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REDUCTION OF ANTINUTRIENTS IN PEARL MILLET (*Pennisetum glaucum)* USING HURDLE TECHNOLOGY

Logasaranya S. M.[1*], Mahesh Kumar S.[1], Periyar Selvam[1]

[1]*Department of Food Process Engineering, SRM University, Tamil Nadu, India.*
*Corresponding author:*logasaranya@yahoo.co.in*

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ABSTRACT

Pearl millet (*Pennisetum glaucum*) is a staple food that supplies a major proportion of nutrition to large segments of the population living in Africa and Asia. The grain is nutritious but has some limitations due to the presence of antinutritional factors. Processing methods such as drying, washing, soaking, germination and autoclaving were used to reduce the antinutrients. The antinutrients analyzed were tannins, oxalates, polyphenols and phytic acid, which were found to reduce post processing. Germination, soaking and autoclaving reduced the level of antinutrients significantly. Hurdle technology was employed to study the combined effect of processing techniques and analyze the synergistic effect on the degradation of antinutrients. The individual treatments were assessed and the most suitable techniques for combination were found to be germination, soaking and autoclaving which were coupled with drying as the end technique to degrade the antinutrients and increase the *In Vitro* Protein Digestibility. Soaking + Germination + Drying combination degraded the antinutrients to the maximum level and increased IVPD from 51.032% to 66.571%.

1. Introduction

Pearl millet (*Pennisetum glaucum*) is a staple food in many parts of India because of its high protein content. The crop is adaptive and can survive in regions that are arid and production is likely to increase due to global warming. The grain is nutritious and is known to have higher protein and energy levels than maize and sorghum. However, the presence of antinutrients limits the absorption into the system. Antinutrients are known to interfere with carbohydrate, mineral bioavailability and protein digestibility (Pushparaj et al., 2001). Maximum utilization of the nutrient potential of the millet is limited by the presence of phytates, phenols, tannins, and enzyme inhibitors (Ramachandra et al., 1977). Domestic treatments had proved to improve the nutritional content of beans (Kataria et al., 1989). Protein digestibility is a measure of susceptibility of protein for the process of proteolysis. Protein with high digestibility is potentially of better nutritional value, as amino acids for absorption on proteolysis is high. Studies have shown that tannins contribute to lower nutritional value of dietary proteins by reducing the bioavailability of proteins to the body. Minerals and proteins bind with the antinutrients thereby retarding the digestibility (Reddy et al., 1994). Polyphenol content of pearl millet is considerably high which affects the mineral bioavailability and protein digestibility of grains.

Studies have proved that processing techniques reduce the level of antinutrients in pearl millet. Various physical and chemical treatments are used to reduce the antinutritional factors such soaking, germination, irradiation, roasting, drying, washing and fermentation. Fermentation had proved to increase the primary nutrients in finger millet (Antony et al., 1996). The antinutritional factors are generally found on the outer portion of the grain (Chavan et al., 1989). Decortication significantly decreases the amount of tannins with a corresponding increase in protein digestibility (Irén Léder, 2004).

Soaking and germination had been found to reduce the antinutrients in pearl millet (Obizoba et al., 1994). Pearl millet does not contain gluten hence it can be advised for patients suffering from Celiac disease. The low Glycemic Index (GI) of the grain is known to help in dealing with diabetes. The application of a single processing technique is frequently insufficient for the effective treatment hence combination of treatments is preferred.

Hurdle technology is used to reduce the level of antinutrients in the millet by employing multiple processing techniques that can reduce the antinutrients to the maximum level. After the primary processing, the results were analysed and the most suitable combinations were used to reduce the level of antinutrients. Soaking, germination and autoclaving were the most effective treatments against antinutrients hence they were applied in sequence. Drying was used as the end technique in all the combinations. In Vitro Protein Digestibility (IVPD) was used to assess the enhanced level of protein in the grain after various treatments and to analyze the amount of protein that can be absorbed by the body.

