantly when the alkaline pH was raised upto pH 11, while there was least effect of particle size on the WHC of protein isolates. Fig 4.8c illustrates the values of WHC of protein isolates by varying IpH and particle size while fixing the SpH. WHC of protein was shown to be highest at pH 3, then drop around pH 4-4.5, and then increase again. It was due to the reason that WHC is greatly affected by the solubility of protein and it is least soluble near isoelectric point.

4.4.3 Oil Holding Capacity (OHC)

Fig. 4.9a shows the values of OHC of protein isolates by varying SpH and IpH while fixing the particle size. It was observed that OHC of protein decreased when SpH was increased while OHC increased on raising the IpH upto pH 5. The best OHC was obtained at combination of pH 9 SpH and 5 IpH.

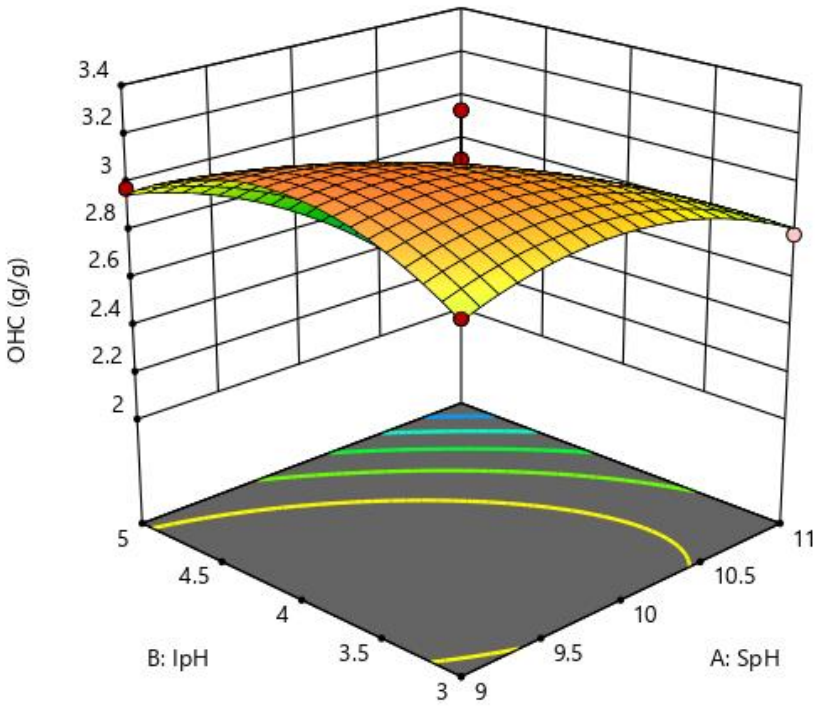

Fig 4.9a Response surface plot for the effect of IpH and SpH on OHC of protein

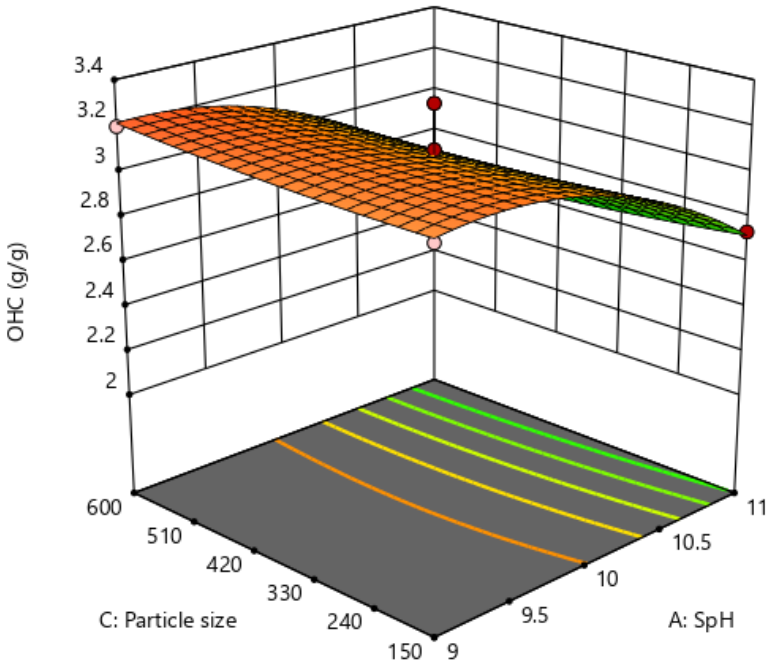

Fig 4.9b Response surface plot for the effect of particle size and SpH on OHC of protein

