International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 23-09-2020; Accepted: 08-10-2020: Published: 23-10-2020
www.botanyjournals.com
Volume 5; Issue 5; 2020; Page No. 415-420

Impact of soaking, germination and fermentation on phytate in Lentil (*Lens culinaris* Medik.)

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Abstract
Lentil (*Lens culinaris* Medik.) is a rich source of iron and protein but the presence of phytate changes their solubility, functionality, absorption and digestibility**.** The study aimed to investigate the impact of the wet processings as soaking (24 h), germination (72 h) and fermentation (24-72 h) on phytate degradation and consequently, enhancement of iron and protein content in lentil samples. There was considerable reduction in phytate during soaking (11.49%-18.44%), germination (19.70%-36.62%) and fermentation (23.79%-87.29%). On the contrary, there was significant enhancement in their iron contents i.e. (31.33%–92.89%), (57.07%– 279.14%) and (186.82%–759.71%) during soaking, germination and fermentation respectively. Similarly, protein increased considerably, i.e. (5.91%–9.95%), (15.39%– 24.45%) and (17.86%–66.74%) during soaking, germination and fermentation, respectively. The molar ratio of phytate/iron was >1 in dry seeds and <1 after wet processings and lowest after fermentation (72 h). The enhancement initiated with soaking, increased during germination and optimised by fermentation.

Keywords: antinutrient, phytate, iron, soaking, germination, fermentation

1. Introduction
Lentil (*Lens culinaris* Medik.), an Indian origin legume makes a significant part of human diet since the aceramic (non-pottery) Neolithic times because of its high nutritional value [1]. It is a dietary source of of protein (26 g), carbohydrates (60 g), cholesterol-lowering soluble fiber (3.1 g), folate (479 µg), vitamin B_1 (0.87 mg), and minerals, like iron (7.54 mg), calcium (56 mg), phosphorus (451 mg), etc. making it valuable and cheaper for human and animal consumption [2]. It is rich and inexpensive source of proteins of plant origin making it meat substitute in many parts of the world, especially in the Indian subcontinent having large vegetarian population [3, 4]. Apart from this it helps in reducing or preventing chronic diseases like, cardio-vascular diseases, cancers, overweight, obesity and diabetes [5]. Due to this consequence, people's interest has emerged in functional food with nutraceutical value; its consumption has been increasing all around the world [6].
But unfortunately, in spite of its high nutritional value, its acceptability as staple foods is limited because it contains anti-nutritional factors such as, phytic acid, saponins and tannins, gossypol, lectins, etc. [7].
Phytic acid (myo-inositol hexa phosphoric acid, IP6) is the main storage form of phosphate accounting for 60-90% of the total phosphorus in seeds [8]. Because of its negative charge, it acts as chelator of divalent cations (Fe^{2+}, Zn^{2+}, and Mg^{2+}), thereby reducing its bioavailability [9, 10]. In particular deficiency of iron is utmost concern as it causes Iron deficiency anemia (IDA) especially affecting females (women, adolescent girls) of developed and developing countries [11, 12]. National Family Health Surveys (NFHS-II) have reported that diet based on cereals and legumes can reverse the IDA trend in pregnant women (70%), children (70-80%) and adult men (24%) [13]. Lentil appears to be promising staple food to combat deficiency of iron as well as protein if its nutritional quality is improved. Thus, it becomes important to increase the bioavailability of iron and other nutrients in lentil by reducing the antinutritional effect of phytate.
In this regard, phytase [myo-inositol hexakis (di hydro gen phosphate) phosphor hydro lase [EC 3.1.3.8], a phytate degrading enzyme naturally present in plant and microorganisms catalyzing the hydrolysis of phytate into lower myo-inositol phosphate esters, is attracting the attention of researchers [14, 15]. Workers are looking for approaches to increase the activity of naturally present (endogenous) phytase in order to lower down the anti-nutritional effect of phytate [16, 17]. Wet food processing methods like soaking, germination and fermentation have been reported to reduce phytate level in foods supplemented with legumes because during processing, phytase is either synthesized or activated [18, 19]. Therefore, the aim of the present work was to analyze the effect of different wet processings (soaking, germination and fermentation) on lentil seed samples for lowering phytate and simultaneously increasing the bioavailability of iron and protein.

