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Standardization and Stabilization of Millet Milk by Enzyme and Its Physicochemical Evaluation

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Millets are nutritionally rich and occupy an important place in the diet of people in many regions of the world. Although millets are nutritionally superior to cereals, their utilization as a food is mostly confined to the traditional consumers. So, the present study was undertaken to standardize millet milk from barnyard millet, little millet, kodo millet and finger millet by enzymatic extraction method. Aqueous extract of millet milk was treated with α amylase and pasteurized at 75ºC for 15 minutes. The pasteurized millet milk was evaluated for physical and nutritional parameters. Results showed that the physical properties of developed millet milk have met the requirement of plant-based milk in terms of viscosity (2.32±0.02 to 2.82±0.03). Protein content of millet milk varied from 1.38±.0.03 to 1.12±.0.02 g. Total polyphenols (205.72±0.13 mg/100 ml) and

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total antioxidant activity (81.64±1.77%) were high for finger millet milk and total flavonoid content was high for barnyard millet milk (96.25±1.88 mg/100 ml). Enzymatic treatment significantly reduced the anti-nutritional factor (phytic acid, tannin and trypsin inhibitor activity) content in millet milk. The enzymatically developed product had high *In vitro* protein (69.28±0.28 to 85.57±1.39%) and starch digestibility (69.75±0.56 to 63.36±0.12 mg maltose/g). From the results, it was concluded that the current approach provides a convenient way for the production of nutritionally sound millet milk at the household and industrial level.

Keywords: Millet milk; enzyme; physicochemical evaluation; total polyphenols; antioxidant activity.

1. INTRODUCTION

In recent years, health concerns of consumers changed their perception and preference for food. This led to the development of functional foods that provide additional health benefits along with nutritional benefits. One such development is the production of non-dairy functional drinks as the dairy drinks (cow milk) may lead to allergy, lactose intolerance and hypercholesteremia [1].

These non-dairy beverages can be produced by extraction plant materials such as soy, nuts, rice and so on along with water. These plant materials can be homogenized and thermally treated to improve the suspension of particles and to increase shelf life [2].

The first commercial plant-based milk substitute is soymilk. Besides soy, other plant materials such as oat, almond, coconut, rice and quinoa can also be used for developing non-dairy milk substitutes [3].

Millets contain a good amount of starch, protein, lipids, vitamins and minerals. Additionally, minerals such as magnesium, manganese and phosphorus are present in a significantly higher amount than other cereals. They also contain significant quantities of essential amino acids, particularly sulphur containing amino acid. It contains 6- 13% crude protein and 1.9 -14% total minerals [4].

Millets are the cheapest raw material source for the development of non-dairy beverages. They offer unique advantages for health being rich in micronutrients, particularly minerals and B vitamins as well as nutraceuticals [5].

Millets contain 56.07 to 62.13 g of starch and have gelatinization temperature in the range of 64 to 75ºC [6]. This poses a problem during the heat processing of millet milk as the liquid milk sets into a gel at high temperature. Hence, the purpose of the work is to optimise a method which maintains the consistency of millet milk at high temperature. The process should employ hydrolysis of starch (converting starch into maltodextrins) to restrict gelatinization. The process can be either chemical (H_2SO_4, HCl, HNO_3, and H_3PO_4 at temperatures below the gelatinization temperature) or enzymatic method (amylase).

Application of enzymes for the preparation of non-dairy beverages (rice milk and other cereal-based beverages) was reported by Deora and Deswal [7]. The enzymatic method involves the application of liquefying enzyme, preferably during gelatinization since it has been found that when starch is gelatinized the semi-crystalline nature of granules becomes amorphous and the starch becomes digestible by amylases [8]. The enzymatic liquefaction process has been shown to increase the yield and also reduces the viscosity. The process also results in the increase of the number of total solids by the action of amylases on starch resulting in the production of maltodextrins [9].

Therefore, the present study was aimed to optimize the enzymatic processing method for the development of millet milk and to analyse nutritional characteristics of developed millet milk.

2. MATERIALS AND METHODS

Barnyard millet (*Echinochloa esculenta*), little millet (*Panicum miliare*), kodo millet (*Setaria italica*) and finger *millet (Eleusine coracana*) were purchased from the local market in Madurai, Tamil Nadu. Germinated millets were processed by enzymatic method and developed millet milk were evaluated for physical (pH, TSS, viscosity, whiteness index, sedimentation rate)and nutritional parameters(starch, sugar, protein, total polyphenols, total flavonoids, total antioxidant

activity, tannin, phytic acid, trypsin inhibitor activity, *invitro* protein and starch digestibility, calcium and iron).

