Enzymatic Treatment and Use of Starters for the Nutrient Enhancement in Fermented Flour of Red and White Varieties of Finger Millet (*Eleusine coracana*)

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Two varieties of finger millet (*Eleusine coracana*)—a tannin-containing red variety, CO13, and nontannin white variety, CO9—processed by treatment with enzymes (cellulase and hemicellulase) and fermentation with starters (from previously fermented finger millet batter), achieved the desirable goals of reduced fermentation time (12 h), increased acidity (2.2 to 2.4%), enhanced in vitro protein digestibility (IVPD) (14 to 26%), and mineral availability compared to 48 h uncontrolled natural fermentation (Usha Antony and Chandra, 1998). Fermentation with starters alone increased titratable acidity (1.02 to 1.88%), IVPD (5.5 to 22%) and mineral availability, and decreased phytate (23 to 26%) and tannin (10.8 to 40.5%) in the millets. Enzymatic treatment (3 h, 50 °C) did not significantly alter the pH, phytate, tannins, IVPD, or HCl−mineral extractability but enhanced fermentative changes. Overall, the changes were marked when the 48 h starter was used and the improvements in nutrient availability was greater in the CO13 variety.

Keywords: *Enzymatic treatment; cellulase; hemicellulase; use of starters; Eleucine coracana; finger millet fermentation; nutrient enhancement; in vitro protein digestibility; HCl−mineral extractability*

INTRODUCTION

Developing food processing methods that maximize utilization of the available resources is crucial to developing economies such as India. Given the high nutrient and health potential of finger millet and its enhancement by fermentative processing (Usha Antony et al., 1996; Usha Antony and Chandra, 1997, 1998), the need to explore methods that further enhance/accelerate the process has been underscored. Finger millet is high dietary fiber and minerals (especially calcium) and has a protein content equivalent to other cereals. However, the dietary fiber in the seed coat binds tannins, phytates, minerals, and proteins, resulting in poor protein digestibility and mineral availability (Malleshi and Hadimani, 1993). Therefore, it would be desirable to maximize the availability of protein and minerals in finger millet based products.

Traditional food processing usually involves the use of endogenous enzymes activated by germination or produced by microorganisms during fermentation. The use of exogenous enzymes from plants, animals, or microbes to improve existing reactions or initiate new reactions is more recent. A number of enzymes such as amylases, cellulases, and hemicellulases are used in the processing of cereals such as wheat, rye, barley, etc. in the manufacture of breads and beers to improve the texture, volume, viscosity, water holding capacity, shelf life, etc. (Tucker and Woods, 1995). Cellulases and hemicellulases can act on the dietary fiber and release the bound nutrients.

Fermentation can be spontaneously initiated without the addition of microorganisms or controlled by the use of specific cultures or starters from a previous batch of fermented product. Highly competitive and well-adapted multiple strain starters effectively accelerate acidification of the substrate (Nout et al., 1988; Nout and Rombouts, 1992).

A combination of enzymatic pretreatment and directed fermentation, is expected to provide a double advantage of accelerating the fermentation and enhancing protein and mineral availability in millet. Such approaches to processing finger millet have not been reported so far and hence have been adopted in the present study. Two *starters* were used to accelerate and enhance the fermentation. Their selection was based on the analyses of the microbial population and metabolic changes during fermentation of the millet (Usha Antony and Chandra, 1997).

MATERIALS AND METHODS

Millets. Two varieties of finger millet—a tannin-containing red variety, CO13, and a nontannin white variety, CO9—were obtained in bulk from the Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Finger millet (red, market variety) was purchased from the local market.

Enzymes. Enzymatic pretreatment was carried out using cellulase and hemicellulase (from *Trichoderma longibrachium*) obtained from Super Organic Research Laboratories, Advanced Biochemical Limited, Thane (W), Maharashtra, India. Both enzymes had GRAS status and complied with the FAO/WHO and FCC III recommended specifications for food-grade enzymes of microbial origin.

Chemicals. All chemicals used for the study were of analytical grade, unless otherwise mentioned. Phytic acid, carboxy methyl cellulose, and birchwood xylan were from Sigma Chemical Company, St. Louis, MO. Pepsin and pancreatin were from Central Drug House (Bombay, India).

Analyses. pH and titratable acidity were done as described by Egan et al. (1981).

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Phytate was estimated by HPLC as described by Usha Antony and Chandra (1998) using the method of Camire and Clydesdale (1982) with a HPLC system (CT-10A Shimadzu Model, Japan) fitted with a Shimpack ODS-C18 column with RI (R16A) detector and Chromatopac integrator (Shimadzu, Japan). Sodium acetate (0.005 M) served as the mobile phase, with a flow rate 1.5 mL per min at 30 °C.

