Intl. J. Food. Ferment. Technol. 8(2): 185-190, December 2018

DOI: 10.30954/2277-9396.02.2018.8

RESEARCH PAPER

Reduction of Phenolics, Tannins and Cyanogenic Glycosides Contents in Fermented Beverage of Linseed (*Linum usitatissimum*)

N. Nivetha, V.C. Suvarna* and R.U. Abhishek

Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bengaluru 560 065, Karnataka, India

*Corresponding author: suvarnavc@gmail.com

Paper No.: 220 **Received:** 03-06-2018 **Revised:** 20-09-2018 **Accepted:** 13-11-2018

ABSTRACT

Linseed (*Linum usitatissimum)* is a highly nutritious oilseed with exceptionally high contents of α-linolenic acid (ALA), dietary fiber, good quality protein and phyto-estrogens. It is the richest known vegetable source of omega-3 fatty acid and various minerals. But it also contains anti-nutritional factors like phenolics, tannins and cyanogenic glycosides that are known to inhibit the activity of digestive enzymes and interfere with availability of nutrients especially minerals like calcium and iron. Hence, an attempt was made to reduce anti-nutrients by different processes like soaking, roasting, fermentation *etc*. Lactic acid bacterial strains were isolated from linseed, screened and isolate LAB-3 was selected based on high titrable acid production (0.08%), desired sour-sweet taste and good flavor after fermentation. Probiotic cultures *viz*., *Lactobacillus acidophilus*, *Bacillus mesentericus* and *Saccharomyces boulardii*; wine yeast *Saccharomyces ellipsoideus* and lactic acid bacterial isolate LAB-3 were used in fermentation of linseed beverage. The quantity of anti-nutritional factors *viz*., total phenolics, tannins and cyanogenic glycosides were estimated. Significant reductions in total phenolics and tannins were observed in *L. acidophilus* and isolate LAB-3 treatments. Fermented beverage had 58% reduced phenolics and 66% reduced tannins compared to raw seed (control). This significant reduction in phenolics and tannins were observed with lactic acid bacteria than yeasts. The highest per cent reduction in cyanogenic glycosides was observed with *L. acidophilus* inoculation (66% followed by isolate LAB-3 (65%) and *B. mesentericus* (58%). The observed antinutritional factors reduction ability may be due to enzymes like linamarase and β-glucosidase production by tested microorganisms. It is concluded that, microbial intervention can be exploited very conveniently to reduce the anti-nutrients contents and to improve nutritional quality of linseed.

Keywords: Linseed, lactic acid bacteria, fermented beverage, reduction of antinutritionals

Linseed also known as "flax" is one of the oldest cultivated crops grown by man for oil, food and fiber. It is the most important industrial oilseed crop of the world and is cultivated over an area of 3.02 million hectares with a production of 2.57 million tones and productivity of 852 kg /ha (Nag *et al.* 2015). India ranks first in terms of area under linseed cultivation with 4.68 lakh ha and third in production with 1.68 lakh tones and 349 kg/ha productivity. Linseed has potential health promoting nutritional profile and the richest known source of α-linolenic acid (omega-3 fatty acid). It has exceptionally high dietary fiber, excellent protein profile and phytoestrogens. α-Linolenic acid is beneficial for cardiovascular diseases and is known to reduce blood lipids (Zhao *et al.* 2004). Fiber reduces constipation, keeps better bowel movement and acts as hypocholesterolemic agent. Lignans decrease free oxygen radicals and possess anticancer properties (Giada, 2010). These properties make it more favorable for food technologists to explore and develop novel nutraceutical foods.