2. Materials and methods
2.1 Materials
Dry seeds samples were procured from three sources, (1) Farmer, (2) Local market and (3) Supermarket and designated as S_1, S_2 and S_3, respectively.

2.2 Methods
2.2.1 Wet processings
Three wet processing methods were applied to study their impacts on phytate, iron and protein contents in the selected samples. All the experiments were done in triplicates.

2.2.1.1 Soaking
Lentil seeds (100 g/sample) were cleaned, washed and soaked in 4-5 volumes of double distilled water (ddw) at (~25 ºC) for 24 h under ambient laboratory condition. At the end of the period, the water was drained and the seed

samples were soak dried on blotting paper (Whatman) and crushed in mortar and pestle.

2.2.1.2 Germination
The soaked samples (24 h) were allowed to germinate in wet muslin cloth for 72 h (3 days). They were sprinkled with fresh ddw every day. The seeds with radicle were picked up, soak dried on blotting paper (Whatmann) and crushed.

2.2.1.3 Fermentation
Powdered lentil seed samples (S_1, S_2 and S_3) and sterilized ddw (1:5, w/v) were allowed to ferment separately in 250 ml conical flasks for 24 h, 48 h and 72 h at 30±2 °C in incubator.

2.2.2 Quantitative Estimation
The phytate, iron and protein were quantitatively estimated to examine the effect of wet processings on the selected samples along with the control (dry raw seeds).

2.2.2.1 Phytate
Phytate contents of the raw, soaked, germinated and fermented suspension of lentil seeds (S_1, S_2 and S_3) were measured by Wade method [20]. In detail, 100 g/sample seeds were grounded to powder separately in a mixer. 1 g/sample (unprocessed and processed) of ground powder was thoroughly mixed with 10 ml of 2.4% HCl in 25 ml test tube. Sample tube was shaken at 220 rpm in a shaker (RS-12RDX) for 16 h and was centrifuged at 8000 rpm (RM-12C BL) at room temperature for 20 min. The crude acid extract was transferred to 25 ml test tube containing 1 g Nacl. The content was shaken at 350 rpm for 20 min to dissolve the salt and left to settle at 4 °C for 60 min. The mixture was centrifuged at 8000 rpm for 20 min at room temperature and clear supernatant was collected for further analysis. 3 ml of the super nantant and 1 ml of modified Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfosalicylic acid in ddw) in a 25 ml test tube was vortexed and absorbance was read at 500 nm double beam UV/VIS spectrophotometer (Systronics, 119). The phytate concentration was calculated using the standard curve.

2.2.2.2 Iron
Iron contents of the raw, soaked, germinated and fermented suspension of lentil seeds (S_1, S_2 and S_3) were determined by a modified bathophenathroline method, measuring only the non-haem iron [21]. 1 g of raw and processed seeds were grounded and mixed with 20 ml of ddw in 25 ml test tube. The mixture was homogenized for 30 seconds. 3 ml of the homogenate was transferred to another 25 ml test tube, added 10 ml of acid reagent (6 M HCl and 1.2 M trichloroacetic acid, 1:1 v/v) and mixed well. The mixture was heated in oven at 65 °C for 20 h, cooled and centrifuged at 8000 rpm for 20 min. 0.2 ml of clear supernatant was taken in test tube and mixed with 1.8 ml of freshly prepared color reagent, made by adding (1) bathophenanthroline reagent (62.5 mg bathophenanthroline disulfonic acid, 0.25 ml of thioglycolic acid and 24.75 ml ddw), (2) 4.5 M sodium acetate and (3) ddw in 1:20:20 (v/v) ratio. Following the color development, the absorbance was read at 535 nm. The iron concentration was calculated with reference to the standard curve.