Tannins were estimated spectrophotometrically after reaction with vanillin−HCl (Price et al., 1978) as described in Usha Antony and Chandra (1998). Values were expressed as catechin equivalents in milligrams/100 grams of dry flour.

HCl Extractability of Minerals. Ca, P, Fe, Cu, Zn, and Mn were extracted into 0.03 N HCl (10 mL) and estimated as described earlier (Sripriya et al., 1997) by inductively coupled plasma emission spectroscopy (ARL-3410, ICP with minitorch, Germany). Percentage extractability of each mineral was calculated as described by Chompreeda and Fields (1984):

$$\frac{\text{mineral extracted} \times 100}{\text{total mineral}} = \%\ \text{extractability}$$

Zinc bioavailability was assessed by calculating the phytate/zinc and phytate × calcium/zinc molar ratios and comparing with critical values suggested by Fordyce et al., (1987).

In vitro protein digestibility (IVPD) was determined as described in Usha Antony and Chandra (1998) by the method of Lorri and Svanberg (1993) after digestion with pepsin and pancreatin. The total and undigested nitrogen were estimated by the Kjeldahl method (AOAC, Helrich, 1990) using a Tecator Kjeltec system (1028, Eden Prairie, MN). Percentage protein digestibility was calculated as

$$\frac{(\text{total nitrogen} - \text{undigested nitrogen}) \times 100}{\text{total nitrogen}}$$

Activities of Cellulase and Hemicellulase. The specific activities of the enzymes were determined by standard methods (IUPAC, 1984; Bailey et al., 1992) using carboxy methyl cellulose and birchwood xylan, respectively, as substrates.

Fermentation of Millet Flour Using Starters. Fifty grams of millet flour (market variety), mixed with 100 mL of distilled water and covered with aluminum foil, was allowed to ferment spontaneously in a 500-mL Erlenmeyer flask at 30 °C for 48 h as described in Usha Antony et al. (1996). At 18 and 48 h, the batter was collected and added as a starter at a 2% (v/v) level to 100 mL of fresh batters of CO9 and CO13 (1:2 w/v). These were allowed to ferment at 30 °C for 12 h, oven dried at 70 °C, powdered, and stored airtight at 5 °C until analyses were performed.

Enzymatic Treatment of Millet Flours. To 50 g of millet flour in 100 mL of distilled water were added 25 mg of cellulase and 5 mg of hemicellulase. The solution was mixed well, incubated at 50 °C for 3 h, oven dried in Petri plates at 70 °C, powdered, and stored in airtight containers at 5 °C until analyses were performed.

Directed Fermentation by Combining Enzymatic Pretreatment and Starter. Millet batters were treated with 25 mg of cellulase and 5 mg of hemicellulase for 3 h, 50 °C, followed by inoculation with 2% (v/v) 18 or 48 h starters, fermented at 30 °C for 12 h, dried, and stored as above.

Untreated, Unfermented Control. Fresh millet batters served as untreated, unfermented controls. All processing of millet flours was carried out in triplicate.

Statistical Analyses. For all parameters studied the mean and standard deviation of three independent estimations were calculated and tabulated. Analysis of variance (ANOVA) was applied to test the significance of the difference in the parameters resulting from the different treatments (Clarke, 1994).

RESULTS AND DISCUSSIONS

(a) Effect of Using Starters. Previous work on naturally fermented millet indicated that at 18 h, batters reached the highest bacterial counts and contained all the major genera of lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, and *Pediococcus*), and they presented the maximum of amylase activity, while at 48 h *Pediococcus* was dominant (Usha Antony and Chandra, 1997). Hence, 18 and 48 h batters were used as starters to accelerate the fermentation.

The use of starters accelerated the fermentation by decreasing pH to 4.5 and increasing titratable acidity to 1.72−1.88 in both varieties within 12 h (Table 1), whereas such values were achieved only after 48 h of natural fermentation without starters (Usha Antony and Chandra, 1997). Starters also effectively decreased the phytate and tannin contents and enhanced the protein and mineral availability of the millets (Tables 1 and 2). Zn bioavailability, in terms of phytate/Zn and Ca × phytate/Zn, although considerably decreased did not fall within the critical levels of 10 and 50 mM/100 g of dry diet, respectively (Fordyce et al., 1987). The 48 h starter was more effective than the 18 h starter, suggesting that the nature of the microbial consortia strongly influences the hydrolytic activity.

(b) Effect of Enzyme Treatment. The specific activities of cellulase and hemicellulase were 0.05 (μmol of glucose) (mg of protein)$^{-1}$ min^{-1} and 30 (μmol of xylose) (mg of protein)$^{-1}$ min^{-1}, respectively. Preliminary experiments with varying levels of enzyme treatment (5, 10, 25, and 50 mg) of the millet, gave a reducing sugar release of 19 mg/g and 10 mg/g of millet flour with 25 mg of cellulase and 5 mg of hemicellulase, respectively, in 3 h. Hence, these two enzyme levels were selected for the treatment of millets.