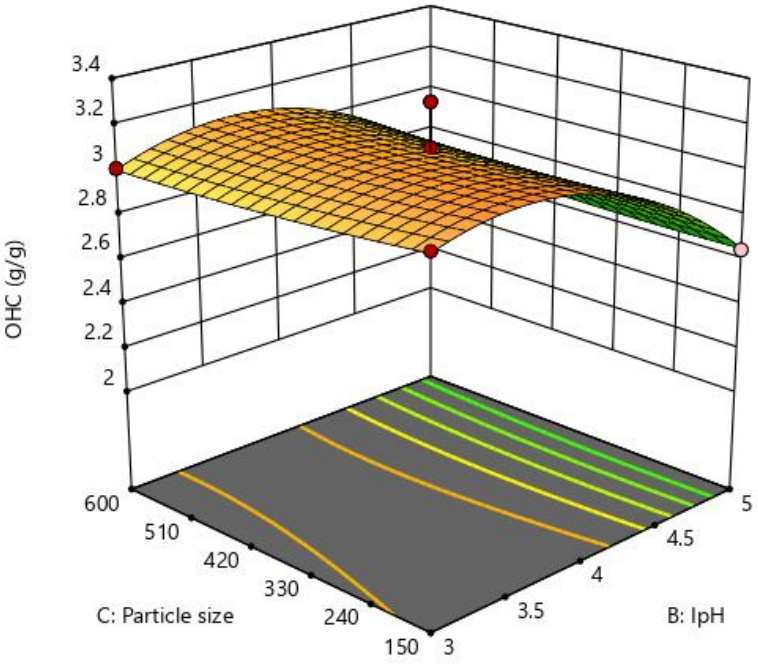

Fig 4.9c Response surface plot for the effect of particle size and IpH on OHC of protein

Fig. 4.9b shows the values of OHC of protein isolates by varying SpH and particle size while fixing the IpH. It was demonstrated that OHC initially increased but started declining when pH was raised from 9 to 11. No significant effect of particle size was observed. Fig. 4.9c shows the values of OHC of protein isolates by varying IpH and particle size while fixing the SpH. The highest OHC was noted near pH 4-4.5 and then OHC started declining as IpH was raised. There was no visible effect of particle size on the OHC of protein.

4.4.4 Foaming Capacity (FC)

Fig. 4.10a shows the values of FC of protein isolates by varying SpH and IpH while fixing the particle size. The FC increased with raising pH upto pH 11 and the maximum value was observed at pH 11. The FC rose at IpH 3 but dropped at pH 4-4.5 before increasing again up to pH 5. The high net charge on protein molecules decreases hydrophobic groups and promotes protein solubility with water and oxygen, increasing foaming capability at strongly acidic and alkaline pH.