2.1 Germination of Millet Grains

Millets were cleaned and rinsed with tap water twice and soaked in water. Soaking time was optimized as 18 hrs, 13 hrs, 20 hrs, 16 hrs respectively for barnyard millet, little millet, kodo millet and finger millet based on its water absorption capacity. The soaked grains were drained, and tied tightly with a muslin cloth and kept in dark for germination. The germination process was carried out at room temperature (~25ºC) for about 24 hours for all millets. The germinated millet grains were dried at 45ºC for 8 hours to obtain the final moisture content of about 7-8%.

2.2 Processing of Millet Milk

Preliminary experiments were conducted to standardize the processing of millet milk. Millets and water were taken at the ratio of 1:6 and the mixture was blended for 15 min to obtain the millet milk slurry. Then, it was filtered through a muslin cloth and the filtrate was subjected to heat-stable alpha-amylase enzyme at 0.5% concentration. Enzyme added millet milk was incubated at 75ºC for 60 minutes. The enzyme was inactivated by heating the milk at 100ºC for 15 minutes. The product was pasteurized at 75ºC for 10 minutes and stored at 4ºC for further analysis.

2.3 Determination of Quality Attributes

2.3.1 pH, TSS and viscosity

The pH of the millet milk samples was measured with a digital pH meter at 25ºC. Total soluble solids were estimated by digital refractometer. Viscosity of the samples was analysed by Brooke field viscometer using spindle no 62 at 100 rpm.

2.3.2 Whiteness index

The colour values of developed millet milk were measured using Hunter colour lab meter. Whiteness index (WI) was calculated based on L*(Lightness), a*(Red to green), b*(Blue to yellow) value (10).

$$WI = 100 - \sqrt{\left(100 - L^{*}\right)^{2} + a^{*2} + b^{*2}}$$

2.3.3 Sedimentation index

Sedimentation rate was determined through phase separation analysis using analytical centrifuge at 1000 rpm for 30 min at 24ºC. Weight of sediment was determined and expressed as g/volume of centrifuge tube [10].

2.3.4 Nutritional compositions

Nutritional composition such as total sugars and protein [11], starch, iron and total phenols (spectroscopic method), calcium by titrimetric method [12] total flavonoids by aluminium chloride method [13], total anti-oxidant activity by DPPH method [14] and anti-nutritional factors such as trypsin inhibitor activity, tannin and phytic acids by spectroscopic method [15] were determined. *In vitro* protein digestibility was determined by the three-enzyme method [16]. *In vitro* starch digestibility was determined by using pancreatic alpha amylase [17].

2.4 Statistical Analysis

Analysis was carried out in triplicate. The statistical design used for analysis is a single factor completely randomized design using AGRES software for windows version 7.0. Means with a significant difference ($P<0.05$) were compared by least significant difference.

3. RESULTS AND DISCUSSION

Physical parameters such as pH, acidity, total soluble solids (TSS), viscosity, whiteness index and sedimentation rate of developed millet milk were given in Table 1.

The lowest pH value was observed for kodo millet milk (6.2±0.09) and the highest pH value was observed for little millet milk (6.7±0.15). Acidity of the enzyme treated millet milk was in the range of 0.50 to 0.83%. When pH of millet milk increases, the acidity milks were decreases and *vice versa* for all the millet milk. Abou-Dobara [18] studied the chemical composition of rice milk. It was reported that the pH and acidity of rice milk were 6.75 and 0.12 respectively. TSS of barnyard millet, little millet and kodo millet milks were 2±0.06 and finger millet milk had TSS value of 3±0.42.

Viscosity of millet milks were 2.75±0.01, 2.32±0.02, 2.44±0.07 and 2.82±0.03 at 100 rpm for barnyard millet, little millet, kodo millet and finger millet respectively. The higher moisture

content of the millet milk resulted in lower viscosity. Jiang et al. [19] evaluated the viscosity of short time germinated soy milk. The result indicated that the germinated soy milk had viscosity of 2.55 cp at 100 rpm. According to the results of Jeske, et al. [10], plant-based milk substitutes (rice milk) contained viscosity in the range of 2.21 to 2.77 cp.

