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RESEARCH PAPER

Microbiological and biochemical characterization of experimentally produced *Sura*-a traditional fermented millet based alcoholic beverage of Kullu District of Himachal Pradesh, India

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Abstract

Microbiological and biochemical characterization of experimentally produced *Sura*- an alcoholic beverage produced and consumed by people in Lug valley of Kullu district (Himachal Pradesh) is reported here. *Sura* is a fermented alcoholic beverage prepared in Kullu district of Himachal Pradesh, India. Peculiarity of this beverage is that no specific inoculum is used its preparation. An additive *dheli* (36 herbs) is added after 10 days of natural fermentation. The identification of the predominant microflora in this traditional fermented beverage revealed that *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Enterococcus pentosaceus* were the main fermenting lactic acid bacteria and *Saccharomyces cerevisiae*, *Saccharomyces fibuligera* and *Pichia kudriavzevii* were the major yeasts involved in its fermentation. During *Sura* fermentation protein content increased from 6.36% (w/w) to 12.8% (w/w), while total carbohydrates decreased from 60% (w/w) to 11% (w/w), starch from 55.3% (w/w) to 16.32% (w/w). Reducing sugars, amylaseand protease activity increased with the progress in fermentation. Increase in the level of B vitamins (B_{12} and B_3) was quite significant from the initial levels. The methanolic extract of *dheli* showed an antioxidant activity of 26%, thus proving the efficacy of *sura* as a potential functional beverage.

Keywords: *Dheli,* LAB, *Sura*, yeasts, alcoholic beverage, functional beverage, traditional beverage

Traditional cereal-based fermented alcoholic beverage with a pleasant sweet-sour, bread-like taste like *boza* is consumed in some areas of Turkey, Albania and Romania (Gotcheva *et al.* 2000). *Thumba* and *jaanr* are some of the popular millet based fermented alcoholic beverages prepared and consumed in Himalayan regions of India (Tamang, 1998). *Sura* is amillet (*Eleucinecoracana*) based fermented alcoholic beverage prepared in *Lug* valley of Kullu district, Kangra and Mandi districts of North western Himalayas of Himachal Pradesh, India (Thakur *et al.* 2004, Joshi *et al.* 2015). In its preparation, no specific inoculum is added and the natural microflora present on the substrate and the vessels carry out starch hydrolyzing (saccharolytic) and ethanol forming (fermentative) activities. This beverage is unique from other millet based beverages in that a herbal additive (*dheli)* made of more than thirty six traditional herbs is used in its preparation. *Dheli* also provides bioactive compounds as well as stimulatory effect (Thakur *et al.* 2004). The microorganisms involved in fermentation and biochemical characteristics of this beverage are not known in literature till date. The objective of this study was to characterize the microbiological and biochemical characteristics of *sura* fermentation.The results obtained are described in this paper.

Materials and Methods

Finger millet (*Eleucine coracana*) locally called as *kodra or kached* flour, is the basic raw material needed which was procured from the local village in *Lug* valley in Kullu district of Himachal Pradesh, India. *Dheli,* the additive used in the prepration of *sura*, was procured from the local villagers and its method of preparation was studied as practiced by the locals.

Preparation of sura

To make *Sura* under laboratory conditions, millet flour was first knead with water and then, dough was left for initial natural fermentation for 10 days in a container. In secondary fermentation, the slurry made of this naturally fermented dough was spread on the hot plate and cooked to make half baked '*roties*' (pancakes). These '*roties*' are cooled, made into small pieces and then mixed with powered *dheli* and water. The whole mixture is put into an earthen pot and left for fermentation for 8-10 days. The earthy colored liquid finally obtained is called *sura.* During fermentation of *Sura* samples were drawn every 24h and were analyzed for microbiological and biochemical aspects.