2. Materials and methods

Pearl millet samples were purchased in bulk from Chennai market. The raw material was then cleaned and extraneous materials were removed.

DRYING

The raw pearl millet samples were subjected to heating in a tray drier under constant temperature. The temperatures include 40°C, 50°C, 60°C, 70°C and 80°C. The dried samples were then cooled in a dessicator, ground and packed in an aluminium pouch. The samples were stored under dry conditions to avoid moisture absorption.

SOAKING

The raw pearl millet samples were subjected to soaking in distilled water in the ratio 1:3. The soaking times were 6, 12,18,20,22 and 24 hours. The soaked samples were then dried at 50°C for 3 hours and ground. The powdered samples were stored in air- tight aluminium pouches.

GERMINATION

The millet samples were initially soaked in water for 12 hours. After soaking, the samples were lined on sterile petri dishes lined with filter paper and sprouted for various time durations. The germination times are 20, 24, 28, 32, 36 and 40 hours. The sprouted samples were then dried at 50°C for 3 hours and ground. The powdered sample were sieved and stored in air- tight aluminium packets.

AUTOCLAVING

The raw samples were weighed and autoclaved at a pressure of 15psi for 15 minutes. The dry samples were allowed to cool in a dessicator, ground and stored for further analysis. The powdered samples were stored in air- tight aluminium pouches.

WASHING

The raw sample was soaked in water for 2 minutes and washed with distilled water. The washing times are 2, 4, 6, 8 and 10 minutes. After the washing procedure the samples were dried in tray-drier at a temperature of 50°C for 3 hours and ground. The powdered samples were stored in air- tight aluminium pouches.

EXPERIMENTAL TREATMENTS

The combination of individual processing techniques tested were,

Soaking + Autoclaving + Drying
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Germination + Autoclaving + Drying
Germination + Soaking + Drying

Experiments were performed in triplicates and samples were

taken immediately after processing and stored in air tight pouches.

2.1.Analytical methods

2.1.1.Estimation of tannins

Determination of tannins based on the method of A.O.A.C (1975). Accurately weighed 0.5 g of the powdered material was transferred to a 250ml conical flask. Add 75ml water. Heat the flask gently and boil for 30 minutes. Centrifuged at 2,000 rpm for 20 minutes and collect the supernatant in 100 ml volumetric flask and make up the volume. Transfer 1ml of the sample extract to a 100ml volumetric flask containing 75 ml water. Add 5 ml of Folin-Denis reagent, 10ml of sodium carbonate solution and dilute to 100 ml with water and shaken well. Read the absorbance at 700 nm after 30 minutes. The

tannin concentration was determined by the standard graph of tannic acid solution.

2.1.2.Estimation of oxalates

Oxalate was determined by using the method of Sanchez-Alonso and Lachica (1987). Exactly one gram of the sample was placed in 250 ml volumetric flask, 190 ml of distilled water and 10 ml of 6M HCl were added. The mixture was then warmed in a water bath at 90°C for 4 hours and the digested sample centrifuged at 2,000 rpm for 5 min. The supernatant was then diluted to 250 ml. Three 50 ml aliquots of the supernatant was evaporated to 25 ml, the brown precipitate was filtered and washed. The combined solution and washings were then titrated with concentrated ammonia solution in drops until the pink colour of methyl orange changed to yellow. The solution was then heated in a water bath to 90°C and the oxalate was precipitated with 5% CaCl2 solution was allowed to stand overnight and then centrifuged, precipitate was washed with hot 25% H_2SO_4, diluted to 125 ml with distilled water and titrated against 0.05 M KMnO4.

