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Optimization of protein extraction from velvet beans

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Abstract
Protein deficiency is a growing global concern and this work was conducted in order to find out an alternative for protein requirement that is economically feasible. A numerical optimization of velvet bean protein isolation method was employed. Response surface methodology (RSM) was used to optimise the extraction factors (concentration of alkali, alkali/feed ratio, extraction temperature) to maximize both the extraction yield and protein content using alkali extraction and acid precipitation method. The optimum conditions were as follows: alkali concentration 0.579%, alkali to feed ratio 50ml and temperature 45.684 ^{0}C. Under these conditions, the maximum predicted protein content and extraction yield were 81.460% and 24.862%, respectively. Considering the protein content and yield velvet bean can be considered as a good plant-based protein alternative source.

Keywords: Mucuna bean, protein isolate, protein alternative, response surface methodology, optimization process

1. Introduction
Malnutrition is a rising global burden, especially affecting millions of pregnant women and children (Asrat, 2021) [4]. According to the Global Hunger Index 2020, 14 percent of India's population is undernourished (Singh *et al*. 2021) [29]. World population being increased and forecast to exceed 9 billion by 2050, every nation has to find alternative to support the escalating global demand for protein (Billen *et al*. 2015) [10]. Plant-based diets have recently become more economical and versatile replacements for animal protein on a worldwide scale. From time out of mind, legumes continue to be the world second prioritized sources of staple food after cereals earning them the moniker “poor man’s meat” (Cheng *et al*. 2019) [12]. Several legumes have been examined and offered as protein replacements for human consumption to far, among which soybean being the most economically important legume (Betancur-Ancona *et al*. 2008; Bhat & Karim, 2009) [8, 9].
Due to strong demand for vegetarian protein, costs have risen disproportionately (Bhat & Karim, 2009) [9]. In this context, underutilised legumes offer significant potential for addressing nutritional needs and ensuring food security (Nayak *et al*. 2022) [22]. Velvet beans (*Mucuna pruriens*), also known as buffalo beans, cowitch, dopa bean, kappikachhu, is one such underutilised legume (Vadivel & Pugalenthi, 2010) [33].
It has a protein concentration of 23-35% which is comparable to other pulses such as soybean (37-42%), lima bean (20.69-23.08%) and horse gram (18.5 to 28.5%) (O'Keefe *et al*. 2015; Prasad & Singh, 2015; Yellavila *et al*. 2015) [23, 24, 35]. *M. pruriens* var. *utilis* seed contain crude protein 31.44%, crude carbohydrate 52.56%, crude lipid 6.73%, crude fibre 5.16% and ash 4.11% (Lim, 2012) [20]. Except for sulfur-containing amino acids and tyrosine, they include all necessary amino acids. Fatty acids such as linoleic, palmitic and oleic acids are also present in them, making it compactable to other oil seeds and nuts (Lampariello *et al*. 2012) [19].
Velvet beans were used as a food source by certain ethnic groups and were used in Ayurveda for the treatment of Parkinson’s disease, reducing the risks of certain cardiovascular diseases, nervous disorders, menstruation disorders, arthritis and can be employed as a powerful aphrodisiac (Lampariello *et al*. 2012; Cheng *et al*. 2019; Suryawanshi *et al*. 2020) [19, 12, 30].
However, literature suggests that the use of velvet beans as a protein source for monogastric animals is limited due to the presence of antinutritional factors such as phenolic content (3.1-4.9%), tannin (0.03-0.06%), l-Dopa (5.6%-6.8%), phytic acid (0.31-0.71%), saponin (1.15-1.31%) hydrocyanic acid (58mg/kg) as they can interfere in nutrient assimilation, reduce digestibility when consumed in high quantities (Carew & Gernat, 2006; Pugalenthi *et al*. 2005) [11, 25]. From studies it is clear that velvet bean seeds can be consumed by humans if properly

cooked (Amira *et al*. 2014) [2].

Proteins isolates are used as a functional ingredient mainly to increase nutritional quality as well as to provide desirable sensory characteristics such as structure, texture, flavour, and colour to formulated food products. Commonly used soybean, whey and wheat protein isolates have dietary restrictions and preferences (related allergenicity, Halal requirements, vegetarianism etc.), so the consumers and food manufacturers are looking for alternative protein sources (Asgar *et al*. 2010; Ladjal-Ettoumi *et al*. 2016) [3, 18]. Alkaline extraction and isoelectric pH precipitation is one of the most commonly used techniques for the production of edible protein as it is simple and can be carried out at a low price (Mechmeche *et al*. 2017) [21].