2.2.2.3 Protein
The protein contents of the raw and processed lentil samples were analysed by Brad fords method [22].

2.2.3 Determination of molar ratio of phytate/iron [PA]: [Fe]
The molar ratio between phytate and iron [PA]: [Fe] was calculated according to the following equation:

$$\text{Molar ratio} = \frac{\text{Wt.}_{PA} / MW_{PA}}{\text{Wt.}_{Fe} / MW_{Fe}}$$

Where, Wt._{PA} = calculated phytate (PA) content; MW_{PA} = molecular weight of phytate (660 g/mole); Wt._{Fe} = calculated iron (Fe) content; MW_{Fe} = Molecular weight of iron (Fe = 56 g/mole)

2.3 Statistical Analyses
Data were statistically analysed using Statistics Package for the Social Sciences, SPSS-X Chicago, USA. All analyses were performed at $p \leq 0.05$.

3. Results and Discussion
Lentil, a legume rich in iron and protein has potential to alleviate serious health problems like IDA. However its bioavailability (i.e., availability to human) is reduced due to presence of phytate, thereby lowering its nutritional value. Considering these facts, in the present investigation effect of wet processings *viz*., soaking, germination and fermentation (24–72 h) on level of phytate, iron and protein in lentil samples (S_1, S_2 and S_3) was studied to identify the most suitable wet operation for enhancing its nutritive value. Similar work has been carried out in various cereals, millets and legumes [23, 24].

3.1 Phytate
In the present work the amount of phytate in raw varied widely in all the three samples with maximum in S_1 (77.42 mg/100 g), followed by S_3 (52.69 mg/100 g) and S_2 (68.20 mg/100 g) **(**Table 1). It has been reported that phytate is formed during seed maturation and its distribution depends on various factors, such as cultivars, climatic conditions, location, irrigation conditions, fertilizer application, type of soil and storage [25, 26].

So, the variations recorded in the three investigated samples could be due to one or several factors, as reported by previous authors.

Further in the present investigation, following the wet processings, the reduction in phytate level was found in all the samples. The declined amount of phytate during soaking, germination and fermentation (24 –72 h) were 11.49%, 22.76% and 32.25 - 48.16% in S_1, 18.44%, 36.62% and 66.30 - 87.29% in S_2, and 15.78 %, 19.70% and 23.79 - 53.84% in S_3, respectively (Fig.2). The significant decrease in phytic acid ranging from 19.70% – 36.62% in germinated lentil samples are comparable to the results obtained in African oil bean [27] and mungbean [28].

Comparing phytate contents in control samples with that of processed samples, some interesting observation were evident: (i) amount of phytate hydrolysed is independent of the quantity present in the raw seed samples and (ii)

fermentation is the most effective biological process for the reduction of phytate, which is enhanced along with increase in fermentation time, i.e. maximum after 72 h (Fig. 1).

Variations in reduction of intrinsic phytate level during fermentation is governed by several factors like capability of endogenous bacterial strains and plant species to dephosphorylate phytate, protein stability, pH and the optimum temperature for the activity of the enzyme, etc. [29, 30]. The probable natural reason of reduction in phytate content during germination may be attributed to (i) supply of important nutrients to the growing seedlings through hydrolysis of reserve nutrients and metabolites [31] and (ii) activation of the pre-existing enzyme or *de novo* synthesis of phytase [32] and (iii) cumulative action of endogenous and microbial phytases [33, 34].