Calculation:

1 ml 0.05 M KMnO4 = 2.2 mg Oxalate

Total amount of protein = (concentration) x (total volume of extract)

(1)

2.1.3.In vitro protein digestibility

In vitro protein digestibility was determined by calculating the difference between the amount of nitrogen in the sample before and after hydrolysis with pepsin (AOAC, 1965). Two hundred milligrams of whole seed or dehulled finger millet flour was incubated with 50 ml of 0.2% pepsin in 0.075 N HC1 for 24 h at 37°C. Digestion was performed in duplicate. The digests were filtered through Whatman No. 2 filter paper, and the residue was washed with warm water on the filter. Nitrogen in the residue was estimated by the micro- Kjeldahl method. IVPD was obtained by calculating the difference between the amount of total nitrogen in the sample before and after in vitro digestion with pepsin. Kjeldahl nitrogen was multiplied by the factor 6.25 to obtain crude protein.

2.1.4.Determination of protein content

The protein content was determined by the Lowry's method. BSA was used as the standard. Take 0.2 ml of BSA working standard in 5 test tubes and make up to 1 ml using distilled water. The test tube with 1 ml distilled water serves as blank. Add 4.5 ml of Reagent I and incubate for 10 minutes. After incubation, add 0.5 ml of reagent II and incubate for 30 minutes. Measure the absorbance at 660 nm and plot the standard graph. Estimate the amount of protein present in the given sample from the standard graph (Wilson and Walker 2000).

2.1.5.Determination of phytic acid

Phytic acid was determined by the procedure of Lucas and Markakas (1975). Two gram of sample was weighed into a 250 ml conical flask. One hundred ml 2% concentrated hydrochloric acid was used to soak sample for 3 hours and then filtered with a Whatmann No. 1 filter paper. Fifty ml of the filtrate and 10 ml of distilled water were added in each case to give proper acidity. Ten ml 0.3% ammonium thiocyanate solution was added into the solution as indicated and titrated with standard Iron II Chloride solution containing 0.00195 g Iron/ml, end point observed to be yellow which persisted for 5 min. The percentage phytic acid was calculated thus:

$$\% \text{ Phytic acid} = y \times 1.19 \times 100 \quad \text{where,}$$

$$y = \text{titre value} \times 0.00195 \text{ g}$$

(2)

2.1.6.Determination of polyphenols

The concentration of phenolic in sample extracts was determined using Singleton et al., 1999 method. Ethanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of ethanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45˚C for 45 min. The absorbance was determined using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance

was obtained. The same procedure was repeated for the standard solution of tannic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of Gallic acid equivalent (mg of GAE/g of extract). (Milan and Tanković, 2011).

2.1.7.Statistical analysis

Each determination was carried out on three separate samples and analyzed in triplicates. Regression and statistical analysis were performed using SPSS. Significance was accepted at $P \leq 0$.

3. Results and discussions

3.1. Effect of processing on the Protein and Tannin content of pearl millet

The processing techniques had shown to reduce the level of antinutrients in pearl millet. Germination and soaking have been the most effective methods. The reduction of tannins was observed the most in germination followed by soaking. The level of tannins is inversely proportional to the protein content. Germination the seeds for 36 hours had the highest quantity of protein. Soaking the grains for 24 hours reduced the tannin content effectively while drying had very less effect on the increase in protein content. Drying at 70°C showed low tannin content but increase in temperature can lead to the denaturation of protein hence temperature beyond 80°C was not preferred. High pressure cooking had also shown reduction of tannins and increase in protein but the levels were lower than that of germination. Washing the pearl millet had very little changes with respect to the tannin content.