The colour of food is one of the first properties observed by consumers, influencing choice and preference [20]. The WI for bovine milk is the highest at 81.89% [10]. It was observed that the whiteness index of the enzyme-treated millet milks were 55.80±1.75, 61.38±1.92, 40.48±0.71 and 62.81±1.06% for barnyard millet, little millet, kodo millet and finger millet milk respectively. This may be due to colour of millet grains and enzymatic treatment

Sedimentation index (i.e. accelerated sediment formation) was a measure of the stability of particles in the beverages. Denser particles sediment while the lighter ones cream on top of the liquid. Among the enzyme-treated millet milk, a significant difference (P>0.05) were found among the millet. Finger millet milk had a lower sedimentation rate of 0.93±0.07 g/40 ml and little millet milk had a higher sedimentation rate of 1.13±0.05 g/40 ml. The difference in sedimentation rate may be due to the difference in viscosity. Jeske, et al. [10] evaluated the sedimentation rate of commercially available plant-based milk and the range of sediment is varied from 0.20 ± 0.19 to 4.22 ± 2.91 mm.

Table 2 represents the starch, total sugars and protein content of enzyme-treated millet milks. Raw millet grain contains 56.07 to 62.13 g/100 ml of starch. The starch content of enzyme-treated millet milk was in the range of 5.07 to 7.13 g/100 ml. The reduction in starch content may be due to the use of alpha-amylase enzyme during processing. Pineli, et al. [21] estimated the starch content of enzyme-treated quinoa milk as 5 g/100 ml.

Total sugar content of enzyme-treated millet milk was in the range of 3.1 to 4.2 g/100 ml with the lowest total sugar content (3.1±0.08) for little millet milk and the highest total sugar content (4.2±0.10) was observed in kodo millet milk. The total sugar content of malt extract treated cowpea - peanut milk increased from 0.024% in the unhydrolyzed sample to 2.43% in the malt-extract treated sample [22].

Protein content of enzyme-treated millet milks were 1.24±0.04, 1.12±.0.02, 1.19±0.03 and 1.38±.0.03 g/100 ml for barnyard millet, little millet, kodo millet and finger millet milk respectively. Makinen, et al. [2] estimated that the protein content of commercially available fourteen non-dairy plant-based milk substitutes. It was inferred that the rice milk had the lowest protein content of about 0.1 g/100 ml whereas soy milk had the highest protein content of about 3.7 g/100 ml.

Table 1. Physicochemical evaluation of enzyme treated millet milks

Millet milks	pH	Acidity (%)	Total soluble solids	Viscosity (cP)	Whiteness index (%)	Sedimentation rate(g/40 ml)
Barnyard millet	6.4±0.14^{bc}	0.77±0.09^{c}	2±0.06^{b}	2.75±0.01^{b}	55.80±1.75^{b}	1.07±0.01^{c}
Little millet	6.7±0.15^{a}	0.50±0.02^{a}	2±0.05^{b}	2.32±0.02^{d}	61.38±1.92^{a}	1.13±0.05^{d}
Kodo millet	6.2±0.09c^{b}	0.83±0.01^{d}	2±0.04^{a}	2.44±0.07^{c}	40.48±0.71^{c}	1.02±.0.03^{b}
Finger millet	6.5±0.13^{a}	0.68±0.09^{b}	3±0.42^{a}	2.82±0.03^{a}	62.81±1.06^{a}	0.93±0.07^{a}

Values are means of 3 replicates. Means in the same column followed by different superscripts are significantly different at P<0.05

Table 2. Chemical constituents of enzyme-treated millet milk

Millet milks	Starch (g/100 ml)	Total sugars (g/100 ml)	Protein (g/100 ml)
Barnyard millet	6.41±0.04^{c}	3.6±0.07^{c}	1.24±0.04^{b}
Little millet	5.07±0.03^{d}	3.1±0.08^{d}	1.12±.0.02^{c}
Kodo millet	6.96±0.20^{b}	4.2±0.10^{a}	1.19±0.03^{b}
Finger millet	7.13±0.04^{a}	3.9±0.09^{b}	1.38±.0.03^{a}

Values are means of 3 replicates. Means in the same column followed by different superscripts are significantly different at P<0.05

Nutraceutical properties of enzyme-treated millet milks were shown in Table 3. The order of increase in total polyphenols content of enzyme-treated millets milks were Kodo millet (115.54±0.71) < barnyard millet (190.93±0.64) < little millet (190.93±0.64) < finger millet (205.72±0.13). The concentration of phenolic compounds increased due to cell wall-degrading enzymes, which became active during germination and modified the cell wall structure of the grain. The significance of this lies in the fact that phenolic compounds such as hydroxyl cinnamates (e.g., ferulic and p-coumaric acids) are bound to non-starch polysaccharides in grain cell walls through associations such as ester and ether bonds. The bound phenolic compounds get liberated by the action of cell wall-degrading enzymes (mainly esterases) on these bonds [23].