Microbiological analysis of *dheli* and *sura*

One gram of fermented sample of *sura* (wet) and powdered '*dheli*' were serially diluted from 10^{-1} – 10^{-8} with physiological saline. 0.1 ml of diluted sample was spread plated on YPD Agar (yeast extract 2%, peptone 1%, dextrose 1% and agar 2%) and MRS agar for isolation of yeasts, moulds and lactic acid bacteria as per standard methods (Dewan and Tamang, 2007). The plates were incubated at 30°C for yeasts and lactic acid bacteria and 28°C for moulds, respectively.

Microbial profile/succession during the fermentation of sura

One gram of sample, withdrawn every 24 h of fermentation was serially diluted and appropriate dilution were plated on YPD agar and Man, Rogosa and Sharpe (MRS) agar plates for isolation of yeasts, moulds and lactic acid bacteria. The number of colonies of yeasts and LAB that appeared on plates were counted and expressed as log cfu (colony forming units) g^{-1} of the sample.

Identification of lactic acid bacteria and yeasts

LAB colonies were picked on the basis of differences in colony morphology during succession studies in fermentation. Genomic DNA was extracted from LAB by alkaline lysis method (Sambrook *et al.* 1989). Extraction of genomic DNA from yeast was done by Bust and Grab method described by Harju *et al.* (2004).

Identification of isolates to species level was done by PCR amplification of the 16S rDNA gene with lactic acid bacterial universal primers (Thomas, 2004). For identification of yeast isolates, the divergent D1/D2 domain of the 26S ribosomal gene was amplified using the universal primers UniF63 and UniLR3 (Minegishi *et al.* 2006). Sequencing was conducted at DNA sequencing facility at ViikkiBiocentre, University of Helsinki, Finland and were compared to those in the NCBI GenBank by BLAST.

Biochemical characterisation of *sura*

Analytical methods: The pH of *sura* samples during the 20 days fermentation period was determined. Total acidity was determined by titrametric method (Amerine *et al.* 1980). Protein content of various *sura* samples was analysed according to the method of Lowry *et al.* (1951). Total carbohydrate, reducing sugars and starch were estimated by methods of Dubois *et al.* (1956), Miller (1959), and Hedge and Hofreiter (1962), respectively. Amylase activity was assayed by the method of Bernfield (1955) while protease activity in the *sura* sample was estimated according to Manachini *et al.* (1988).

Vitamin and amino acid analysis: The water soluble B-vitamins i.e B_1 (Thiamin), B_2 (riboflavin), B_3 (Niacin), B_6 (Pyridoxine), B_9 (Folic acid) and B_{12} (Cyanocobalamin) were assayed in the fermented beverage samples at initial and final stage of fermentation according to Snajdrova *et al.* (2004). The amino acid analysis was based on the treatment of aqueous amino acid solutions with ethyl chloroformate (ECF) using Vapor-phase

acid hydrolysis method of Schilling *et al.* (1996). Derivatization of amino acids was done according to Hûsek (1991) and gas chromatography was carried out.

Volatile analysis of *sura* by gas chromatography: Ethanol and other metabolites (acetic acid) were determined with the help of Agilent Systems Gas Chromatograph with Flame Ionization Detector (GC-FID) Michro-9100, from Netel Chromatographs, Thane, India..

Functional aspects of *sura*

Flavonoid content: Total flavonoid contents were measured with aluminium chloride colorimetric assay (Kumar *et al*., 2008).

Free radical Scavenging Activity Assay

The free radical scavenging activity of methanolic extracts and the standard L-Ascorbic Acid (vitamin C) was measured in terms of hydrogen donating or radical scavenging ability using DPPH-RSA method (Blois, 1958).

Results and Discussion

During *sura* fermentation, the samples were analyzed at an interval of 5 days over a period of 20 days.

Microbiological analysis of '*dheli*' (Herbal additive): The microbiological analysis of '*dheli*' revealed it to be a consortium of different microorganisms which mainly comprised of lactic acid bacteria (3.6×10^6 cf u/g^{-1}), yeasts (1.7×10^7cfu/g^{-1}) and moulds (1.2×10^3 cf u/g^{-1}). LAB isolated from *dheli* are *Enterococcus faecium* (JX141329) and *Lactobacillus* sp. Among the yeasts, *Saccharomyces fibuligera* (JX141337) and *Saccharomyces cerevisiae* were the major yeasts identified. Fadahunsi *et al.* (2013) have also been identified *Lactobacillus brevis* and *Saccharomyces cerevisiae* fresh and stored samples of *Burukutu* and *Pito*.