To the best of the understanding, there are no published papers reported to date on the numerical optimization of velvet bean protein extraction process. So the aim of the study was to employ response surface methodology (RSM) to optimise the extraction factors (concentration of alkali, alkali/feed ratio, extraction temperature) to maximize both the extraction yield and protein content of velvet beans.

2. Methodology

2.1 Materials and method

Velvet beans (*Mucuna pruriens* var. *utilis*) (white variety of 5 kg) was purchased from local market in Ludhiana city, Punjab. Seeds were sorted, cleaned, removing the damaged ones, were soaked in distilled water in ratio of 1:20 (w/v) in a room temperature for 12hr. The soaked water was discarded and the beans were dehulled manually. The cotyledons were dried in hot air oven at 50±2 ^{o}C until constant weight was reached. It was then grounded using mixer (Sujata, mittal electronics, India), sieved (BSS36 mesh screen) to obtain fine powder and was stored in zip lock pouch at room temperature for further analysis. All chemicals and reagents used in the study were of analytical grade and were procured from standard manufacturer.

2.2 Extraction of velvet bean protein

Protein was extracted from raw velvet bean flour by alkali extraction and acid precipitation method, with reference to S. Banerjee *et al*. (2022) [6] with slight modification as shown in fig. 1. Raw velvet bean flour was added to independent variables used for protein extraction aqueous solution of sodium hydroxide (NaOH) as alkali with variable concentration (0.0, 0.5, 1.0% w/v), alkali to feed dilution value (10:1, 30:1, 50:1) and variable extraction temperature (30, 50, 70 ^{o}C) with constant extraction period of 1h. The mixture was then centrifuged (Remi PR-24, India) at 12000×g for 30 min. The extracted protein was precipitated using glacial acetic acid by lowering the pH to 4.0, again centrifuged at 12000×g for 30 min. Extracted protein was washed with distilled water and was dried in tray drier (Fini X 72, India) at 50 ^{o}C ± 2 ^{o}C and stored at room temperature for further analysis.