Enzyme treatment did not alter the pH, although the titratable acidity increased in both varieties of millet. The decrease in phytic acid content was not significant ($p < 0.05$). The tannin content of CO13 decreased significantly ($p < 0.05$), probably due to the release of the fiber bound tannins. The IVPD and mineral extractability showed no appreciable change (Tables 1−3). Therefore, the enzyme treatment is inferred to have affected the binding of the crude fiber constituents to phytate, tannins, and protein and enabled better microbial fermentation compared to untreated controls (Tables 1−3).

(c) Combination of Enzymes and Starters. Enzymatic pretreatment followed by accelerated fermentation with the two starters markedly decreased the pH, increased titratable acidity, and enhanced the IVPD (Table 1). The enhanced protein digestibility may be due to the release of protein from the seed by the enzymatic breakdown of dietary fibers, which is known to bind phytate and tannins, and get extracted into the HCl and methanol prior to estimation. Mineral extractability was not enhanced to anticipated levels, the increase was similar to the millets processed by starters alone.

Zinc bioavailability is important in developing food supplements for growing children. Since the phytate/Zn and Ca × phytate/Zn ratios are influenced by the phytate levels, only a considerable reduction of the phytate levels can appreciably lower the ratio to desired levels for adequate Zn availability (Fordyce et al., 1987). The microbial phytase activity during fermentation possibly needs to be supplemented with exogenous addition of phytase along with the cellulase and hemicellulase for synergistic activity.

The effect of processing was greater on the tannin-containing red variety (CO13) compared to the nontan-

Table 1. Acidity, Phytic Acid, Tannin, and IVPD in Processed CO9 and CO13 Finger Millet Flour[a]

processing method	pH		percent titratable acidity (g of lactic acid/100 g of flour)		phytic acid (g/100 g)		tannins (g/100 g)		IVPD (%)	
	CO9	CO13	CO9	CO13	CO9	CO13	CO9	CO13	CO9	CO13
untreated unfermented control	6.0 ± 0.0	5.3 ± 0.0	0.42 ± 0.08	0.63 ± 0.02	0.679 ± 0.054	0.693 ± 0.022	0.0	0.74 ± 0.03	65.7 ± 2.1	61.4 ± 1.8
untreated, fermented with 18 h starter	5.2 ± 0.0	5.25 ± 0.1	1.14 ± 0.07	1.02 ± 0.02	0.647 ± 0.024	0.631 ± 0.128	0.0	0.66 ± 0.08	71.2 ± 3.0	74.4 ± 2.6
untreated, fermented with 48 h starter	4.5 ± 0.1	4.6 ± 0.0	1.88 ± 0.1	1.72 ± 0.11	0.522 ± 0.092	0.517 ± 0.112	0.0	0.44 ± 0.06	75.6 ± 2.8	83.7 ± 3.1
enzyme-treated unfermented	5.9 ± 0.1	5.3 ± 0.0	0.65 ± 0.05	1.09 ± 0.08	0.666 ± 0.093	0.625 ± 0.064	0.0	0.64 ± 0.01	61.5 ± 2.5	63.1 ± 2.2
enzyme-treated, fermented with 18 h starter	4.3 ± 0.1	4.4 ± 0.1	2.37 ± 0.01	2.18 ± 0.02	0.678 ± 0.041	0.585 ± 0.037	0.09 ± 0.01	0.77 ± 0.07	76.1 ± 3.0	77.3 ± 2.3
enzyme-treated, fermented with 48 h starter	4.5 ± 0.4	4.6 ± 0.2	2.3 ± 0.03	2.43 ± 0.22	0.674 ± 0.052	0.532 ± 0.008	0.10 ± 0.02	0.76 ± 0.06	74.5 ± 3.5	89.5 ± 2.4

[a] All values are on dry weight basis. Values are mean ± SD of three independent determinations.

