CHAPTER-III
MATERIALS AND METHODS

This chapter deals with the selection of raw materials for the study, procedure followed for determination of proximate composition of pigeon pea grains, oil and enzymatic pre-treatments, determination of protein content and cooking quality of treated dhal.

3.1 Location

All the experiments were carried out in the Department of Processing and Food Engineering, College of Agricultural Engineering & Technology, Junagadh Agricultural University, Junagadh.

3.2 Selections of Variety and Procurement

BSMR 736 variety was procured from Marathawada Agriculture University, Parbhani (Maharashtra) for the experimental work.

3.2.1 Cleaning and grading

The pigeon pea seeds were sorted to remove extraneous materials such as dust, dirt, stones, chaff, immature grains, and insect eaten and broken seeds. The cleaned seeds were then graded by manually. Cleaned and well graded seeds haves were weighed 1 kg using digital weighing balance (Mettler, model PE 360) with an accuracy of ± 0.01g in plastic bags for each enzymatic pre-treatment.

3.2.2 Moisture content

The initial moisture content of pigeon pea seed sample was determined by hot air-oven methods reported by Sahay and Singh (1994). One stage procedure was followed for samples with moisture content less than 13 %. The samples about 9 to 10 g each were weighed and placed in to 2 to 3 petri dishes. The samples in a petri dish were placed inside the hot air oven (Scientronic-universal oven) at 100 ± 1 °C till it got constant weight (about 3 h). The samples were cooled and weighed. The difference in the initial and the final weights of the sample was taken as the moisture content using following formula.
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\[
\text{Moisture content (\% w.b.)} = \frac{\text{Initial wt. of sample} - \text{Oven dried wt. of sample}}{\text{Initial wt. of sample}} \times 100 \quad \ldots (3.1)
\]

3.3 Proximate Composition of Pigeon Pea Grain

The proximate composition of pigeon pea grains, \textit{viz.}, carbohydrate, protein, fat, crude fibre and ash content were determined at 12.45 \%, (w.b.) moisture content as described in the subsequent sections here under.

3.3.1 Carbohydrate

Carbohydrate was determined by using Phenol sulphuric acid method reported by Sadasivam and Manickam (1992). 0.1 g of pigeon pea dhal powder was hydrolysed by keeping in water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. Working standard was then pipette out into 0.2, 0.4, 0.6, 0.8 and 1 ml in series of test tubes and make up to 1 ml. Sample was analysed in duplicate for 0.1 & 0.2 ml in separate tubes. Blank was set by 1 ml of water. 1 ml of phenol and 5 ml of sulphuric acid 96 \% was then added to each tube, shaken well and colour was read at 490 nm by using absorption type UV-VIS spectrophotometer (Systronics Model: UV-VIS 108). Standard graph was then used for calculating amount of carbohydrate.

\[
\text{Total carbohydrate (\%) = \frac{\text{Graph factor} \times \text{Optical density} \times \text{Total volume} \times 10^{-6}}{\text{Aliquot taken}} \times 100} \quad \ldots (3.2)
\]

3.3.2 Protein

Protein content of pigeon pea grains as well as pigeon pea dhal was estimated as per the method suggested by Lowry \textit{et al.} (1951). Weigh 0.1 g of sample (Plate 3.1), centrifuge it with 10 ml of buffer and use the supernatant for protein estimation. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tube. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank. Add 5 ml of reagent C to each tube including the blank. Mix well and allow standing
for 10 min. Then add 0.5 ml of reagent D, mix well and incubate at room temperature in the dark for 30 min. Blue colour is developed. Take the reading at 660 nm using spectrophotometer. Draw a standard graph and calculate the amount of protein in the sample.

\[
\text{Protein (\%)} = \frac{\text{Graph factor } \times \text{ Optical density } \times \text{ Total volume of buffer (ml)}}{\text{Volume taken from extracts (ml)} \times \text{ weight of sample (gm)}} \times 100
\]  

\[\ldots (3.3)\]