Table 1. Effect of Drying on the Antinutrients and *In Vitro* Protein Digestibility (%) of Pearl millet

TEMPERATURE (°C)	PROTEIN (g/100g)	TANNINS (%)	OXALATES (mg/100g)	PHYTIC ACID (mg/100g)	POLY-PHENOLS (mg/100g)	IVPD (%)
UNPROCESSED	**7.213 ± 0.189**	**21.896 ± 0.014**	**2.42 ± 0.103**	***686 ± 0.014**	**304 ± 0.156**	**51.032 ± 0.024**
40°C	7.397 ± 0.119	21.311 ± 0.026	1.69 ± 0.00	679 ± 0.145	297 ± 0.293	52.417 ± 0.149
50°C	7.471 ± 0.519	20.940 ± 0.008	1.54 ± 0.103	650 ± 0.261	284 ± 0.137	53.963 ± 0.317
60°C	7.619 ± 0.361	20.647 ± 0.035	1.54 ± 0.00	627 ± 0.797	277 ± 0.419	54.414 ± 0.213
70°C	7.728 ± 0.174	20.392 ± 0.035	1.32 ± 0.00	604 ± 0.148	270 ± 0.145	54.890 ± 0.119
80°C	7.998 ± 0.213	19.978 ± 0.004	1.1 ± 0.00	582 ± 0.452	262 ± 0.352	55.287 ± 0.172

Values are means ± SD of three independent determinations Significant difference ($p \leq 0.05$) exist

3.2.Effect of processing on the Phytic acid content of pearl millet

The unprocessed pearl millet had considerable quantity of phytic acid. Phytic acid plays a significant role in deciding the nutritive value of pearl millet. The sprouted samples had higher levels of protein when compared to the control. Sprouts had low concentration of phytic acid and the lowest level was observed at 36 hours at 30°C.

A significant reduction in phytic acid level was observed by soaking the sample for 6 hours and the maximum at 24 hours.

Reduction in phytic acid concentration during soaking is due to the hydrophilic nature of phytates (Duhan et al., 1989).

Treating the millet sample at high pressure (15psi), were found to lower the level of phytic acid to a significant level while washing had negligible changes in the phytic acid content. The reduction of phytic acid after autoclaving could be due to the breakdown of phytic acid at high temperature. Drying at 80°C had effect on the levels of phytic acid but the preferred temperature was 70°C to avoid browning of pearl millet.

Table 2. Effect of Soaking on the Antinutrients and *In Vitro* Protein Digestibility (%) of Pearl millet

SOAKING TIME (hours)	PROTEIN (g/100g)	TANNINS (%)	OXALATES (mg/100g)	PHYTIC ACID (mg/100g)	POLY-PHENOLS (mg/100g)	IVPD (%)
UNPROCESSED	**7.213 ± 0.189**	**21.896 ± 0.014**	**2.42 ± 0.103**	**686 ±0.0149**	**304 ± 0.156**	**51.032 ± 0.024**
6	7.668 ± 0.213	21.224 ± 0.011	1.11 ± 0.103	653 ± 0.341	290 ± 0.268	52.129 ± 0.251
12	7.821 ± 0.124	20.909 ± 0.019	0.88 ± 0.00	617 ± 0.145	268 ± 0.134	52.972 ± 0.0139
18	8.119 ± 0.279	20.789 ± 0.016	0.88 ± 0.103	597 ± 0.296	251 ± 0.335	53.867 ± 0.233
20	8.327 ± 0.147	20.409 ± 0.005	0.66 ± 0.103	585 ± 0.167	242 ± 0.148	55.259 ± 0.161
22	8.551 ± 0.824	19.769 ± 0.010	0.44 ± 0.00	569 ± 0.172	221 ± 0.162	56.924 ± 0.217
24	8.671 ± 0.159	19.486 ± 0.014	0.44 ± 0.00	542 ± 0.214	208 ± 0.110	58.526 ± 0.156

Values are means ± SD of three independent determinations. Significant difference ($p \leq 0.05$) exist

3.3.Effect of processing on the Polyphenol content of pearl millet

The polyphenolic content in the sample reduced significantly with germination, drying, soaking and autoclaving. Autoclaving the sample decreased the level of polyphenols from 404 mg/100g to 286 mg/100g. Soaked and sprouted samples also had reduced polyphenolic values.