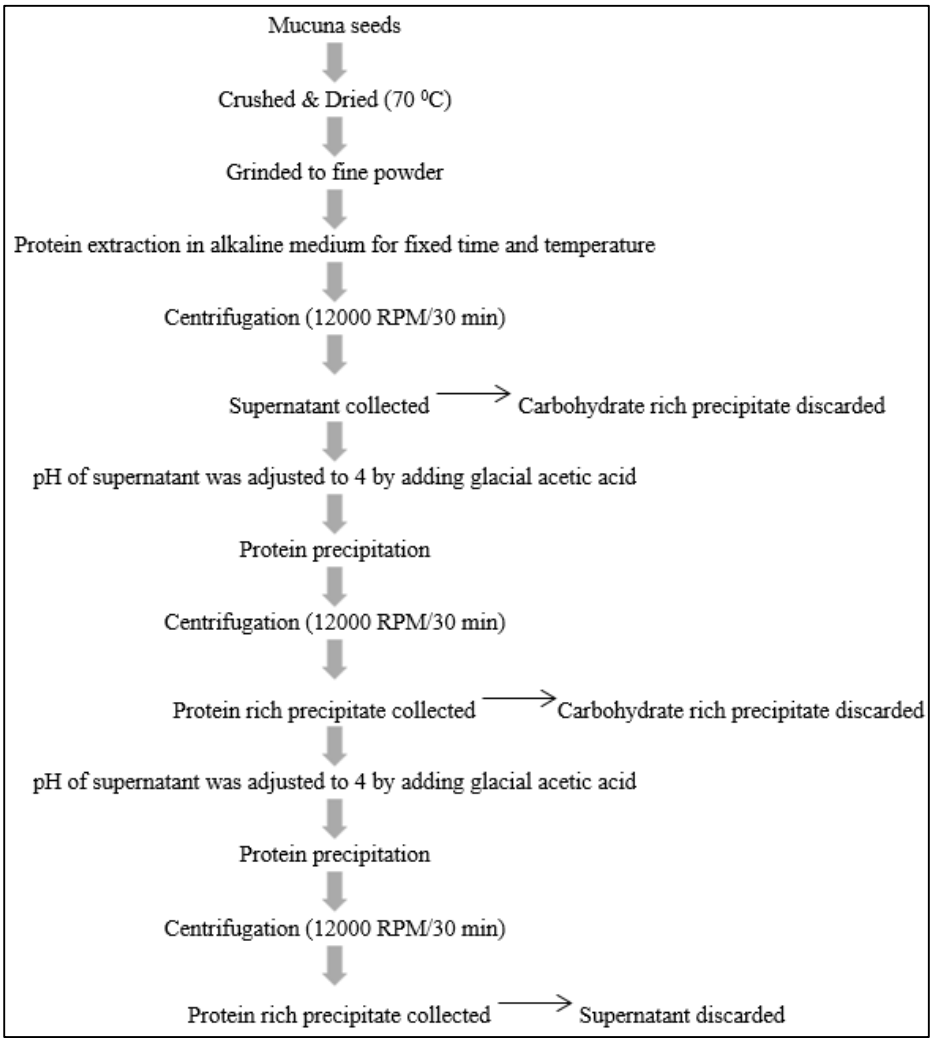

Fig 1: Protein extraction protocol

2.3 Total protein content estimation
Crude protein of protein isolates was determined by the Kjeldahl procedure using a conversion factor of 6.25.

2.4 Determination of protein recovery
According to Shao *et al*. (2014) [27], the content of protein was determined by the amount of protein in each fraction compared with the amount of protein in the raw material.

Protein yield % = $\frac{M_1}{M_0} \times 100\%$; where M_0 is the total protein in raw flour and M_1 is the total extracted protein.

2.5 Statistical analysis
The experimental design was conducted using the Design Expert software (version 13.0, Stat Easy Inc., Minneapolis, USA). The effect of independent variables i.e., NaOH, alkali to feed dilution value, extraction temperature were investigated to optimise with range and centre point values where protein content (Y_1) and extraction yield (Y_2) used as the responses as in Table 1 using RSM. A Box–Behnken design (BBD) was employed in this regard.

Table 1: Constraint for Treatment Conditions

Treatment	Coded	Units	Factor levels		
			-1	0	+1
NaOH	X_1	Percentage	0.0	0.5	1.0
NaOH to feed ratio	X_2	Milli liter	10	30	50
Temperature	X_3	Degree Celsius	30	50	70

3. Results and Discussions
3.1 Optimization of the MBPI extraction conditions
The results of 17 runs using BBD design are presented in Table 2. Results showed that the experimental and predicted values are in close agreement. The extraction yield and protein content are found to be in range of 0.75 to 30% and 26.26 to 87.54% respectively seemed to be varied depending on the conditions given.

Table 2: Box-Behnken design of the levels of factors, program and test results of RSM

Independent variables			Responses			
X1: NaOH (%)	X2: Alkali to feed (ml)	X3: Temp (°C)	Y1: content (%)	Y1: Predicted value (R^2)[a]	Y2: Yield (%)	Y2: Predicted value (R^2) [a]
1	10	50	52.52	49.87	20	21.08
0.50	10	70	48.36	46.85	14.84	15.75
0.50	50	70	63.70	59.69	15.54	17.64
0.50	10	30	37.93	41.94	12.31	10.21
0.5	30	50	87.54	87.55	13.15	15.49
0	10	50	32.09	32.24	5	4.67
0	30	30	46.65	42.48	1.12	3.55
0.5	30	50	87.50	87.55	18.84	15.49
0.50	30	50	87.62	87.55	16.03	15.49
0	30	70	37.50	38.86	0.75	0.26
0.5	30	50	87.54	87.55	14.15	15.49
1	30	70	67.01	71.18	24.17	21.74
1	30	30	72.95	71.59	16.96	17.97
1	50	50	82.87	82.72	30	30.33
0.5	30	50	87.54	87.55	15.30	15.49
0.50	50	30	67.11	68.62	25	23.66
0	50	50	36.26	38.91	11.40	10.32
Raw flour			23.34			

[a]Values were from Design Expert version 13 software package

3.2 Model fitting for optimisation of protein yield
The results of analysis of variance (ANOVA) indicate that the contribution of quadratic model for the protein yield (Table 3) was significant. It can be seen that the variable with the highest effect on extraction yield was X_1 and X_2. However, the quadratic term of X_3^2, X_2^2 as well as the interaction terms X_1X_2, X_1X_3 and X_2X_3 were found insignificant (p>0.05). The lack of fit p value of 0.1742 (p>0.05) indicating the experimental data fitted well to the model and adequate for predicting the extraction yield. The value of R^2 was 0.9396 while the R^2 adj was 0.8618, indicating a high degree of correlation between the experimental and predicted values (fig 2). The maximum yield 30% was found under the experimental conditions of X_1 = 1%, X_2 =50ml and X_3 = 50 °C.