**3.3.3 Oil**

Oil content was determined by soxhlet method (Socs plus, Model: SCS 06 AS DLS) for fat content of weigh 2 g of pigeon pea grains powder and transfer it to the cellulose thimble (Plate 3.3). Take the initial weight of the beaker, where the fat or oil is extracted. Fix the thimble to the thimble holder; place the thimble into the beaker. Add 80 ml of solvent (petroleum ether, boiling point temperature 40-60 °C) and insert the beaker with solvent in the extraction system. Ensure proper sealing of beaker. Connect the water inlet and start the water flow through the system. Switch on the power and set 80 °C temperature for first step of one hour’s boiling and 120 °C for second step of half hour’s evaporation of solvent. When the complete evaporation of solvent is done take out the beaker from the system, take out the thimble from the beaker. Keep the beaker inside the hot air oven for few minutes to remove solvent vapours. Take the final weight of the beaker and calculate the oil content of sample taken in thimble using following equation. Calculate fat content of pigeon pea grains powder.

\[
\text{Oil content of powder (\%)} = \frac{(\text{Weight of flask + oil}) - \text{Weight of flask (g)}}{\text{Weight of sample (g)}} \times 100
\]  

\[\ldots (3.4)\]

**3.3.4 Crude Fibre**

Fibretherm Gerhardth plus instrument was used for estimation of crude fibre content (Plate 3.4). Pigeon pea dhal sample was treated with 1.25 % H\textsubscript{2}SO\textsubscript{4} followed by 1.25 % NaOH and washed thoroughly with distilled water after each treatment. Neutral residue left over was dried, weighed and then ignited into muffle furnace.
From the loss in weight of residue, the percentage of crude fibre was calculated using following formula given by Ranganna (1986).

\[
\text{Crude Fibre, } \% = \frac{W_2 - W_3}{W_1} \times 100
\]  

\[\text{... (3.5)}\]

Where,

\[W_1 = \text{Initial sample weight, g}\]
\[W_2 = \text{Weight of digested sample, g}\]
\[W_3 = \text{Weight of ash, g}\]

3.3.5 Ash

Ash content was determined by keeping 5 g of sample placed in silica dish in muffle furnace (Meta Instruments, Mumbai) at 600 °C until grey coloured ash is occurred to a constant weight (Plate 3.5). The ash was weighted after short time of cooling (Anon., 2012). The ash content was calculated by

\[
\text{Ash (\%)} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \times 100
\]

\[\text{... (3.6)}\]
3.4 Dry Milling Method Followed as Control

Generally, the dry method is followed throughout the Indian subcontinent for milling of pigeon pea. Hence, for the comparison of enzymatic pre-treatment, the dry milling method was considered as control. The optimum operating speed and feed rate of the dehusking machine were 1420 rpm and 64 kg/h, respectively. The cleaned and size graded grains were pitted through dehusking roller machines. Thereafter, mustard oil was used for oil treatment and applied @ 0.5 kg oil per 100 kg pigeon pea grains (Patel et al., 2000). For 1 kg pigeon pea grains 5 g mustard oil was mixed and kept in glass bottle for 36 h for diffusion of oil (Plate 3.6). After 36 h, the water was sprayed (50 g/ 1 kg grain) on the grain and heaped for 12 h. subsequently after tempering, the grains were dried in a mechanical dryer (tray dryer) at 60 °C up to a moisture content of 10 ± 0.5 % (w.b.). This sequence of operation was repeated three to four times. The samples were then milled in a dehusking machine at optimum operating conditions. During each dehusking operation, husk, powder and broken were
Materials and methods

Separated from dehusked split dhal for the analysis of hulling efficiency. Dehusked splits obtained in this operation were considered as ‘second grade’ because their edges were not sharp and usually rounded-off by scouring. The process flow chart following during the experiment of milling of pigeon pea grains is given in Fig. 3.1

![Flow chart for dry milling method of pigeon pea grains](image-url)

Fig. 3.1 Flow chart for dry milling method of pigeon pea grains
3.5 Selection of Enzymes

Enzyme is a biocatalyst and they speed up biochemical reactions without being consumed in the process. All enzymes are proteins. The most distinguishing property of enzyme in its catalytic action is its specificity and selectivity. Enzymes are selective for their substrates and speed up only a specific reaction. Therefore, the selection of enzymes was based on the chemical composition and binding substances present between husk and cotyledon of pigeon pea grain.