Microbial fermentations are employed to improve nutritional value by reducing the anti-nutritional factors thus enhancing bioavailability of minerals and starch. The cyanide content decreases during the fermentation of cassava roots and leaves by more than 70% by bacterial enzyme linamarase, allowing the hydrolysis of cyanogenic glycosides. Certain lactic acid bacteria commonly associated with fermentations are resistant to high cyanide concentrations in the range of 200 to 800 ppm (Kobawila *et al*. 2005). Boonaop *et al.* (2009) studied cassava root fermentation by yeast and they found that *Saccharomyces cerevisiae* decreased hydrocyanic acid of cassava chips from 3.4 to 0.5 per cent and 68.6 to 47.3 per cent in fresh cassava roots after 132 hrs of fermentation. Adeyemo and Onilude (2013) used *Lactobacillus plantarum* isolates of local foods to ferment soybean for reduction of anti-nutritional factors. Significant reduction in tannin content was recorded during fermentation from 1.93 to 0.12 mg g^{-1}. Zarate *et al.* (2014) reviewed the role of lactic acid bacteria in tannin biodegradation in foods. Hence, an attempt was made to prepare a nutraceutical fermented beverage of linseed using prebiotic (honey) and probiotic cultures (*Lactobacillus acidophilus*, *Bacillus mesentericus*, *Saccharomyces boulardii), Saccharomyces ellipsoideus* and lactic acid bacterial isolates.

Kajla *et al*. (2014) reported that linseed contains anti-nutrients having adverse effects on health and well-being of human beings. Cyanogenic glycosides are the major anti-nutrients and are fractioned into linustatin, neolinustatin and linamarin. Among cyanogenic glycosides linustatin (213-352 mg/ 100 g) is a major component, accounting for 54-76% of the total cyanogenic glycoside content. The content of neolinustatin and linamarin is 91-203 mg/100 g and 13.8-31.9 mg /100 g respectively. Cyanogenic glycosides release hydrogen cyanide, a potent respiratory inhibitor upon hydrolysis and are present in the range between 9 – 178 mg kg^{-1} of linseed products. It is clear from observations of Cresseya *et al.* (2013) that products having linseed as an ingredient contain the highest concentration of total hydrocyanic acid (>50 $mgkg^{-1}$). The minimum lethal dose of HCN taken orally is approximately 0.5-3.5 mg /kg body weight or 35 - 245 mg for a 75 kg person (Shahidi and Wanasundara, 1997). Trypsin inhibitors, phenolic compounds and tannins are also reported to be present in linseed. El-Beltagi *et al*. (2007) reported that linseed's total phenolic content ranges from 130-220 mg gallic acid equivalents (GAE) per 100 g of seeds. Raw linseeds contain 146.3 ± 0.5 mg of tannin /100 g. Tannins are known to decrease feed intake, growth rate, feed efficiency and protein digestibility in experimental animals. Tannins also inhibit enzyme activities like trypsin, chymotrypsin, amylase and lipases. Tannins are known to decrease the protein quality of foods and interfere with dietary iron absorption. Tannins form insoluble complexes with proteins and tannin-protein complexes may be responsible for anti- nutritional effects (Gemede and Ratta, 2014). Efforts made to reduce the phenolics, tannins and cyanogenic glycosides contents in Fermented Beverage of Linseed (Linum usitatissimum) were made and the results have been described in this communication.

MATERIALS AND METHODS

Preparation of fermented linseed beverage using probiotic cultures and lactic acid bacterial isolate

Lactic acid bacteria were isolated from linseed surface by enrichment method followed by standard plate count method using De Man, Rogosa and Sharpe agar medium (De Man *et al.* 1960). Lactic acid bacterial strains (five) were selected and further characterized for effective fermentation based on acid production and sensory characteristics of fermented beverage. Probiotic organisms (*Lactobacillus acidophilus*, MTCC-10307, *Bacillus mesentericus* and *Saccharomyces boulardii), Saccharomyces ellipsoideus* and lactic acid bacterial isolate (LAB-3) were used for the preparation of fermented linseed beverage and analyzed for anti-nutritional factors.

Linseed was fermented by following the protocol, standardized for the concentration of ingredients. Roasted linseed powder was mixed with sugar, honey and water at required concentrations. The mixture was simmered for 30 seconds and 8% inoculum was added and incubated at 30°C for two days for preparation of linseed fermented beverage. After fermentation beverage was filtered, pasteurized at 60°C for 30 min, cooled and stored in refrigerator (Nivetha *et al*. 2017). The entire process is shown diagramatically in Fig. 1.