Table 3: ANOVA for quadratic model: estimated regression model of relationship between response variable (yield) and independent variables (X_1, X_2, X_3)

Source	Sum of Squares	DF	Mean Square	F-value	p-value
Model	902.66	9	100.30	12.09	0.0017*
X_1	602.39	1	602.39	72.61	< 0.0001*
X_2	138.03	1	138.03	16.64	0.0047*
X_3	0.0010	1	0.0010	0.0001	0.9915
X_1X_2	0.0064	1	0.0064	0.0008	0.9786
X_1X_3	14.36	1	14.36	1.73	0.2297
X_2X_3	35.94	1	35.94	4.33	0.0759
X_1^2	18.63	1	18.63	2.25	0.1777
X_2^2	69.72	1	69.72	8.40	0.0230*
X_3^2	29.36	1	29.36	3.54	0.1020
Residual	58.07	7	8.30		
Lack of Fit	39.25	3	13.08	2.78	0.1742
Pure Error	18.82	4	4.71		
Cor Total	960.74	16			
R^2	0.9396				
Adj R^2	0.8618				
C.V.	18.98				

*Significant (p<0.05)

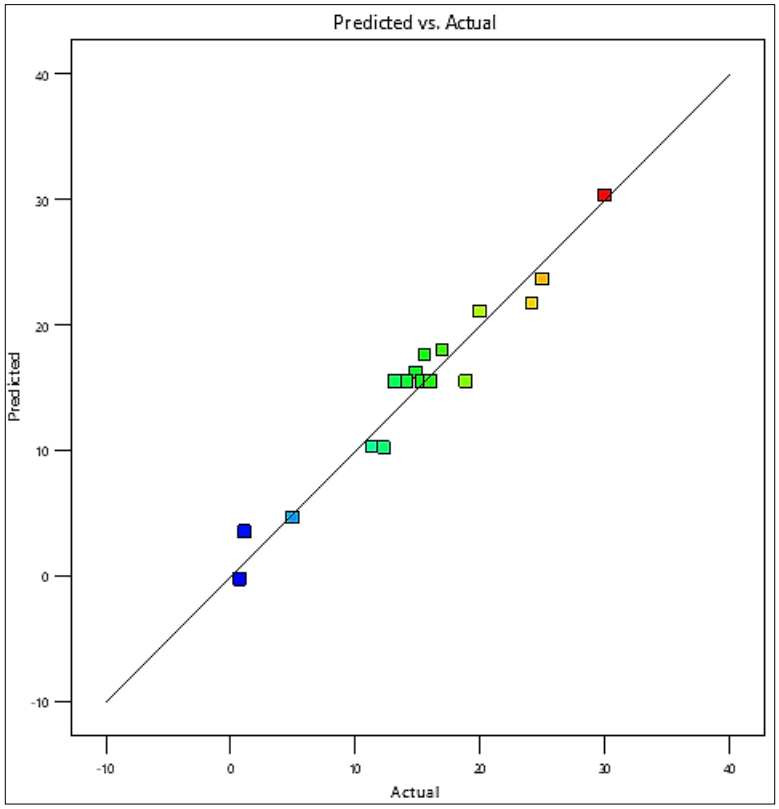

Fig 2: Actual value v/s predicted value of protein yield

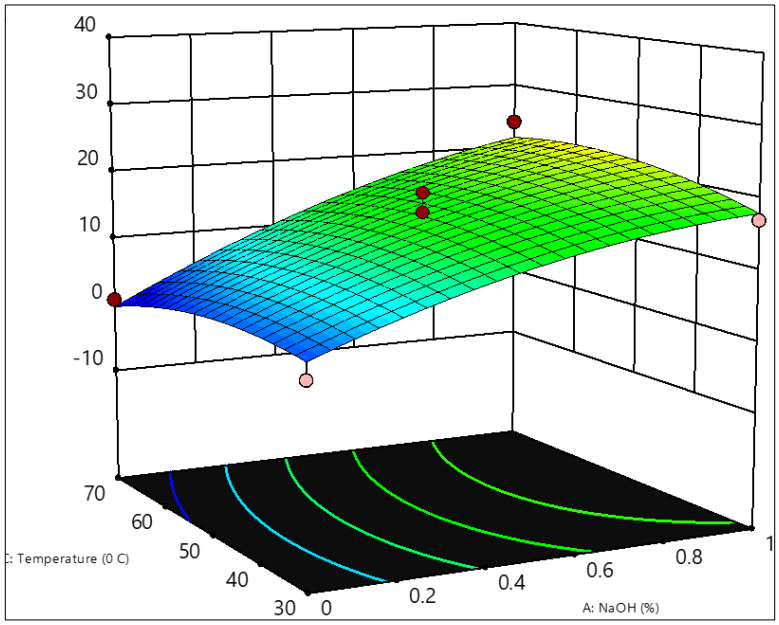

Fig 3: 3D plot for protein yield (Y_2) as a function of alkali concentration (X_1) and extraction temperature (X_3)

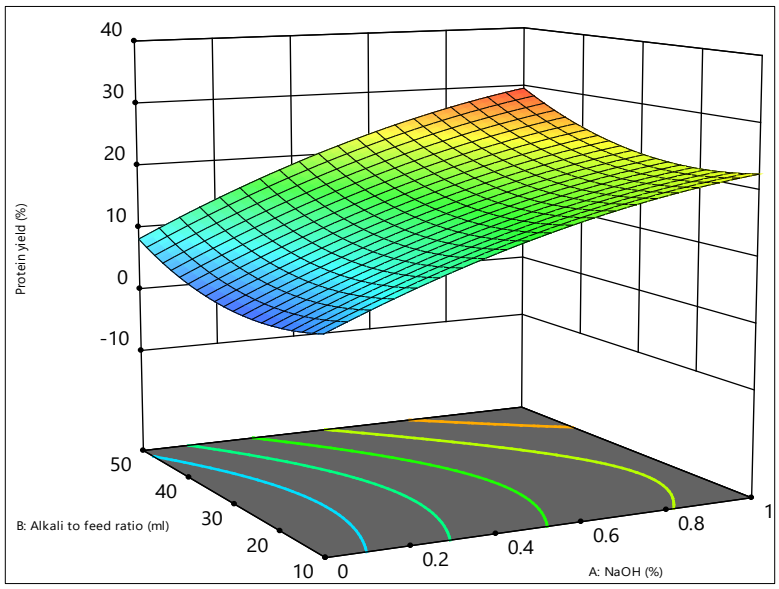

Fig 4: 3D plat for protein yield (Y_2) as a function of alkali concentration (X_1) and alkali to feed ratio (X_2)

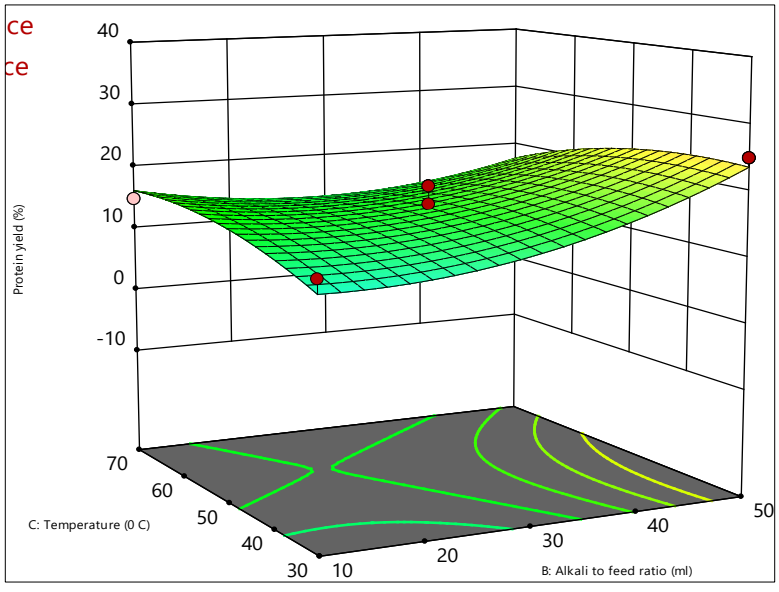

Fig 5: 3D plot for protein yield (Y_2) as a function of alkali to feed ratio (X_2) and extraction temperature (X_3)