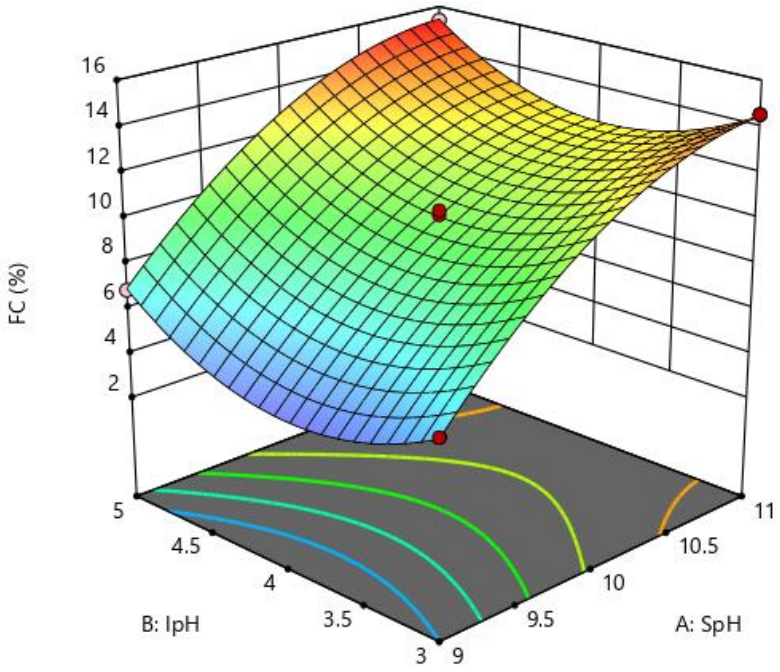

Fig 4.10a Response surface plot for the effect of SpH and IpH on FC of protein

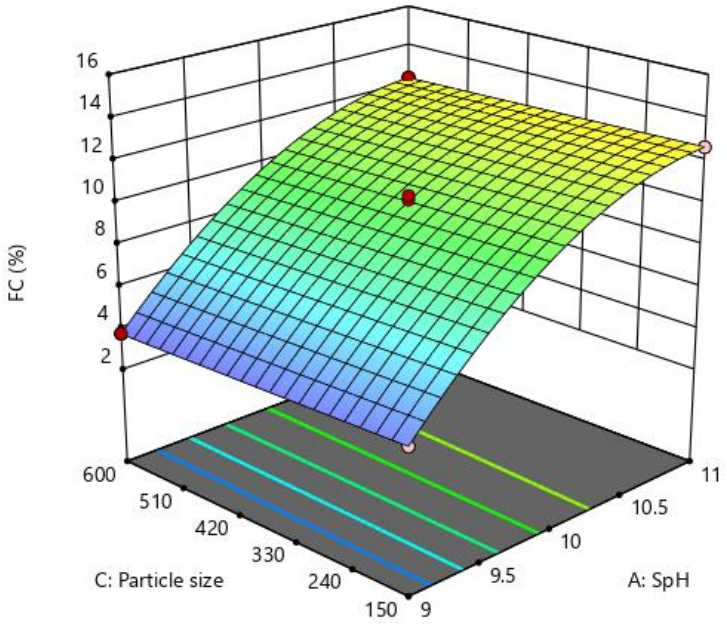

Fig 4.10b Response surface plot for the effect of SpH and particle size on FC of protein

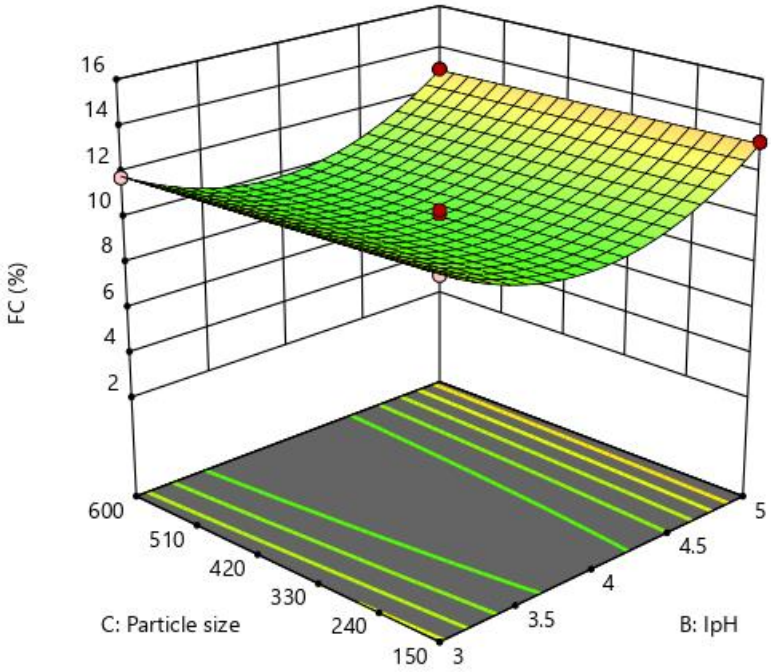

Fig 4.10c Response surface plot for the effect of IpH and particle size on FC of protein