The xylanase enzyme being widely used as bio leaching agent for lignin isolation. Cellulase and pectinase break down cellulose to beta-glucose and pectinase to pectinic acid and finally pectic acid. The xylanase, cellulose and pectinase are the key enzymes that break the binding materials leading to increase the dehulling efficiency.

3.5.1 Procurement of enzymes

Commercial food grade enzymes selected for enzymatic treatment were obtained from their manufactures. The xylanase was procured from Advanced Enzyme Technologies Ltd., Thane (Maharashtra). While Cellulase and Pectinase enzymes were obtained from HiMedia Laboratories Pvt. Limited, Mumbai (Maharashtra) as shown in Plate 3.7.
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Plate 3.7 Enzymes in plastic bottle packages

3.5.2 Enzymatic pre-treatment

The enzyme solution was prepared at the standardized proportion of all the three selected enzymes. The process flowchart of enzymatic pre-treatment is given in Fig. 3.2 for milling of pigeon pea. In case of enzymatic pre-treatment, the degumming may be due to the action of different enzymes used for pre-treatment, i.e., xylanase, pectinase and cellulase (2: 1: 1). The combination of these three enzymes at different proportion (Sangani et al., 2014) reacts on the gums which is basically a polymer of sugar and acid. By the action of enzymes, the polymeric compound degrades to monomeric units resulting in loosening of the husk.

pH adjustment: The distilled water was mixed with 37 % hydrochloric acid to adjust pH 4 to 6 with an interval of 0.5 pH (Yoo, 2007). Digital pH meter (Eutech instrument, Model: 700) was used to adjust required pH range (Plate 3.9).

Preparation of enzyme solution: The required amount of pH water solution was calculated using the formula 3.7 and required amount of enzymes were weighed and dissolved in pH water solution. The enzymes were dissolved completely before adding to the pigeon pea grains. The procedure for calculation of weight of the three enzymes required for preparation of different enzyme concentrations are given in Appendix-A.

\[ W_1 \frac{(M_f - M_i)}{(100 - M_f)} = \ldots (3.7) \]

Weight of water added (g) = \[ W_1 \frac{(M_f - M_i)}{(100 - M_f)} \]

Where,

\( W_1 = \) Initial weight of sample (g)

\( M_i = \) Initial moisture content, % (w.b.)
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\[ M_f = \text{Final moisture content, } \% \text{ (w.b.)} \]

For each experiment, 1 kg grains was taken in a stoppered glass bottle and calculated amount of enzyme solution was mixed to increase the initial moisture content from 12.45 % (w.b.) to the desired level moisture content 26.0 % (w.b.). Glass bottle after putting its stopper was shaken with enzyme solution for 3 minutes for equilibrium moisture. The samples were incubated in a humidity oven at 35-55 ± 0.5 °C and incubation time (4-12 h). After incubation, the samples were dried in a tray dryer at 60 °C to inactive enzyme and to reduce the moisture content to 10 ± 0.5 % (w.b.). Enzyme treated samples in thin layer (25 mm) were spread on a wire mesh tray.
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**Distilled water (183.10 ml)**

- **pH adjustment (4.0 to 6.0 pH)**
  - **HCL 36.46 %**

- **Addition of enzymes (175.1 to 437.75 mg for Concentration of 20 to 50 mg/100 g dry matter)**

  - **Enzyme solution**

  - **Addition of enzyme solution (183.10 ml)**

- **Moisture equilibrium 26.0 % (w.b.) m.c**

- **Enzyme concentration (20 to 50 mg/100g dry matter)**

  - **Enzyme treated sample**

  - **Incubation for hydrolysis**
    - **Temperature 35 to 55°C**
    - **Incubation time 4 to 12 h**