Table 3. Effect of Germination on the Antinutrients and *In Vitro* Protein Digestibility (%) of Pearl millet

GERMINATION TIME (hours)	PROTEIN (g/100g)	TANNINS (%)	OXALATES (mg/100g)	PHYTIC ACID (mg/100g)	POLYPHENOLS (mg/100g)	IVPD (%)
UNPROCESSED	**7.213** ± 0.189	**21.896 ± 0.0143**	**2.42 ± 0.103**	**686 ± 0.0149**	**304 ± 0.156**	**51.032 ± 0.024**
20	8.471 ± 0.176	21.221 ± 0.098	0.88 ± 0.00	651 ± 0.269	286 ± 0.132	56.251 ± 0.072
24	8.508 ± 0.974	20.776 ± 0.026	0.88 ± 0.0	627 ± 0.317	261 ± 0.219	56.682 ± 0.112
28	8.673 ± 0.175	20.201 ± 0.057	0.88 ± 0.103	586 ± 0.524	243 ± 0.135	57.853 ± 0.143
32	8.747 ± 0.110	19.967 ± 0.079	0.66 ± 0.103	559 ± 0.411	239 ± 0.211	58.274 ± 0.092
36	8.964 ± 0.845	19.317 ± 0.053	0.66 ± 0.00	521 ± 0.128	212 ± 0.259	60.129 ± 0.187
40	9.012 ± 0.114	18.993 ± 0.004	0.44 ± 0.103	501 ± 0.169	197 ± 0.249	62.728 ± 0.212

Values are means ± SD of three independent determinations, Significant difference ($p \leq 0.05$) exist

3.4.Effect of processing on the Oxalate content of pearl millet

The oxalate content in pearl millet is less when compared to other antinutrients. Germination and soaking the samples showed the lowest levels of oxalates while washing did not have any significant changes. Autoclaving resulted in reduction from 2.42 mg/100g to 1.10 mg/100g. Increase in temperature caused mild reduction in the level of oxalates in pearl millet sample.

3.5.Effect of processing on the *in vitro* protein digestibility (IVPD) of pearl millet

In Vitro Protein Digestibility of pearl millet increased after germination, the increase in digestibility was observed with increase in germination time. Germination time greater than 36 hours cannot be employed due to the formation of cyanide higher than the permissible limit (Panasiuk *et al.*,1984). Germination has been found to reduce antinutrients like phytic acid in millets (Khetarpaul et al.,1990). Soaking the samples for 24 hours significantly increased the digestibility of protein. High pressure cooking had pronounced improvement in the digestibility.

3.6.Effect of combined processing on the *in vitro* protein digestibility (IVPD) and antinutrients

The samples that were subjected to combination of techniques showed better degradation of antinutrients. Soaking + Germination + Drying, had the highest value of IVPD and low values of antinutrients, followed by Germination + Autoclaving + Drying.

Table 4. Effect of Washing on the Antinutrients and *In Vitro* Protein Digestibility (%) of Pearl millet

WASHING TIME (minutes)	PROTEIN (g/100g)	TANNINS (%)	OXALATES (mg/100g)	PHYTIC ACID (mg/100g)	POLYPHENOLS (mg/100g)	IVPD (%)
UNPROCESSED	**7.213** ± 0.189	**21.896 ± 0.0143**	**2.42 ± 0.103**	**686 ± 0.0149**	**304 ± 0.156**	**51.032 ± 0.024**
2	7.213 ±0.189	21.896 ± 0.0143	2.42 ± 0.103	686 ± 0.014	304 ± 0.156	51.032 ± 0.024
4	7.225 ± 0.121	21.861 ± 0.0167	2.42 ± 0.00	681 ± 0.165	302 ± 0.153	51.347 ± 0.125
6	7.250 ± 0.173	21.837 ± 0.0241	2.42 ± 0.00	673 ± 0.213	296 ± 0.314	51.621 ± 0.219
8	7.311 ± 0.146	21.796 ± 0.0173	2.20 ± 0.00	669 ± 0.137	291 ± 0.239	51.842 ± 0.168
10	7.338 ± 0.129	21.753 ± 0.0142	2.20 ± 0.103	658 ± 0.184	288 ± 0.157	51.998 ± 0.153