3.2 Iron

In the control, the iron quantified was 4.10 mg/100 g, 2.11 mg/100 g and 3.67 mg/100 g in S_1, S_2 and S_3, respectively. The increment of iron in S_1 was more during fermentation (10.04 –11.79 mg/100 g) than germination (6.44 mg/100 g) and soaking (5.80 mg/100 g). Thus, there was increment in iron level by 113.17% to 186.82% after 24 h to 72 h fermentation, 57.07% on germination and 41.46% during soaking. It increased by 92.89%, 279.14% and 361.13%–759.71 after soaking, germination and fermentation in sample S_2 (Fig. 2).In sample S_3, the iron content increased as 4.82 mg/100g, 6.45 mg/100g and 7.66 mg/100g–12.93 mg/100g after soaking, germination and fermentation (24–72 h). From the observations, it is apparent that there is significant increase of iron by 31.33%–92.89%, 57.07%–279.14% and 186.82%–759.71% during soaking, germination and fermentation (72 h) and thus, it was inferred that fermentation is the most efficient process for enhancing iron. Similar observations were found in mungbean [35], pearl millet [36] and in moth bean cultivars [37]. The significant increase was observed in available iron by 64.6%, 67.8%, 75.8% and 81.3% in chickpea, green gram, cowpea and lentil, respectively, on germination, over the control [38]. The registered enhancement in the present work is probably due to an increase in phytase activity during different wet processings [39]. They were of the view that due to gradual increase in phytase activity, more bonds between the minerals-enzyme- protein got hydrolysed, liberating the minerals, therefore, increasing the available iron. Fermentation leads to lowering of pH, as organic acids are produced by bacteria, which favor hydrolysis of phytate and their intermediates. The hydrolysed complexes make them more soluble and readily available for absorption in the intestine.

3.3 Protein

The protein content was lowest (37.55 mg/100 g) in S_3 in comparison to S_2 (44.90 mg/100 g) and S_1 (46.86 mg/100 g), which significantly ($p<0.002$) increased after soaking, followed by germination and fermentation (Table 1). The increment was more prominent in S_2 followed by S_3 and S_1 after wet processing. The increase in protein contents ranged from 15.39% to 24.45% in the germinated seeds, which is comparatively higher i.e. 7.33% to 12.60% as reported in lentil [40]. The protein enhancement has been attributed to various explainations: (i) synthesis of proteins during soaking and the hormonal changes [41], (ii) synthesis of enzyme proteins by germinating seed or a compositional change following the degradation of other constituents [42] and (iii) loss in dry weight, particularly carbohydrates through respiration during germination [43]. During fermentation (24–72 h), enhancement was more pronounced i.e. 17.86–30.81% in S_1, 42.71–66.74% in S_2 and 23.78–45.61% in S_3 (Fig. 3), which could be due to combined action of intrinsic as well as microbial phytases. More enzyme will release more protein from complexes, formed between phytate and protein, therefore, increasing the availability of protein [39].

3. 4 Molar ratio of phytate/iron [PA]: [Fe]

The inhibitory effect of phytate on the bioavailability of iron was determined by measuring the molar ratios of phytate and iron. In control, the ratios, observed as 1.59, 2.78 and 1.21 in S_1, S_2 and S_3 respectively. It gradually decreased ranging from 1.15-0.781, 0.787-0.461 and 0.160-0.0414 during soaking, germination and fermentation in the analysed samples (Fig. 4).

The decrease was more in S_2 (2.78 - 0.041) in comparison to S_1 (1.59-0.289) followed by S_3 (1.21-0.160). The molar ratio of phytate to iron is assumed to provide an indication of the available iron in the food and diet [44].

Phytate begins to lose its inhibitory effects on iron absorption, when the ratio is less than one [45]. The molar ratios of phytate and iron in the dry raw seeds were found to be more than one (>1) in all the analysed samples indicating inhibitory effect on iron absorption. The lowest ratio was obtained during 72 h fermentation in all the analysed samples, predicting the efficient degradation of phytate by phytase.