Soymilk prepared from germinated soybean of different cultivars contained total polyphenol content of 3.94± 0.04 to 5.52±0.27 mg of gallic acid equivalent/ml of soymilk. This distinct difference in TPC is mainly due to the genetic variation of the soybean cultivars [24]. Similar results were found in the present study.

Total flavonoid content of millet milks varied from 75.93±1.13 to 96.25±1.88 mg/100 ml of catechin equivalent. The higher concentration of total flavonoids in the millet milk may attribute to the activity of several enzymes during germination and processing. This may degrade macromolecules and develop new compounds due to liberation and solubilization of bound components [25]. Xu and Chang [26] reported total flavonoid content of soymilk processed by different cooking methods. It was found that raw soymilk had 0.13± 0.00 mg/g and steam cooked soymilk had 0.17±0.01 mg/g.

It was observed that the radical scavenging activity of enzyme-treated millet milks were in the range of 62.80±1.58 to 81.64±1.77%. The higher antioxidant activity was exhibited by finger millet followed by little millet, barnyard millet and kodo millet. The total antioxidant activity of millet milk is probably due to the presence of total polyphenolic content of the millet milk. Blended fruit juice - soymilk beverage prepared by Rodríguez-Roque, et al. [27] evaluated the total antioxidant activity. It was reported that the total antioxidant activity of soymilk beverage was 78.2%. Pradeep and Guha [28] reported that the percentage of DPPH inhibition is directly correlated with the amount of polyphenols in millet milk.

Anti-nutritional factors like tannin, phytic acid and trypsin inhibitors mostly exist in water-soluble form. It is recognized that traditional food processing treatments like soaking and malting/germination may significantly reduce the anti-nutrient content of cereal grains and improve their nutrient bioavailability [29].

The highest amount of tannin was observed for little millet milk which is 1.49±0.42 mg TAE/100 ml. The tannin content of the enzyme treated millet milk was lower compared to raw millet grains. This may be due to germination and enzyme treatment of millet milk. Various studies have reported the reduction in tannin content in *Bauhinia purpurea* during soaking. Raw seed contained 2.35 g of tannin and soaking in water for 6 h significantly reduced the tannin content to 0.68 g/100 g [30]. In the present study, soaking was taken as the preliminary step for germination which may attribute to leaching of tannin into water.

Trypsin inhibitor inhibits the function of trypsin enzyme and causes pancreatic hypertension and dietary loss of cysteine. Trypsin inhibitors are the proteins that interfere with nutrient absorption by reducing the activity of proteolytic enzymes (trypsin and chymotrypsin). Trypsin inhibitor activity of barnyard millet, little millet, kodo millet and finger millet milks were 1.76±0.20, 3.61±0.12, 3.01±1.52 and 3.66±0.29 TIU/mg of protein respectively. Joshi and Varma [31] found that trypsin inhibitor activity of soybean has reduced after treatments like soaking, dehulling, germination and roasting. The reduction of TIA was observed in germinated soybean (50±8.2 TIA/mg) compared to raw soybean (79.2±8.7 TIA/mg) Soaking increases the permeability of cell membrane which increases the amount of anti-nutrient leaching.

The phytic acid content of barnyard millet, little millet, kodo millet and finger millet milk were 17.31±0.33, 15.49±0.55, 12.24±0.40 and 12.61±0.75 mg/100 ml respectively. The increased phytase activity during the germination may be the reason for the reduction of phytic acid in sprouts, since phytase hydrolyzes phytic acid. During the incubation process with amylase, the millet milk was subjected to 75ºC which may reduce the amount of phytic acid content in all the samples irrespective of raw material as phytate is a heat liable compound [32].

Table 3. Nutraceutical properties of enzyme-treated millet milks

Millet milks	Total polyphenols (mgGAE/100 ml)	Total flavonoids (mgCEE/100 ml)	Total antioxidant activity (%RSA)
Barnyard millet	175.61±1.34^{c}	96.25±1.88^{a}	69.89±1.37^{c}
Little millet	190.93±0.64^{b}	93.58±0.34^{a}	72.35±0.73^{b}
Kodo millet	115.54±0.71^{d}	75.93±1.13^{c}	62.80±1.58^{d}
Finger millet	205.72±0.13^{a}	86.37±2.52^{b}	81.64±1.77^{a}

Values are means of 3 replicates. Means in the same column followed by different superscripts are significantly different at P<0.05