  - **Drying (Tray dryer)**
    - **60 °C, 10 ± 0.5 % (d.b.) m.c.**

  - **Dehusking and splitting**
    - **Using dhal mill**

  - **Separation**
    - **Broken, husk, unhulled grains, powder**

  - **Dhal**

Fig. 3.2 Flow chart for enzymatic pre-treatment and milling of pigeon pea
3.6 Milling of Sample

Enzymatic pre-treated and control samples of 1 kg weight having about 10 ± 0.5 % moisture content (w.b.) were milled using laboratory dehusking machine/dhal mill. Samples were milled at the standard settings of the machine, i.e., 1420 rpm operating speed and 64 kg/h feed rate. After milling, all the fractions were collected in polyethylene bag. Each of the samples was milled separately and care was taken to obtain all the fractions without any loss using a cleaning brush.

3.6.1 Dehulled Sample Separation

The different fractions of the milled product were separated by suitable sieves and hand picking such as whole dehulled grains, split dehulled grains, partly dehulled and unhulled grains, broken, husk and powder (Plate 3.13). A grain was considered completely dehulled when there was no husk adhering to it.

3.6.2 Husk Content

The husk (seed coat) content in whole grain was determined by soaking approximately 10 g of pigeon pea grain in distilled water (2 h at 50 °C). The seed coats were then separated manually from the cotyledons, dried in a hot air oven at 100 ± 5 °C up to initial moisture content of 10.40 % (w.b.) (Bharodia, 2004). The data regarding husk content are presented in Appendix B. The husk content in % was calculated using the following formula 3.8

\[
\text{Husk content, } \% = \frac{\text{Wt. of husk}}{\text{Wt. of pigeon pea grain}} \times 100 \quad \ldots (3.8)
\]

3.6.3 Dehulled Fractions

All the fractions were weighed accurately using digital weighing balance with an accuracy of ± 0.01g. (Mettler, model PE 3600) Following equations were used to calculate dehulled fractions obtained by dehulling treatments Singh et al. (2004).

\[
\text{Coefficient of hulling (Ch)} = 1 - \frac{\text{Wuh}}{\text{Wth}} \quad \ldots (3.9)
\]
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Coefficient of wholeness of kernel \((C_{wk})\) = \(\frac{W_{fp}}{W_{fp} + W_{br} + W_{po}}\) … (3.10)

Where,

\[\begin{align*}
W_{uh} &= \text{Weight of unhulled grain after milling (g)} \\
W_{th} &= \text{Weight of grain used for milling (g)} \\
W_{fp} &= \text{Weight of finished product (g) (Splits and whole dehulled grain)} \\
W_{br} &= \text{Weight of brokens (g)} \\
W_{po} &= \text{Weight of powder (g)}
\end{align*}\]

The hulling efficiency was determined using eq. 3.10

\[
\text{Hulling efficiency (HE)} = Ch \times C_{wk} \times 100 \quad \text{…. (3.11)}
\]

### 3.7 Cooking Time

Pigeon pea dhal samples obtained through various enzymatic treatments and dry milling method (control) were cooked in a beaker on heating mortal (EIE instrument, model : EIE 070318) having a ratio of dhal: distilled water as 1: 10. For determination of cooking time, distilled water was heated to boiling point in a 150 ml beaker and then 15 g dhal was added. During boiling, the level of water was maintained by regular addition of boiling water. Boiling was continued and samples were drawn at 1 min interval to check the level of cooking by pressing between the thumb and the forefinger till no hard core is left (Plate 3.14) as described by Singh et al. (1984), Full cooking time was recorded as the time when 90 % of the dhal were soft enough to masticate (Williams and Singh, 1987).
Plate 3.12 Different fractions of pigeon pea grains

A. Cooking test                              B. Check the level of cooking by pressing between thumb and forefinger

Plate 3.13 sample drawn for cooking test

3.8 Experimental Design

The effects of four independent variables *viz.*, enzyme concentration, incubation time, incubation temperature and tempering water pH on hulling efficiency, protein content and cooking time were studied with variables coded as $X_1$, $X_2$, $X_3$ and $X_4$, respectively. Table 3.1. The levels of parameter values were carefully chosen based on the literature available on the enzymatic hydrolysis of pigeon pea grain. Four response variables, *viz.*, husk removed, hulling efficiency, protein content and cooking time were determined for optimization of the process. Response Surface
Methodology (RSM) was used for designing the experiments. A Central Composite Rotatable Design (CCRD) of 4 variables at 5 levels each with 6 center point combinations were used (Khuri and Cornell, 1987). Altogether, 30 combinations (including 6 replications at the center point and single observation at other points) were chosen according to a Central Composite Rotatable Design. The coded and uncoded variable values of the design are presented in Table 3.3 and Table 3.4.