Analysis of the model shows that the concentration of NaOH was the most significant factors as it had a positive effect on the extraction yield (X_1 = 9.11). As observed in fig 3, the alkali- feed ratio had a major effect on the extraction yield as the yield was improved with increasing ratio from 10 to 50 mL/g. Similar observation was also found by Hadidi *et al*. (2020) [17]. This process can be considered as a diffusion process that involves the transfer of protein molecules from a solid to a solvent and requires a number of aspects (Aguilera, 2003) [1]. This could be due to a higher driving force for protein mass transfer, which increases the diffusivity of the solvent into cells and facilitates protein desorption from cells (Bedin *et al*. 2020) [7]. As the concentration of alkali increases protein yield also increases (fig 4) which could be due to high alkali concentration helps to break down the hydrogen bonds and to dissociate hydrogen from carbonyl and sulphate groups (Shen *et al*. 2008) [28]. The increased surface charge on protein molecules then leads to an enhanced solubility in the solvent system (Sari *et al*. 2015) [26]. The "salting in-effect" is a distinctive behaviour found for moderately increasing salt concentrations. This trend was also observed in Feyzi *et al*. (2015); Wen *et al*. (2021) [15, 34]. The texture can be weakened by alkaline conditions when hydrogen bonds are broken, causing hydrogen ions to separate from carbolic and sulphate groups (Shen *et al*. 2008) [28].

Temperature had negative effect on yield responses (X_3= -0.0113). Extraction yield was found to decrease with increasing the temperature especially from 50 to 70 °C (fig 5). Protein can be subjected to thermal denaturation if the temperature is increased (Hadidi *et al*. 2020) [17]. At 70 °C (Fig 7), which might be due to the presence of higher phytate content in the resulting slurries. The interaction between protein-phytic acid complexes caused the decreasing in protein solubility and consequently led to lower protein extraction yield (Tan *et al*. 2014) [31].

It can be observed that the yield increase with temperature and NaCl concentration, NaCl and alkali to feed ratio but it highly decreases with reduction in NaCl concentration and temperature. These results show the high influence of NaCl concentration on the extraction process. This was also supported by Tanger *et al*. (2020) [32].

3.3 Model fitting for optimisation of protein content

The protein content varied from 32.24 to 87.62%, depending on the process parameters. The F-value of 60.77 and P-values less than 0.0500 indicate that the model is significant. In Table 4, the results of quadratic model of ANOVA analysis show that the velvet bean protein content is more significantly affected by X_1, quadratic terms of X_1, X_2, and X_3. However, interaction terms (X_1X_3 and X_2X_3) were found insignificant ($p > 0.05$). The R^2 value was 0.987 indicating that the models for response variables were very significant ($p<0.0001$). The maximum content (87.54%) was found under the experimental conditions of X_1 = 0.50%, X_2 =30ml and X_3 = 50 °C. The experimental and predicted values are shown in fig 6.

Table 4: ANOVA for quadratic model: estimated regression model of relationship between response variable (content) and independent variables (X1, X2, X3)

Source	Sum of Squares	df	Mean Square	F-value	p-value
X_1	1886.52	1	1886.52	147.75	< 0.0001*
X_2	780.92	1	780.92	61.16	0.0001*
X_3	8.14	1	8.14	0.6376	0.4508*
X_1X_2	171.35	1	171.35	13.42	0.0080*
X_1X_3	2.58	1	2.58	0.2018	0.6669
X_2X_3	47.89	1	47.89	3.75	0.0940
X_1^2	1279.22	1	1279.22	100.19	< 0.0001*
X_2^2	1549.38	1	1549.38	121.35	< 0.0001*
X_3^2	835.94	1	835.94	65.47	< 0.0001*
Residual	89.38	7	12.77		
Lack of Fit	89.37	3	29.79	15515.73	< 0.0001
Pure Error	0.0077	4	0.0019		
Cor Total	7072.97	16			
R^2	0.9874				
Adj R^2	0.9711				
C.V.	5.61				