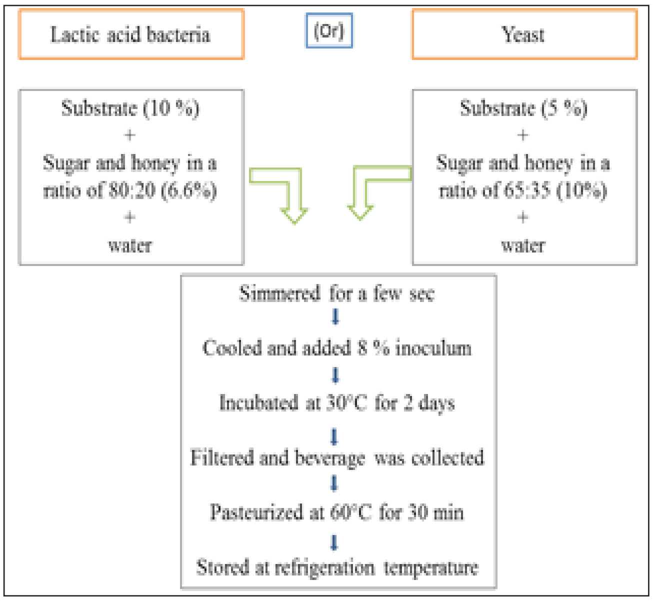

Fig. 1: Preparation of fermented linseed beverage

Determination of pH, titrable acidity, total soluble solids and alcohol content of the fermented linseed beverage

Samples were analyzed for changes in pH, titrable acidity, total soluble solids and alcohol content after fermentation. The pH of the sample was measured using digital pH meter (Digital pH meter type MK-VI, India). Titrable acidity of the samples were measured following the procedure of Srivastava and Kumar (1993) by titrating against 0.1N NaOH and using phenolphthalein indicator and expressed as per cent lactic acid. The total soluble solids of the samples were determined with the help of hand refractometer (ERMA, Japan) having a range of 0 to 32° Brix at room temperature. Alcohol content of the beverage was estimated by chemical oxidation method (Amerine and Ough, 1980).

Determination of total phenolics

Phenolic compounds of beverage were determined following the procedure of Fazeli *et al*. (2011). Standard graph was prepared using gallic acid. Amount of total phenolic compounds in the samples were calculated from the standard graph and expressed as gallic acid equivalents (mg of GAE/g of linseed used).

Determinations of tannins

Tannins were determined using Folin-Ciocalteu method (Tambe and Bhambar, 2014). The tannin content was expressed in terms of mg of gallic acid equivalent (GAE) /g of extract.

Determination of total cyanide content

Total cyanide content of the linseed beverages were estimated by acid hydrolysis method (Haque and Bradbury, 2002). Total cyanides in the linseed beverages (4ml) were extracted using 0.1M H_3PO_4 and extracts were filtered using Whatman No. 1 filter paper and volume was made to 20 ml. Extracts (2 ml) were taken in a glass stopper test tubes, added 4 ml of 4M H_2SO_4, incubated for 50 min in boiling water bath, cooled and incubated at 4°C for overnight. After incubation, 2 ml of this solution was added to 5 ml of 3.6M NaOH and allowed to stand for 5-10 min at room temperature. Aliquots (one ml) of the solutions were added to test tubes containing 7 ml 0.2 M phosphate buffer and 0.4 ml of 0.5% chloramine-T. The tubes were cooled in ice for 5 min and added with 1.6 ml pyridine-barbituric acid solution. The purple colour was allowed to develop up to 90 min and the absorbance was measured at 583 nm. Amount of total cyanide was calculated from the standard graph prepared using potassium thiocyanate.

RESULTS AND DISCUSSION

Isolate LAB-3 recorded higher significant reduction in pH (4.60) more titrable acid production (0.08%) with favorable sour-sweet taste and good flavor (Nivetha

Table 1: Changes in pH, TA, TSS and alcohol content of fermented linseed beverage