Fig. 4.10b shows the values of FC of protein isolates by varying SpH and particle size while fixing the IpH. The FC substantially increased with rising SpH upto pH 11 and there was no positive effect of particle size on FC of protein isolates. Fig. 4.10c shows the values of FC of protein isolates by varying IpH and particle size while fixing the SpH. The FC increased at IpH 3 but fell at pH 4-4.5 before increasing again to pH 5. The highest FC was reported at IpH 5 and particle size 150.

4.4.5 Emulsifying capacity (EC)

Fig. 4.11a shows the values of EC of protein isolates by varying SpH and IpH while fixing the particle size. The highest EC was observed at SpH 11 and IpH 5, it was assessed that EC increased with increasing SpH and IpH.

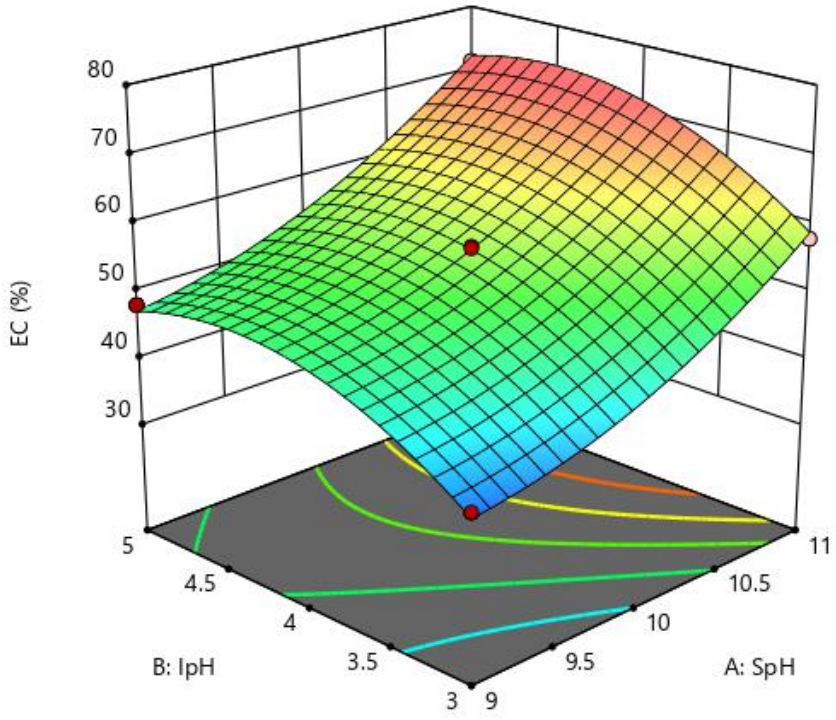

Fig 4.11a Response surface plot for the effect of IpH and SpH on EC% of protein

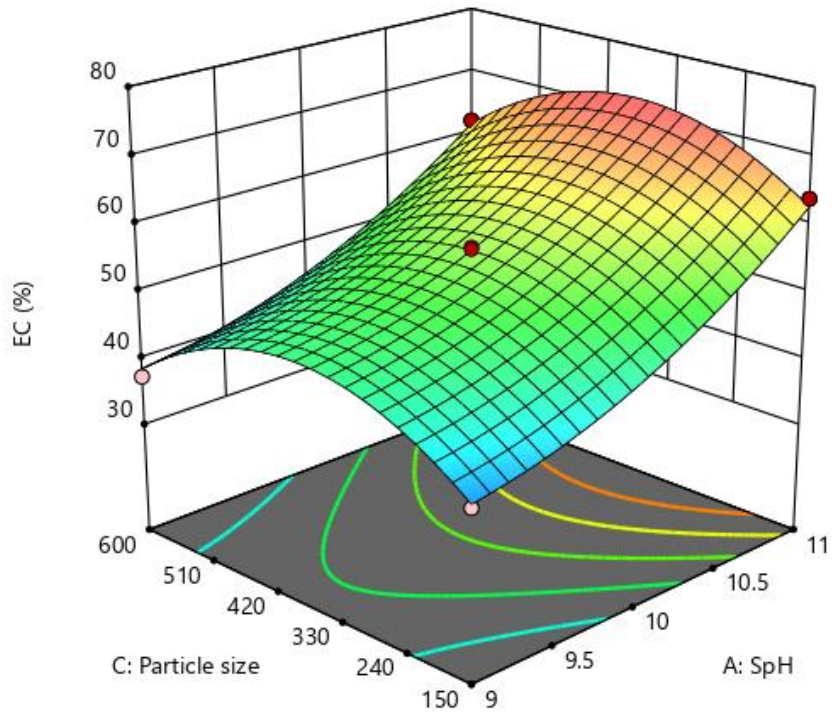