*Significant ($p<0.05$)

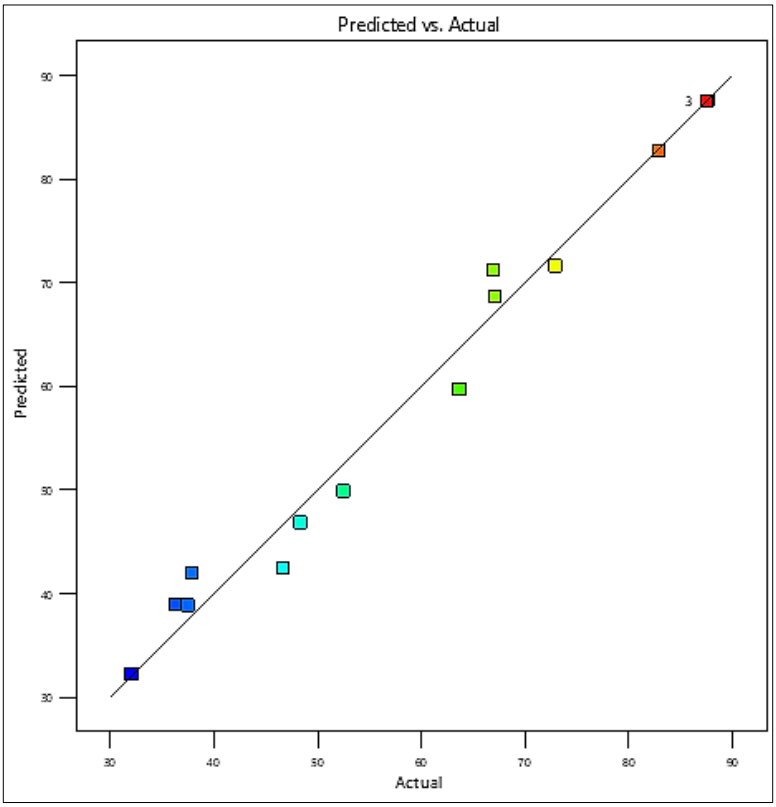

Fig 6: Actual value v/s predicted value of protein content

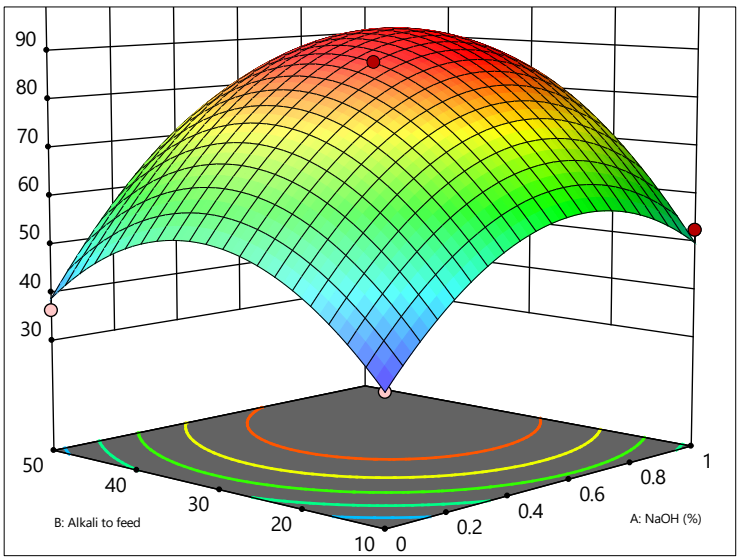

Fig 7: 3D plot for protein content (Y_1) as a function of alkali concentration (X_1) and alkali to feed ratio (X_2)