Microbiological analysis of *sura* fermentation: Initial viable counts of yeasts and lactic acid bacteria were 7.3×10^6 and 2×10^7 cfu/g (wet basis), respectively during spontaneous fermentation but these decreased to 5.5×10^5 and 1.9×10^7cfu/g on the 10^{th} day of non-alcoholic fermentation. On 15^{th} day, counts were 7×10^4 cfu/g for yeasts and 4×10^6cfu/g (wet basis) for lactic acid bacteria, respectively during alcoholic fermentation. The main Lactic acid bacteria (LAB) associated with *sura* fermentation as identified by molecular techniques (16S rDNA) ware *Lactobacillus* sp. (JX141320), *Lactobacillus casei* (JX141324), *Enterococcus* sp., *Pediococcuspentosaceus* (JX141326) and *Enterococcus faecium* (JX141323), *Enterococcus lactis* (JX141327). Major yeasts isolated and identified by sequencing of D1/D2 region of 26S rDNA are *Pichia kudriavzevii* (JX141333), *Saccharomyces cerevisiae* (JX141334). Similar microorganisms have been isolated from fermented alcoholic beverages of North East India earlier by Tamang *et al.* (2012).

Biochemical changes

In case of *sura* fermentation, change in pH was from initial 5.0 to 3.4 during 10 days of primary fermentation, whereas in control the decrease was from initial 5.0 to 3.6. Titrable acidity increased from 0.01% to 0.12% in *sura* and from 0.01% to 0.08% in case of control. The percentage of titrable acidity increased as fermentation progressed in relation to fall in pH (Fig. 1). The correlation between acidity and pH is believed to be associated with both *yeasts* and LAB as LAB are well known for the production of acids especially lactic acid whereas some yeasts were previously reported to produce acid in alcohol fermentation to make a positive contribution to the product flavor (Fleet, 2003). At the same time, low pH and high acidity have advantage as these could eliminate enteropathogens, coliforms and spoilage organisms in *Sura*.

In case of *sura* fermentation, the protein content increased from initial 6.36% (w/w) to 12.8% (w/w) on the 20^{th} day of fermentation, whereas in case of control it increased up to 10.30% (w/w) as shown in Table 1. Basappa *et al.* (1997) have also reported an increase in proteins during fermentation of *ragi* (Finger millet). This bioenrichment through fermentation is beneficial for consumers needing high protein intake. Total carbohydrates were analyzed during *sura* fermentation at an interval of 5 days over a period of

20 days (Table 2). Initial carbohydrates present were 60% (w/w) that decreased to 10.6% (w/w) at the end of 20th day of fermentation. The sugars are rapidly metabolized to acids, ethanol, biomass, carbon dioxide and other metabolites required for the growth of microorganisms with concomitant decrease in total sugars during fermentation (Mensah, 1997).

Table 1: Protein content of *sura* during fermentation

Days of incubation	Protein (% w/w) in *chhang*	Protein (% w/w) in *control
1	6.36±0.21	6.36±0.21
5	8.15±0.20	8.15±0.20
10	6.30±0.47	10.30±0.30
15	12.8±0.80	10.30±0.30
20	12.8±0.80	10.30±0.30

Table 2: Level of total carbohydrates in *sura* during fermentation

Days of incubation	Toal carbohydrates % (w/w) in sura	Toal carbohydrates % (w/w) in *control
1	60.0±0.2	58.6±0.78
5	56.6±0.57	55.6±0.90
10	50.3±0.70	54.7±0.96
15	45.6±0.90	48.3±0.88
20	10.6±0.52	38.3±0.88