Fig 4.11b Response surface plot for the effect of SpH and particle size on EC of protein

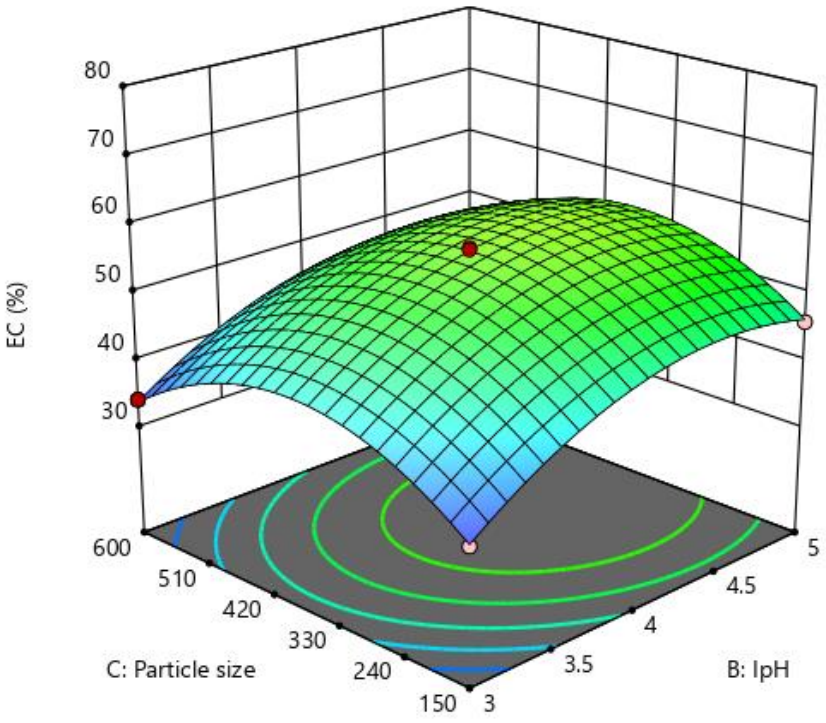

Fig 4.11c Response surface plot for the effect of particle size and IpH on EC of protein

Fig. 4.11b shows the values of EC of protein isolates by varying SpH and particle size. EC initially raised and then started declining as particle size was increased. Fig. 4.11c shows the values of FC of protein isolates by varying particle size and IpH while fixing the SpH. The FC increased initially and then started declining with increasing IpH and particle size.

4.4.6 Viscosity

Fig. 4.12a shows the values of viscosity of protein isolates by varying SpH and IpH while fixing the particle size. The viscosity increased significantly on raising the SpH and IpH. There was no significant effect of particle size on visocity of protein as shown in Fig 4.12b and Fig 4.12c.