Table 2. HCl Extractability of Minerals in Fermented CO9 and CO13 Finger Millet Flour[a]

processing method	percentage HCl extractability of minerals[a]											
	Ca		P		Fe		Zn		Mn		Cu	
	CO9	CO13	CO9	CO13	CO9	CO13	CO9	CO13	CO9	CO13	CO9	CO13
untreated, unfermented control	27.4 ± 2.1	59.7 ± 1.2	58.6 ± 3.1	53.8 ± 2.2	45.0 ± 2.6	29.1 ± 0.8	21.9 ± 2.1	52.2 ± 0.6	42.6 ± 2.2	41.9 ± 1.1	27.2 ± 0.9	28.3 ± 1.5
untreated, fermented with 18 h starter	39.0 ± 1.3	60.1 ± 0.7	71.4 ± 4.3	56.7 ± 0.9	56.7 ± 1.7	50.8 ± 1.3	40.7 ± 1.6	75.2 ± 0.8	60.9 ± 3.0	70.1 ± 1.3	66.5 ± 1.8	42.8 ± 1.9
untreated, fermented with 48 h starter	38.1 ± 1.1	65.7 ± 2.1	81.0 ± 2.0	61.3 ± 1.4	60.5 ± 1.4	71.4 ± 2.1	40.2 ± 0.8	84.6 ± 1.1	67.5 ± 2.1	72.8 ± 0.6	65.4 ± 2.0	42.4 ± 0.4
enzyme-treated, unfermented	36.5 ± 0.8	58.0 ± 2.1	51.9 ± 1.1	59.6 ± 1.4	46.9 ± 1.9	19.3 ± 0.9	18.2 ± 0.7	57.7 ± 1.0	37.8 ± 0.9	42.2 ± 0.7	30.1 ± 2.7	29.2 ± 0.7
enzyme-treated, fermented with 18 h starter	32.6 ± 1.2	65.4 ± 0.9	52.4 ± 2.5	67.5 ± 0.6	61.1 ± 1.3	53.9 ± 0.5	28.9 ± 1.9	82.1 ± 0.8	62.9 ± 1.3	73.5 ± 1.2	56.6 ± 1.4	54.7 ± 1.6
enzyme-treated, fermented with 48 h starter	33.8 ± 0.7	72.6 ± 1.3	78.1 ± 1.3	69.7 ± 1.2	66.1 ± 1.3	70.9 ± 0.8	32.9 ± 1.7	82.7 ± 0.1	67.7 ± 1.7	80.6 ± 0.9	65.9 ± 1.7	56.6 ± 1.8

[a] Values are on dry weight basis. Values are mean ± SD of three independent determinations. [b] Total mineral content: [CO9 (mg/100 g)] Ca, 365.3 ± 4.5; P, 210.0 ± 1.9; Fe, 6.65 ± 0.9; Zn, 2.21 ± 0.07; Mn, 7.44 ± 0.51; Cu, 3.55 ± 0.41; [CO13 (mg/100 g)] Ca, 383.0 ± 2.5; P, 240 ± 1.1; Fe 6.42 ± 0.33, Zn 2.23 ± 0.08; Mn, 5.84 ± 0.01; Cu, 2.69 ± 0.12.

Table 3. Phytate/Zinc and Calcium × Phytate/Zinc Molar Ratios in Fermented CO9 and CO13 Finger Millet Flour[a]

processing method	phytate/zinc		calcium × phytate/zinc (mM/100 g)	
	CO9	CO13	CO9	CO13
untreated, unfermented control	30.4	30.8	273.1	294.4
untreated, fermented with 18 h starter	29.0	28.0	260.5	267.6
untreated, fermented with 48 h starter	23.4	23.0	206.6	219.8
enzyme-treated, unfermented	29.9	27.8	268.6	265.7
enzyme-treated, fermented with 18 h starter	30.4	26.0	273.1	248.5
enzyme-treated, fermented with 48 h starter	30.2	23.9	271.3	228.4

[a] Values are on dry weight basis. Values are calculated from the mean of three independent determinations.

nin white variety (CO9). Presence of phenolics and tannins can influence the type of endogenous seed microflora and flour composition, and therefore the susceptibility of the flour to fermentative changes.

Analysis of variance (ANOVA) of the data allowed the comparison of the different treatments to which the millet varieties were subjected. The processing methods significantly ($p < 0.05$) affected pH, titratable acidity, phytate, tannins, and IVPD in the red CO13 variety. In CO9, only pH, titratable acidity, and phytate were significantly affected ($p < 0.05$). Enhanced protein availability, as mentioned earlier, is crucial in diets of people whose intake of this nutrient is otherwise low. The present method may be further refined and/or modified to develop specific products that serve as supplementary health foods or probiotic foods. However, scaling up, product trials and palatability studies are a prerequisite. The specific benefits that can be obtained from the processed millet also need to be confirmed by suitable human trials.

CONCLUSIONS

To conclude, processing of finger millet by combination of enzymatic treatment and use of starters effectively directed the fermentation to desired goals of (a) reduced fermentation time, (b) sufficient acidity, (c) enhanced protein digestibility, (d) reduction in phytates and tannins, and (e) increased mineral extractability. The partial retention of phytates and tannins is beneficial for their contribution to health benefits such as antidiabetic, antioxidant, and anticancer effects which have been recently recognized (Thompson, 1993).

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