Reducing sugars increased from initia l 0.11% (w/w) to 3.0% (w/w) at the end of the 20th day of fermentation (Table 3). Mosha and Svanberg (1983) have reported the hydrolysis of starch and oligosaccharides present in the substrates of fermentation resulting in an initial increase in reducing sugars due to the activity of amylases present in the cereal grains and the amylases produced by the microorganisms present in the initial phases of fermentation. As the pH of the ferment decreases, the saccharification of starch by α and β-amylases, and amyloglucosidases is also reduced which results in a gradual decrease of reducing sugar concentration towards the later stages of fermentation (Syu and Chen, 1997). Starch content from an initial 55% (w/w) decreased to 16.32% (w/w), whereas in case of the control the decline was to 37.6% (w/w). A continuous degradation of starch during natural fermentation of *bushera* with concomitant increase in maltose and glucose has been recorded earlier by Muyanja *et al.* (2004).

Table 3: Level of reducing sugars in *sura* during fermentation

Days of incubation	Reducing sugars % (w/w) in sura	Reducing sugars % (w/w) in *control
1	0.11±0.01	0.11±0.01
5	0.13±0.02	0.13±0.02
10	1.20±0.02	0.25±0.02
15	2.50±0.5	1.10±0.02
20	3.00±0.5	1.10±0.02

Amylolytic activity was 0.04 U/g at the start of the fermentation (Fig. 2) which increased to 0.23 U/g of dry matter at the end of 15th day of fermentation and finally, decreased again to 0.04 U/g at the end of 20 days of fermentation. Dry milling has been reported to cause high level of mechanical damage to starch granules, thus making them more susceptible to attack by either endogenous amylolytic enzymes or hydrolytic enzymes of microorganisms (Akigbala *et al.* 1987).

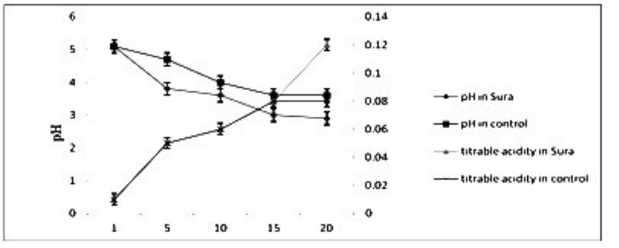

Fig. 1: Change in pH and titrable acidity in sura

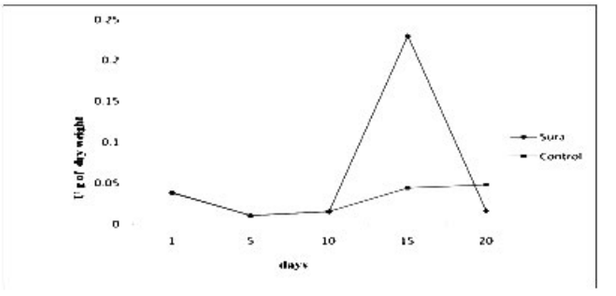

Fig. 2: Amylase activity during sura fermentation

The other reason for decline in amylase activity could be due to the disappearance of amylolytic yeasts that occurs as the ethanol content increases in the fermentation system (Basappa, 2002).

Proteolytic activity, increased from initial 0.004 U/g to 0.07U/g till 20th day of fermentation. In control, it remained almost constant (0.004 U/g) and then, decreased to 0.002 U/g of dry matter at the end of fermentation (Fig. 3). This low proteolytic activity could be either due to increase in ethanol or lowering of pH during fermentation, which might have inhibited proteolytic activity.

Table 4: Vitamin content during *sura* fermentation

Vitamins	Initial sura	Final sura	Final control*
B_2 (mg/100g)	0.032±0.004	0.17±0.01	0.028±0.01
B_3 (mg/100g)	0.77±0.007	1.90±0.02	0.93±0.06
B_6 (mg/100g)	0.12±0.03	0.16±0.03	0.03±0.003
B_9 (µg/100g)	212±0.70	13.1±0.43	9.20±0.43
B_{12} (µg/100g)	2.89±0.10	35.83±1.6	0.28±0.02