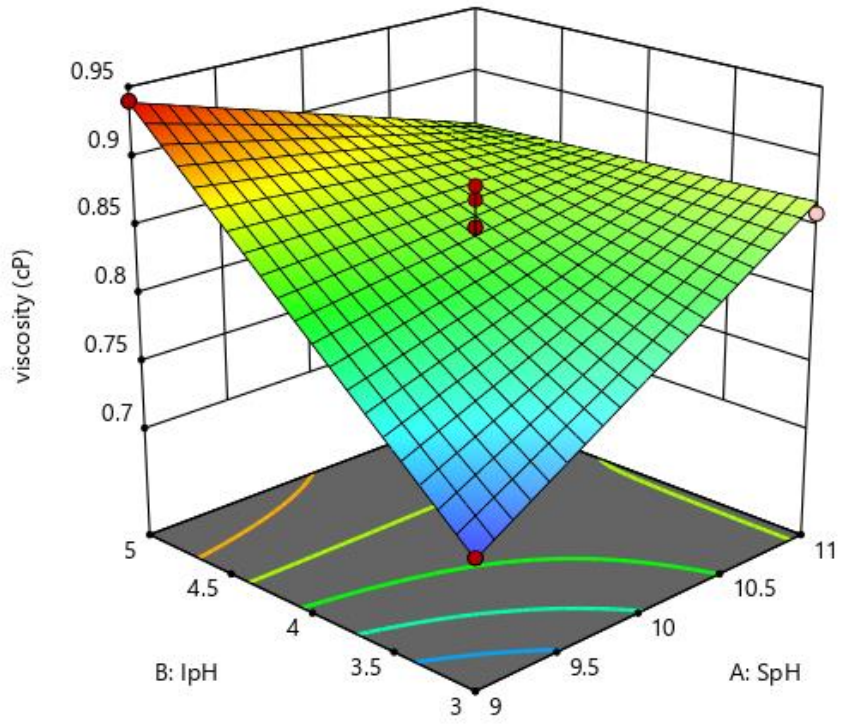

Fig 4.12a Response surface plot for the effect of IpH and SpH on viscosity of protein

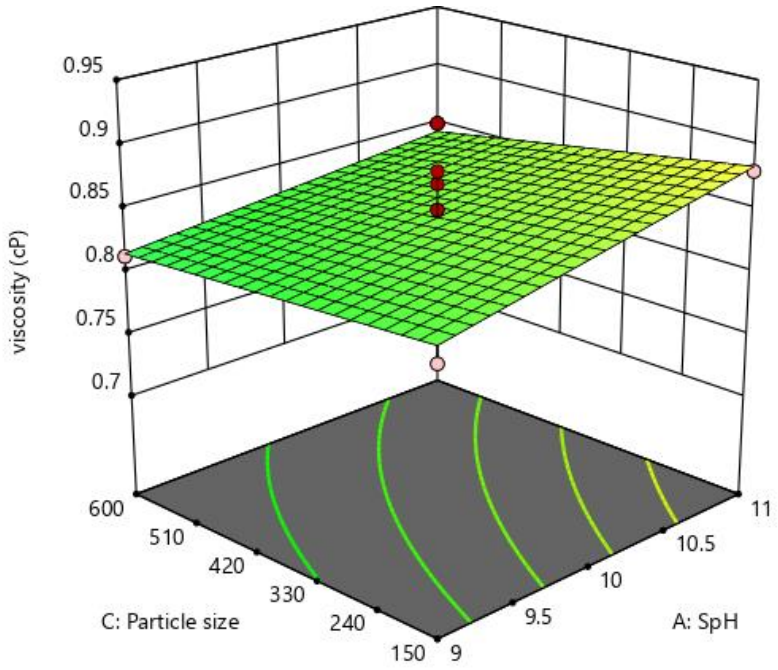

Fig 4.12b Response surface plot for the effect of particle size and SpH on viscosity of protein