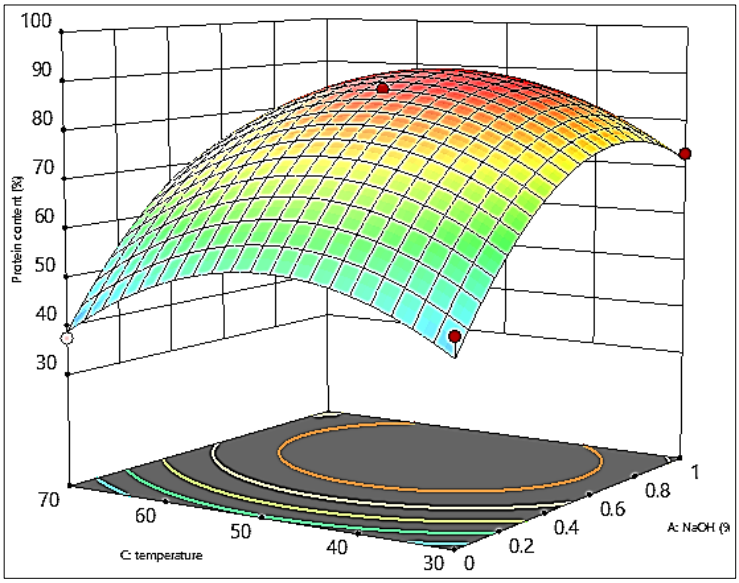

Fig 8: 3D plot for protein content (Y_1) as a function of alkali concentration (X_1) and extraction temperature (X_3)

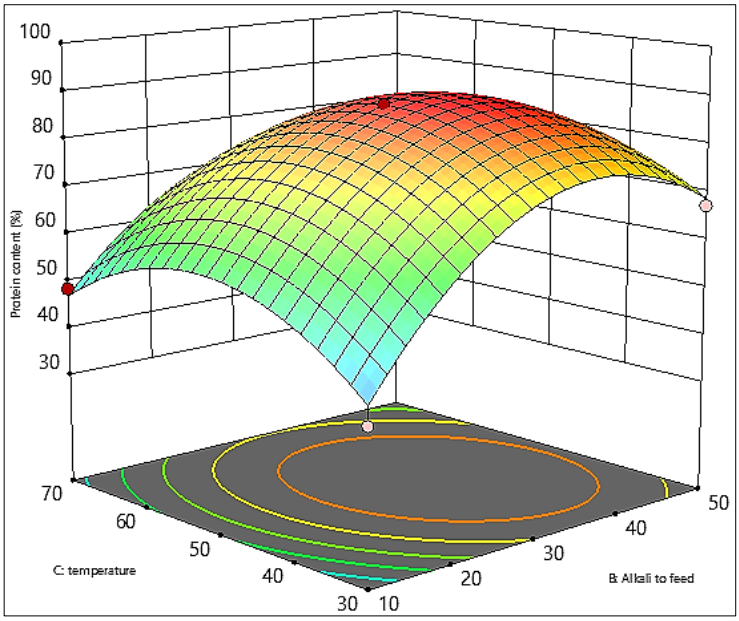

Fig 9: 3D plot for protein content (Y_1) as a function of alkali to feed ratio (X_2) and extraction temperature (X_3)

Alkali concentration had a greater influence on protein content (X_1 = +15.36) as it was found to increase with increasing alkali concentration (fig. 7) which might be due to salting in effect of low NaOH concentration on protein solubility (Eromosele *et al*. 2008) [13]. The results showed that with an increase in temperature from 30 to 50 ºC, the protein content was improved gradually (52.52-87.54%) and then decreased (fig 8). Appropriate heat treatments might partially break down hydrogen and disulfide bonds, resulting in an improvement in protein dissolution rate which may lead to protein isolate with higher protein content when temperature is increased later on heat denaturation of the proteins may have happened (Atra *et al*. 2005; Hadidi *et al*. 2020) [5, 17].

The protein content of velvet bean protein extraction was found to increase as the alkali/feed ratio increases up to about 30 mL/g (46.65-87.54%) and decreases when the ratio goes beyond this (fig 9). This effect may be attributed to an improved driving force for the protein mass transfer, which improves the solvent diffusivity into cells and enhances the protein desorption from the cells (Bedin *et al*. 2020) [7]. Although, at higher alkali/feed ratio, the high-water polarity can cause break down between electrostatic interaction and hydrogen link of hydrophilic side chains of protein molecules (Feyzi *et al*. 2015) [15].

4. Conclusion

The wild legume, velvet bean is an underutilised legume that can be thought to an alternative for plant-based protein source and the results obtained concludes the same. Depending on the optimization results alkali concentration of 0.579%, alkali to feed ratio 50ml and temperature 45.684 ºC was found to have high protein content of 81.460% with yield of 24.862%. Further exploration of the study is required to establish the isolate into a food model so as to reach the benefit of plant-based protein alternative to human.

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