Table 4. Anti-nutritional factors content in the enzyme-treated millet milk

Millet milks	Tannin (mg of TAE/100 ml)	Trypsin inhibitor activity (TIU/mg of protein)	Phytic acid (mg/100 ml)
Barnyard millet	1.26±0.02^{a}	1.76±0.20^{a}	17.31±0.33^{c}
Little millet	1.49±0.42^{c}	3.61±0.12^{c}	15.49±0.55^{b}
Kodo millet	1.47±0.05^{c}	3.01±1.52^{b}	12.24±0.40^{a}
Finger millet	1.38±0.03^{b}	3.66±0.29^{c}	12.61±0.75^{a}

Values are means of 3 replicates. Means in the same column followed by different superscripts are significantly different at P<0.05

Table 5. *In vitro* starch, protein digestibility and mineral content of enzyme-treated millet milk

Millet milks	*In vitro* starch Digestibility (mg maltose/ml)	*In vitro* protein digestability (%)	Calcium (mg/100 ml)	Iron (mg/100 ml)
Barnyard millet	63.36±0.12^{c}	69.28±0.28^{d}	4.17±0.03^{b}	1.32±0.03^{a}
Little millet	69.75±0.56^{a}	72.90±1.16^{c}	5.33±0.05^{b}	0.43±0.01^{d}
Kodo millet	63.50±0.06^{c}	81.95±0.78^{b}	4.66±0.09^{b}	0.78±0.11^{c}
Finger millet	66.75±0.49^{b}	85.57±1.39^{a}	91.31±2.36^{a}	1.54±0.05^{b}

Values are means of 3 replicates. Means in the same column followed by different superscripts are significantly different at P<0.05

The *in vitro* starch digestibility of enzyme-treated millet milks were reported in Table 5. *In vitro* starch digestibility was expressed in terms of mg of maltose/ml of starch. The higher *in vitro* starch digestibility was observed in little millet milk (69.75±0.56 mg/ml) followed by finger millet milk (66.75±0.49 mg/ml), kodo millet milk (63.50±0.06 mg/ml) and barnyard millet milk (63.36±0.12 mg/ml). A significant difference (p>0.05) in *In vitro* starch digestibility was found among the millet milk.

In vitro protein digestibility of barnyard millet milk was 69.28%, little millet milk was 72.90%, kodo millet milk was 81.95% and finger millet milk was 85.57%. Sharma and Gujral [33] evaluated the *in vitro* protein digestibility of various millet flours (finger millet, foxtail millet, barnyard millet, kodo millet, little millet and proso millet). The range of *in vitro* protein digestibility varied from 72.01±0.82 to 80.84±1.34%. *In vitro* protein digestibility of enzyme-treated millet milk was slightly higher than reported results. The highest protein digestibility of millet milk may be due to the reduced amount of anti-nutritional factors in the respective millet milk.

The mineral element constitutes an important group of nutrients required by the human body for optimal functioning. Iron and calcium content of enzyme-treated millet milks were analysed and given in Table 5. The iron content of the samples varied from 0.43±0.01 to 1.54±0.05 mg /100 ml for little millet milk to finger millet milk. The higher amount of calcium (91.31±2.36 mg/100 ml) and iron (1.54±0.05 mg/100 ml) was observed in finger millet milk. The remaining millets had calcium content in the range of 11.27 to 43.31 mg/100 ml.

Krishnan, et al. [34] found the changes in mineral content of finger millet during different processing methods. Raw millet grain contains 372. 6.4

mg/100 g of calcium and the malted finger millets contains 369.35 mg/100 g. A slight reduction was found in malted millet flour compared to other treatment likes decorticated millet and expanded millet.

According to the results of Singhal, et al. [35] most of the plant-based milk substitutes contained a higher amount of calcium and iron content. The highest calcium content is found in almond milk (72 mg/100 ml). But, it was comparatively lower than the calcium content found in finger millet milk (91.31±2.36 mg/100 ml). The iron content of commercial plant milks were in the range of 1.4 to 17.1 mg/100 ml according to the results of Singhal, et al. [35].

4. CONCLUSION

The millet milk developed from enzymatic method shows desirable quality in terms of nutritional parameters. The product had a good amount of calcium and nutraceutical compounds like polyphenols and flavonoids. The preliminary processing steps like soaking and germination effectively reduced the anti-nutritional compounds and thereby increased the total polyphenol and flavonoids content of the developed millet milk. Since the products were treated with an enzyme, the *in vitro* starch and protein digestibility of the product was increased. Millet milks can be served with palm gur or palm sugar for better acceptability. Hence, this millet milk can be a substitute for dairy beverages and can be consumed by all age groups of people.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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