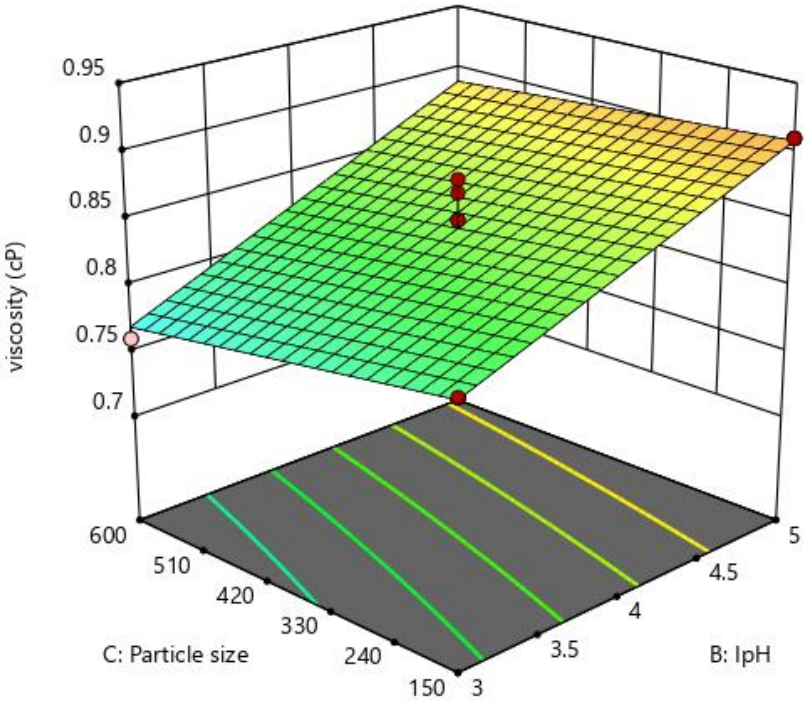

Fig 4.12c Response surface plot for the effect of particle size and IpH on viscosity of protein

4.5 Optimization

The optimization value was obtained by differentiating the quadratic model using Design-Expert software. The fundamental goal of optimization is to determine the amounts of independent variables that will result in the maximum protein yield and functional properties. The combination of SpH 11, IpH 4 and particle size 375 µm were predicted to provide the maximum protein yield of 11.808 %, WHC of 1.5 (g/g), OHC of 2.50 (g/g), FC of 12.56 (%), EC of 69.1 (%) and viscosity of 0.881 (cp).

4.6 SDS page

The molecular structure of DMMI was determined SDS-PAGE. The SDS-PAGE profile of DMMPI and molecular weight standard are presented in Figure 4.13. From Fig 4.13, it can be observed that there is no difference in the molecular weight of DMMPI obtained by pH shift method. So, from Fig 4.13, it can be concluded that the selected SpH 11, IpH 4 and particle size 375 µm do not produce any remarkable changes in the molecular weight of protein

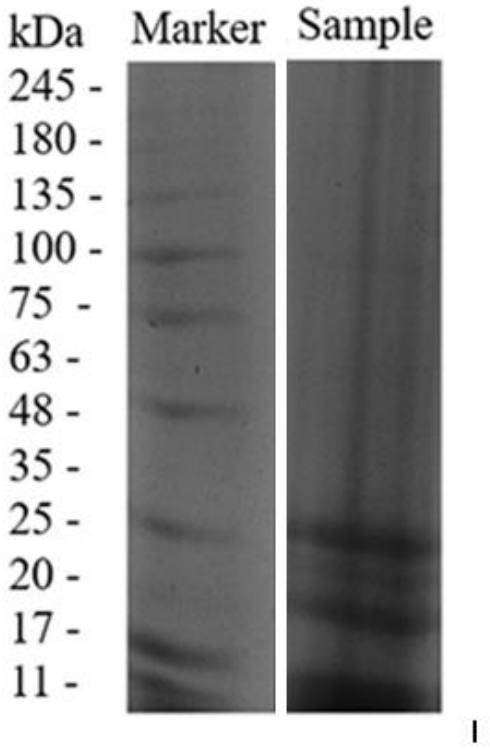

Fig 4.13 SDS-PAGE electrophoretic profile of alkaline extracted DMMPI

4.7 FT-IR

Figure 4.14 shows FTIR spectrum of alkaline solubilization and acidic precipitation protein isolates extracted from the mustard meal. As it is evident from the spectrum, the main peaks for alkali extracted mustard meal protein appeared at 3291, 2925, 2858, 1655, 1539, 1248, 702, and 622 cm−1. These peaks could be attributed to the N–H stretch, carboxyl stretch (C–H), carbonyl stretch (C=O), O–C–N stretch, and stretching vibrations of many organic functional moieties. This suggests the low population of bonds in the high transmitted curve. This could be a result of structural modification induced by high alkaline and acidic conditions.