Treatment details		pH		Titrable acidity (TA)		Total soluble solids (TSS)		Alcohol content (%)
	Inoculum	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	
T_1	*L. acidophilus*	5.96	4.39	0.03	0.22^{c}	9.00	8.00	0.36^{d}
T_2	*B. mesentericus*	5.99	4.38	0.02	0.30^{a}	9.00	7.45	0.10^{e}
T_3	LAB-3 isolate	5.94	4.48	0.03	0.25^{b}	9.00	6.93	0.50^{c}
T_4	*S. ellipsoideus*	5.91	4.20	0.02	0.29^{a}	10.00	8.05	1.24^{b}
T_5	*S. boulardii*	5.89	4.21	0.02	0.30^{a}	10.00	7.80	1.47^{a}

Table 2: Phenolic compounds, tannins and cyanide contents of fermented linseed beverage

Treatment details		Phenolic compounds (mg/ g of gallic acid equivalent)		Tannins (mg/g of gallic acid equivalent)		Total cyanide content (mg/g of sample)	
		Phenolics	**% Reduction**	**Tannins**	**% Reduction**	**Cyanide**	**% Reduction**
T_1	Raw seed	1.97^{a}	—	2.20^{a}	—	5.17^{a}	—
T_2	Roasted seed	1.40^{b}	29^{b}	1.70^{b}	22^{e}	4.76^{b}	08^{c}
T_3	*L. acidophilus*	0.59^{e}	58^{a}	0.62^{f}	64^{a}	1.62^{e}	66^{a}
T_4	*B. mesentericus*	0.65^{e}	54^{a}	0.77^{e}	54^{b}	2.02^{d}	58^{a}
T_5	LAB 3 isolate	0.60^{e}	57^{a}	0.58^{f}	66^{a}	1.66^{de}	65^{a}
T_6	*S. ellipsoideus*	1.02^{d}	27^{b}	1.10^{d}	35^{c}	3.41^{c}	28^{b}
T_7	*S. boulardii*	1.19^{c}	16^{c}	1.15^{c}	33^{d}	3.32^{c}	30^{b}

et al. 2017). Hence, isolate LAB-3 was selected for further fermentation studies. Fermentation of linseed by probiotic bacteria, yeasts and lactic acid bacteria showed a significant reduction in pH. Increase in titrable acidity was also observed in fermented samples and it ranged between 0.22-0.30% as lactic acid (Table 1). Similar results were also reported by Wakil and Kazeem (2012), that an increase in titrable acidity from 0.40 to 1.15% was also recorded during 72 hrs fermentation using *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in cowpea blends. Acid production by microorganisms is one of the methods for reduction of anti-nutritional factors. Total soluble solid content decreased after fermentation and yeasts utilized more sugar than lactic acid bacteria. This may be due to the fermentation efficiency and sugar conversion capacity of different microorganisms. Linseed beverage prepared using *S. ellipsoideus* and *S. boulardii* reported a significantly higher alcohol content of 1.24% and 1.47%, respectively. Fermentation by *Bacillus mesentericus* and lactic acid bacterial fermentations had an alcohol content ranging from 0.1 to 0.5%.

Fermentation of linseed using lactic acid bacteria and yeasts showed significant reduction in anti-nutritional factors including total phenolics, tannins and total cyanides (Table 2). Maximum reduction of phenolic compounds was recorded by *Lactobacillus acidophilus* fermented linseed beverage (58%) followed by isolate LAB-3 (57%) and *Bacillus mesentericus* (54%). The least reduction was observed in *Saccharomyces boulardii* (16%) fermented beverage. These results are in conformation with the observations made by Rodriguez *et al.* (2009) that *Lactobacillus plantarum* was able to degrade some food phenolic compounds and yielding compounds responsible for aroma and antioxidant activity. Isolate LAB-3 showed the highest reduction of tannins (66%) followed by *Lactobacillus acidophilus* (64%). Similar results were reported by Adeyemo and Onilude (2013) in soybean using *L. plantarum*. *S. boulardii* and *S. ellipsoideus* exhibited less reduction in tannin content. This could be due to the fact that yeasts are known to hydrolyze only low molecular weight tannins like gallotannins but not complex tannins (Cerda *et al*. 2003).