### Table 3.1 Treatment details

<table>
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<th>Levels</th>
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<td>Enzyme concentration (X₁)</td>
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</tr>
<tr>
<td>2</td>
<td>Incubation time (X₂)</td>
<td>4, 6, 8, 10 and 12 h</td>
</tr>
<tr>
<td>3</td>
<td>Incubation temperature (X₃)</td>
<td>35, 40, 45, 50 and 55 °C</td>
</tr>
<tr>
<td>4</td>
<td>pH (X₄)</td>
<td>4.0, 4.5, 5.0, 5.5 and 6.0</td>
</tr>
</tbody>
</table>

#### Total number of treatment combinations

\[= (2)^{\text{No. of variables}} + (2 \times \text{No. of variables}) + \text{Central points} \tag{3.12}\]

No of variables: 4

Total number of treatment combinations \[= 2^4 + (2 \times 4) + 6 = 16 + 8 + 6 + \text{Control} = 31\]

Five different levels for each treatment combinations in coded form are: \(-\alpha, -1, 0, +1, +\alpha\)

Where, \(\alpha = 2^{\text{No. of variables}/4} = 2^{4/4} = 2\)

The relationship between the coded and actual values of a factor is given by

\[\text{Coded value } = X_i = \frac{2 \left( X_i - \bar{X} \right)}{R_i} \tag{3.13}\]

Where,

\(X_i = \) Actual setting in the uncoded units of the \(i^{th}\) factor

\(\bar{X} = \) Average of low and high settings for the \(i^{th}\) factor

\(R_i = \) Range between the low and high settings
Table 3.2 Coded and uncoded Parameters Levels

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<td>Enzyme concentration, mg/100 g dry sample (X₁)</td>
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<td>pH (X₄)</td>
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Table 3.3 Different treatment combinations of variables

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<th>X₂</th>
<th>X₃</th>
<th>X₄</th>
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3.8.1 Data analysis and optimization

The CCRD design was used to conduct experiments and the Response Surface Methodology (RSM) was applied to the experimental data using a commercial statistical package, Design Expert – version DX 7.0.0.6. Analysis of variance (ANOVA) was conducted for fitting the model represented by Eq. 3.15 to examine the statistical significance of the model terms. Model analysis with respect to lack-of-fit test and $R^2$ (co-efficient of determination) was done for determining adequacy of model. The co-efficient of variation (CV) was calculated to find the relative dispersion of the experimental points from the prediction of the model. Response surfaces were generated and by using the same software, numerical optimization was done. The most commonly used model for optimization using response surface methodology is a second order polynomial equation (Bas and Boyaci, 2007). The model is of the form:
Table 3.4 Different treatment combinations of variables

<table>
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<th>Treat. No.</th>
<th>Coded variables</th>
<th>Uncoded (Real) variables</th>
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<td>42.5  10  50  5.5</td>
</tr>
<tr>
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$Y_k = b_{k0} + \sum_{i=1}^{3} b_{ki}X_i + \sum_{i=1}^{3} b_{kii}X_i^2 + \sum_{i\neq j=1}^{3} b_{kij}X_iX_j$ .... (3.14)

(k = 0, 1, 2, 3….)

Where, $Y_k$ is the response, $b_{k0}$, $b_{ki}$, $b_{kii}$ and $b_{kij}$ are the constant, linear, quadratic and cross-product regression coefficients, respectively and $X_i$’s are the coded independent variables.

3.8.2 Validity Test

The optimum conditions obtained through statistical analysis was verified by conducting the experiment in triplicates. The average value of dehulling efficiency, protein content and cooking time were considered for the validation.