Values are means ± SD of three independent determination.No Significant difference exist ($p \geq 0.05$)

Table 5. Effect of Autoclaving on the Antinutrients and *In Vitro* Protein Digestibility (%) of Pearl millet

SAMPLE (PRESSURE)	PROTEIN (g/100g)	TANNINS (%)	OXALATES (mg/100g)	PHYTIC ACID (mg/100g)	POLYPHENOLS (mg/100g)	IVPD (%)
UNPROCESSED	**7.213** ± 0.189	**21.896 ± 0.0143**	**2.42 ± 0.103**	**686 ± 0.0149**	**304 ± 0.156**	**51.032 ± 0.024**
15 psi	7.964 ± 0.345	19.521 ± 0.124	1.10 ± 0.103	602 ± 0.462	286 ± 0.132	54.128 ± 0.192

Values are means ± SD of three independent determinations. Significant difference ($p \leq 0.05$) exist

Table 6. Effect of combined treatment on antinutrients and *In Vitro* Protein Digestibility (%) of Pearl millet

SAMPLE	PROTEIN (g/100g)	TANNINS (%)	OXALATES (mg/100g)	PHYTIC ACID (mg/100g)	POLYPHENOLS (mg/100g)	IVPD (%)
UNPROCESSED	**7.213** ± 0.189	**21.896 ± 0.0143**	**2.42 ± 0.103**	**686 ± 0.0149**	**304 ± 0.156**	**51.032 ±0.024**
Soaking + Autoclaving + Drying	11.267 ± 0.168	18.368 ± 0.015	0.44 ± 0.103	523.102 ± 0.167	207 ± 0.214	63.129 ±0.067
Soaking + Germination + Drying	12.314 ± 0.212	18.779 ± 0.024	0.44 ± 0.103	489.171 ± 0.021	197 ± 0.251	66.571 ±0.182
Autoclaving + Germination + Drying	10.652 ± 0.180	19.327 ± 0.214	0.44 ± 0.00	506.954 ± 0.269	278 ± 0.134	64.375 ±0.015
Autoclaving + Soaking + Drying	9.851 ± 0.014	19.659 ± 0.287	0.66 ± 0.00	541.264 ± 0.154	243 ± 0.049	59.126 ±0.017
Germination + Autoclaving + Drying	11.627 ± 0.216	18.225 ± 0.203	0.44 ± 0.103	492.524 ± 0.583	221 ± 0.127	65.121 ±0.219
Germination + Soaking + Drying	11.983 ± 0.312	19.263 ± 0.381	0.44 ± 0.103	502.352 ± 0.552	219 ± 0.082	64.876 ±0.084

Values are means ± SD of three independent determinations. Significant difference ($p \leq 0.05$) exist

The synergistic effect of treatments had greater influence on the digestibility of protein. Autoclaving + Soaking + Drying, had relatively low value of protein digestibility. Germination + Soaking + Drying also showed significant reduction in antinutrients, but the millet is more prone to microbial contamination in this technique as soaking the seeds after germination may produce unfavorable odour and slimy texture. Treatments like soaking and autoclaving were effective in the reduction of phytic acid and polyphenols in pearl millet (Sharma *et al.*,1996). Soaking + Autoclaving + Drying is one the

traditional methods of processing pearl millet, the antinutrient reduction was significant and the IVPD was high.

4. Conclusions

The samples subjected to individual processing treatments had shown reduced levels of antinutrients and increased protein digestibility. It was observed that combination of techniques showed relatively better results than individual techniques. Combinations containing germination as one of the treatments had maximum desirability. Analyzing the combinations, it can be concluded that Soaking + Germination + Drying, gave the most preferred result of low antinutrients and high *in vitro* protein digestibility.

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