Tannins are known to decrease feed intake, growth rate, feed efficiency and protein digestibility in experimental animals. Tannins inhibit enzyme activities like trypsin, chymotrypsin, amylase and lipases. Tannins are known to decrease protein quality of foods and interfere with dietary iron absorption. Tannins form insoluble complexes with proteins and tannin-protein complexes may be responsible for anti- nutritional effects of tannin rich foods (Gemede and Ratta, 2014). Kajla *et al*. (2014) reported that linseed contains anti-nutrients that may have adverse effects on health and well-being of human beings. Cyanogenic glycosides are the major anti-nutrients and are fractioned into linustatin, neolinustatin and linamarin. Among cyanogenic glycosides linustatin (213-352 mg /100 g) is a major component, accounting for 54-76 % of the total cyanogenic glycoside content. The content of neolinustatin and linamarin is 91-203 mg /100 g and 13.8-31.9 mg /100 g, respectively. Cyanogenic glycosides release hydrogen cyanide, a potent respiratory inhibitor upon hydrolysis and are present in the range between 9 – 178 mg kg^{-1} of linseed products. It is clear from the observations of Cresseya *et al.* (2013) that products having linseed as an ingredient contain the highest concentration of total hydrocyanic acid (>50 mg kg^{-1}). The minimum lethal dose of HCN taken orally is approximately 0.5-3.5 mg /kg body weight or 35 - 245 mg for a 75 kg person (Shahidi and Wanasundara, 1997). Trypsin inhibitors, phenolic compounds, tannins are also reported to be present in linseed. El-Beltagi *et al*. (2007) reported that linseed's total phenolic content ranges between 130-220 mg gallic acid equivalents (GAE) per 100 g of seeds. Raw linseeds contain 146.3 ± 0.5 mg of tannin /100 g. This reduction is due to the breakdown and degradation of the anti-nutrients into smaller units by the action of the enzymes mobilized during fermentation period.

Significant reduction in cyanide content of linseed was reported by roasting treatment (8%). Thermo-labile property of cyanogenic glycosides and destruction of cyanide using heat treatments were earlier reported by Feng *et al.* (2003). The highest reduction of cyanogenic glycosides was observed in case of *Lactobacillus acidophilus* (66%), isolate LAB-3 (65%) and *Bacillus mesentericus* (58%,) and the least reduction was observed with yeasts. Reduction of cyanogenic glycosides during fermentation could be due to low pH and enzymes production by microorganisms like linamarase and β-glucosidase. Similar results were reported by Kobawila *et al*. (2005) using lactic acid bacteria and Boonaop *et al*. (2009) using *Saccharomyces cerevisiae* in cassava indicate the important role of microorganisms in nutritional quality improvement. *Lactobacillus acidophilus* and LAB-3 isolate were found to be efficient in fermented beverage preparation with improved nutritional quality.

Microbial fermentations are employed to improve nutritional value by reducing the anti-nutritional factors thus, enhancing bioavailability of minerals and starch (Kobawila *et al*. 2005). Boonaop *et al.* (2009) studied cassava root fermentation by yeast and they found that *Saccharomyces cerevisiae* decreased hydrocyanic acid content of cassava chips from 3.4 to 0.5% and 68.6 to 47.3% (fresh cassava roots) after 132 hrs of fermentation. Adeyemo and Onilude (2013) used *Lactobacillus plantarum* isolates of local foods to ferment soybean for reduction of anti-nutritional factors. Significant reduction in tannin content was recorded during fermentation from 1.93 to 0.12 mg g^{-1}. Zarate *et al.* (2014) reviewed the role of lactic acid bacteria in tannin biodegradation in foods. An attempt to prepare a nutraceutical fermented beverage of linseed using prebiotic (honey) and probiotic cultures (*Lactobacillus acidophilus*, *Bacillus mesentericus*, *S. boulardii)*, *Saccharomyces ellipsoideus* and lactic acid bacterial isolates has yielded good results.

CONCLUSION

The results of this study reveal that lactic acid bacteria and yeasts reduced anti-nutritional factors in linseed significantly, thereby help in producing a good nutraceutical beverage. Fermentation of the linseed through microbial activity led to the hydrolysis and reduction of anti-nutritional factors. The nutritional composition of the beverage is also improved by fermentation. Thus, the growing demand of usage of linseed in diet can be fulfilled by developing linseed products with reduced anti-nutritional factors and improved nutritional qualities through fermentation technology.

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