Table 1: Phytate, Iron and Protein Content of the Raw and Processed Seeds [a]

Processings	Phytate (mg/100gm)			Iron (mg/100gm)			Protein (mg/100gm)		
	S_1[b]	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
Raw seeds (RW)	77.42±3.330[c]	68.20±3.201	52.69±1.318	4.10±5.114	0.305	3.67±0.208	46.86±0.164	44.90±0.192	37.55±0.922
Soaking(SK)	68.53±3.098	55.63±0.937	44.38±1.315	5.80±2.627	4.07±0.353	4.82±0.393	50.01±0.274	49.37±0.658	39.77±0.947
Germination (GR)	59.80±3.330	43.23±0.880	42.31±1.170	6.44±1.350	8.00±0.544	6.45±0.366	54.75±0.295	55.88±0.768	43.33±0.642
Natural Fermentation (FM)									
NF(24 h)	52.45±0.710	22.99±1.226	40.15±1.880	8.74±0.244	9.73±0.515	7.66±0.261	55.23±0.271	64.08±1.458	46.48±0.706
NF(48 h)	45.98±0.108	15.12±0.563	35.25±0.208	10.04±0.235	15.12±0.482	10.00±0.326	59.29±0.306	68.99±0.546	50.29±0.448
NF(72 h)	40.13±0.727	8.87±0.311	24.32±1.000	11.79±0.287	18.14±0.543	12.93±0.210	61.30±0.402	74.87±5.742	54.68±0.781

[a] RW: Raw seeds; SK: Soaked; GR: Germination (72h); NF: Natural Fermentation (24h, 48h & 72h)

[b] Samples: S_1-Farmer; S_2- Local market; S_3- Supermarket

[c] Mean±standard deviation (n=3)

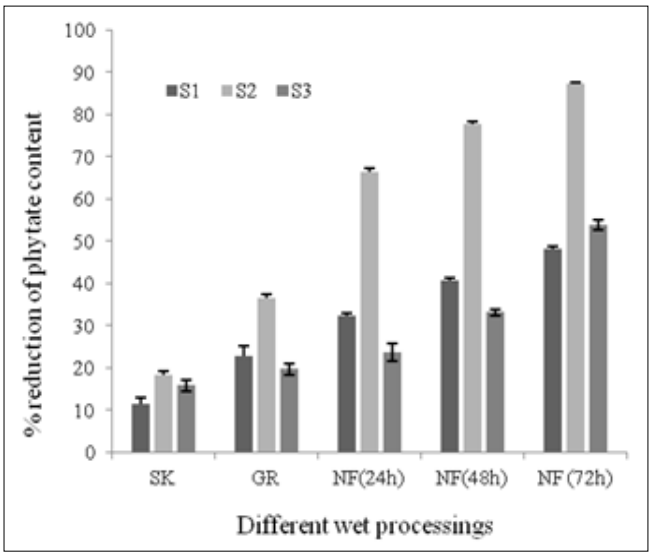

Fig 1: Phytate reduction after wet processing in S_1, S_2 & S_3 Vertical bars indicate standard error of mean (n=3). SK: Soaked; GR: Germination (72 h); NF: Natural Fermentation (24 h, 48 h & 72 h).

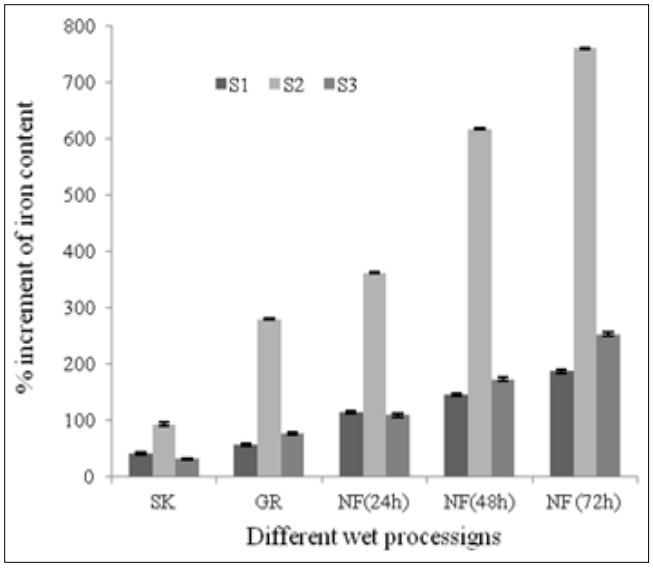

Fig. 2: Iron enhancement after wet processings in S_1, S_2 & S_3. Vertical bars indicate standard error of mean (n=3).