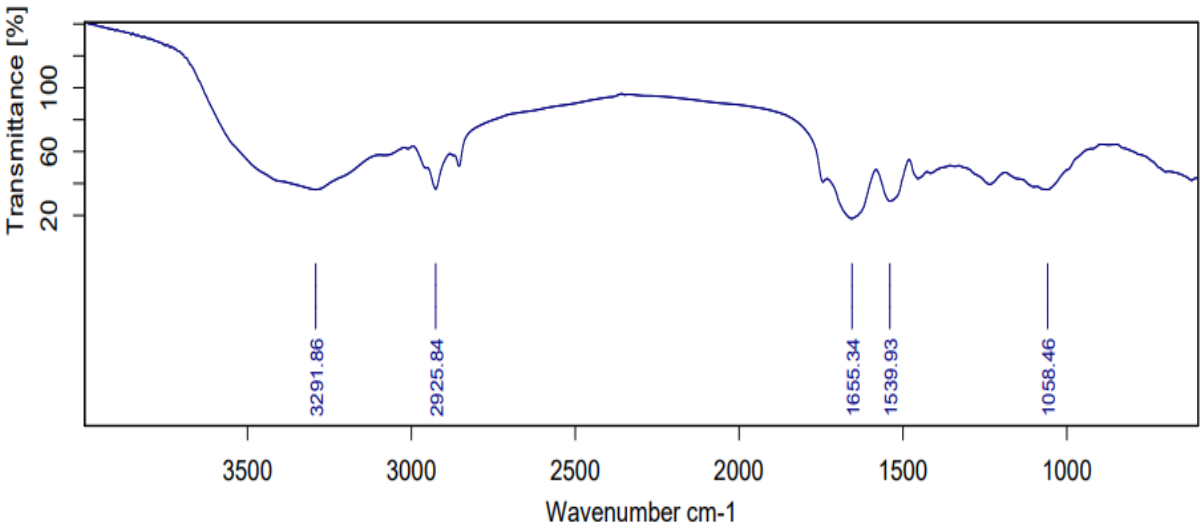

Fig 4.14 Fourier transform infrared spectrometer of DMMPI

Chapter 5

CONCLUSION

Plant-based protein isolates have recently gained popularity in food applications due to their increased sustainability and lower operating costs. The protein content of mustard seeds ranges from 24-30 percent with a good amino acid composition including essential amino acids. Numerous methods have been developed to extract protein from plant sources. The pH shift method is a promising technology for isolating protein from plant sources, it is a simple and easy method with high protein yields and better functional properties of protein isolates. It involves alkaline solubilization and isoelectric precipitation of protein isolates. The method is based on increased protein solubility caused by pH changes, which allows protein separation from insoluble material, thus increasing the extraction yield. The solubility of protein increases at high alkaline pH and and decreases at acidic pH.

Various parameters such as soluble pH (SpH), isoelectric pH (IpH) and particle size were tested for extraction of protein from defatted mustard meal. The pH was optimized using Box Behken Design model of Response Surface Methodology. ANOVA indicated that one linear term (A) and one quadratic term (A^2) were significant factors affecting the protein yield. Protein yield of 11.808% was obtained under the optimum conditions of SpH 11, IpH 4 and 375 µm particle size. The good functional characteristics such as WAC, OAC, EA, FC and FS were observed for protein isolate from mustard meal at the optimum pH values. The results indicated that alkaline extraction method improves the yield and have significant effect on the functional properties of protein isolates.

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