Table 5: Amino acid content during *sura* fermentation

Amino acid (mg/g)	Initial	Final (20 days)	Final Control*
Valine	0.54±0.07	5.2±0.2	ND
Aspartic acid	ND	3.17±0.3	ND
Proline	ND	2.49±0.4	ND
Leucine	0.3±0.05	1.15±0.05	ND

*Control comprised of uninoculated cooked millet slurry incubated similar to *sura.*

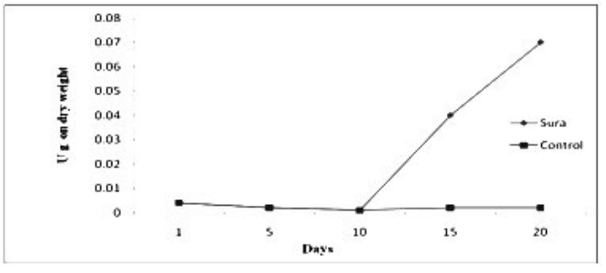

Fig. 3: Proteolytic activity during sura fermentation

*Control comprised of uninoculated cooked millet slurry incubated similar to sura.

Increase in the level of B vitamins (B_{12} and B_3)was quite significant from the initial levels as presented in Table 4, vitamin B_{12} increased from initial 2.89 µg/100 g to 35.8 µg/100 g nearly 12 times increase, compared to the control (0.28 µg/100 g), vitamin B_3 (niacin) also increased from initial 0.77 mg/100g to 1.90 mg/100 g. Basappa *et al.* (1997) have also reported synthesis of vitamin cyanocobalamin B_{12} (40 µg/100g) during the fermentation of cooked *ragi*. The rise in content of various vitamins especially B-vitamins, during fermentation is due to the growth of microorganisms mainly yeasts as these have the ability to produce, vitamins from simple precursors (Soni and Sandhu, 1990). Similarly, amino acids like valine (0.54-5.2 mg/g) and leucine (0.3-0.3 mg/g) showed a remarkable increase of nearly 10 times (valine) and 4 times (leucine) post-fermentation but others like, proline and aspartic acid were detectable only in the final ferment (Table 5). Similar studies have been reported by Basappa *et al.* (1997) during evaluation of nutritional composition of fermented ragi (*chhang*) by *phab*. They reported an increase in some free amino acids in addition to the synthesis of extra amino acids.

During *sura* fermentation, ethanol content was analyzed over a period of 20 days of fermentation. Initially it was nearly 1% (0.92% w/w) on 10th day of spontaneous fermentation and then, increased to nearly 2% (1.84% w/w) and finally on 20th day, was 5% (w/w). No acetic acid was however detected.

Functional aspect (Flavonoids content)

The total flavonoids estimation of methanolic extracts of *sura* showed a value of 1.87% (w/w) for Quercetin. In studies carried out by Patel *et al.* (2010), the ethanolic leaf extracts of medically important plant *Tephrosiapurpurea* Linn (Sarpankh) had Quercetin content in 1.56±0.32% (w/w).

Antioxidant activity

The antioxidant reacts with the stable free radical, DPPH and converts it to 1, 1-Diphenyl-2-Picryl

Hydrazine. The ability to scavenge the free radical, DPPH was measured at an absorbance of 517nm. Ascorbic acid was taken a the reference. The per cent of inhibition exhibited by ethanolic extract of *dheli* was 26% at inhibitory concentration value of 4.3±0.1 μg/ml.

Conclusion

Sura contained a variety of yeasts and lactic acid bacteria that contribute towards the flavor and preservation of this beverage and thus serves as a suitable alternative to milk–based carriers for probiotic bacteria as these have high counts of viable lactic acid bacteria and may serve as a blueprint for development of cereal-based probiotic functional beverages. In addition to being alcoholic in nature, this beverages has substantial nutritional value with significant amounts of B-vitamins and essential amino acids as a result of fermentation. It is also radical scavenging ability of methanolic extract of *dheli* contributes to the antioxidant effect. Thus, it is concluded that these traditionally fermented cereal beverages have potential nutritional vis-à-vis probiotic and functional benefits to the consumers.

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Conflict of interest

Authors declare that there is no conflict of interest.

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