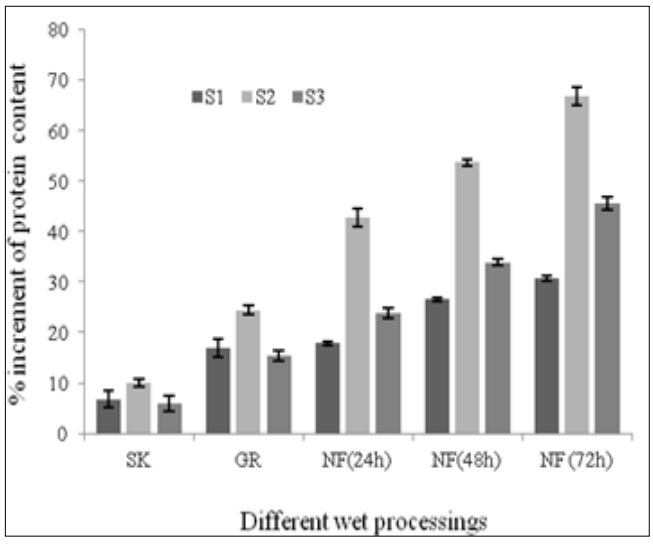

Fig 3: Protein enhancement after wet processings in S_1, S_2 & S_3. Vertical bars indicate standard error of mean (n=3).

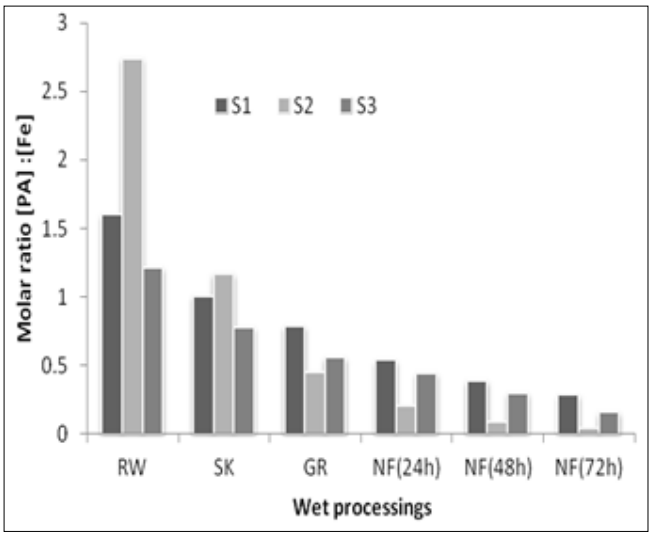

Fig 4: The molar ratios of Phytate and Iron in S_1, S_2 & S_3. RW: Raw seeds; SK: Soaked; GR: Germination (72 h); NF: Natural Fermentation (2 4h, 48 h & 72 h).

4. Conclusion

The findings show that lentil has considerable amount of iron, protein and also antinutrient phytate, which impairs the availability of nutrients to the body. Therefore, most efficient, cost effective wet food processing method should be chosen to minimise the inhibitory effect of phytate. All the wet processing techniques (soaking, germination and fermentation), followed in this investigation gave very encouraging results with respect to the availability of iron and protein in the analyzed samples. Soaking and germination increased the amount of iron and protein, while fermentation maximised them. Negative correlations were found between phytate vs iron and protein. It was found that soaking, germination and fermentation have different efficacies in reducing phytate and simultaneously increasing iron and protein.

5. Acknowledgment

This work was supported by University Grants Commission, (Selection & Awards Bureau), Bahadur Shah Zafar Marg, New Delhi, India (grant numbers F. 15-27/2012(SA-II)**.**

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