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Original Article

PHYSICO-CHEMICAL CHARACTERISTICS OF RUSSIAN TEA FUNGUS: *KOMBUCHA*

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ABSTRACT

Objective: To characterize various physicochemical parameters for Russian tea fungus: *Kombucha*.

Methods: In the present investigation, various physicochemical analyses of *kombucha* such as, estimation of biomass, ascorbic acid, acetic acid, carotenoid contents, antioxidant activity and antibacterial activity against *E. coli, Salmonella* sp and *Staphylococcus aureus* were carried out after growing at different temperature and pH with varied concentrations of 5, 10, 15 and 20% tea powder boiled for 10 min along with 5% sugar and filtered to obtain tea decoction.

Results: The bacteria in *kombucha* were Gram-negative, flexible rods. Both bacteria and yeast were positive for acid and gas production with glucose and sucrose and negative with lactose. A highest biomass content of 33.39 and 32.9 g/l in 5 and 10% tea decoction respectively was observed when grown statically at 25 °C and pH 5.0. Highest carotenoids (92.5 µg/ml) content was found at normal pH without setting, while 85 and 84 µg/ml at pH 4.5 and 5.0 respectively under static condition. A highest DPPH(2,2-diphenyl-1-picrylhydrazyl) inhibition of 76.95% with IC_{50} value 3.26 µg/ml and ascorbic acid content of 33.19% were found with 5% tea at 25 °C and pH 4.5. Acetic acid, which was shown to be one of the antimicrobial agents in *kombucha* had the highest strength of 2.25 g/l in 5% tea grown at 25 °C and normal pH, while 4.5g/l at pH 4.5. The antibacterial activity showed the sensitivity of *Escherichia Coli* with 57% and *Salmonella* sp with 42.9% inhibition for *kombucha* grown at 25 °C and pH 4.5 when compared to positive control.

Conclusion: *Kombucha*, a miracle drink can be exploited furthermore for human well-being through combating various health issues due to changing lifestyle.

Keywords: *Kombucha*, SCOBY, Fermented tea, Antioxidant, Antimicrobial

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INTRODUCTION

"*KOMBUCHA*" is a fermented tea made with a symbiotic culture of bacteria and yeast (SCOBY). The *kombucha* broth has two portions, a floating cellulosic pellicle layer and the sour liquid broth. The liquid broth is known to have acetic acid, ethanol and gluconic acid as major components [1]. It is a traditional fermentation carried out by inoculating previously grown mother culture into fresh tea decoction and incubating statically under aerobic conditions at room temperature for 10-15 d.

Eventually, a pleasantly sour beverage called *kombucha* is obtained [2] which is claimed to have high human prophylactic properties [1, 3, 4]. *Kombucha* is also considered as a probiotic microbial drink, which helps regenerate the bowel flora and is excellent for well-being. The *kombucha* culture feeds on the sugar and in exchange produces other valuable substances such as glucuronic acid, acetic acid, lactic acid, vitamins, amino acids, antibiotic substances and 0.5-1% alcohol.

Kombucha is a highly palatable health beverage due to its beneficial effects such as antibiotic properties, regulation of gastric, intestinal and glandular activities, relief of joint rheumatism, gout and haemorrhoids, positive influence on the cholesterol level, atherosclerosis, toxin excretion and blood cleansing, diabetes, nervousness and aging problems [5]. Because of its highly beneficial nature, the present study was focused on estimation of biomass, ascorbic acid, acetic acid, carotenoid contents, antioxidant activity and antibacterial activity against *E. coli, Salmonella* spand *Staphylococcus aureus* in *kombucha*.

MATERIALS AND METHODS

Mother culture of SCOBY

The symbiotic culture of bacteria and yeast was obtained from a local household brewer from Mangalore, India.

Microbial strains

The bacterial cultures such as *Escherichia coli, Salmonella* and *Staphylococcus aureus* used in the study were collected from the stock of the Department of Studies and Research in Microbiology, Mangalore University, Post Graduate Centre, Kodagu.

Chemicals and reagents

Acetic acid, agar, alcohol (75, 95 and 100%), ammonium dihydrogen phosphate, Ammonium sulphate, Ascorbic acid, α-naphtholamine, beef extract, bromothymol blue, β-carotene, β-naphthol, crystal violet, di-potassium hydrogen phosphate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrous sulphate, gelatin, glucose, Gram's Iodine, hydrogen peroxide, lactose, malachite green, magnesium sulphate, mercuric chloride, methyl red, peptone, phenol red, potassium dihydrogen phosphate, potassium hydroxide, potassium nitrate, potassium chloride, potassium iodide, potassium ferricyanide, safranin, sodium chloride, sodium citrate, sodium thiosulphate, sodium carbonate, sucrose, sodium hydroxide, starch, tetramethyl p-phenylenediamine dihydrochloride, trichloroacetic acid, urea and yeast extract were used in the present study.

All the chemicals and reagents were of extra pure and analytical grade and were procured from Hi-Media, Mumbai, India and Sisco Research Laboratory, Bangalore, India.

Microscopic identification and biochemical tests of *Kombucha*

The symbionts were microscopically identified by preparing a semi-permanent slide using lactophenol cotton blue stain. The bacteria were identified based on morphological characteristics such as color, margin, elevation, transparency, texture, form, shape [6], Gram staining technique and biochemical tests such as indole, methyl red, Voges-Proskauer, citrate utilization, catalase, urease, H_2S production, starch and gelatin hydrolysis, carbohydrate fermentation using glucose, sucrose and lactose.

Brewing *Kombucha*

Different concentrations (5, 10, 15 and 20%) of tea decoction were prepared by boiling appropriate amount of tea powder for 10 min and then strained. The concentration of sugar was maintained at 5% for all the above tea concentrations. 100 ml aliquot was poured into sterile 250 ml Erlen Mayer's flask and inoculated with 5-10% of mature *kombucha*. This acidifies the tea which is important for maintaining a selective environment that favours the SCOBY and prevents potential contaminants from developing. This was followed by aseptic addition of mother culture at 1% (g/wet wt) level and maintaining at room temperature until SCOBY grows completely like a pellicle.

Optimization of temperature and concentration of tea for the growth of SCOBY

The above mentioned different concentrations of tea decoction were made and *kombucha* was inoculated with 1% mother culture. The flasks were incubated for 7 d under both static and rotary conditions at various temperatures from 25 to 45 °C with an interval of 5 °C. After incubation, all the samples were subjected to various biochemical and growth tests such as radical scavenging activity using DPPH free radical, estimation of ascorbic acid and acetic acid by titration methods, carotenoid estimation by spectrophotometric method, antimicrobial activity and biomass determination with appropriate standard procedures.

Optimization of pH for the growth of *SCOBY*

After determining the optimum temperature and concentration of tea decoction, pH was set from 3.0 to 7.5 with an interval of pH 0.5. After incubating for 7 d all the samples were subjected to various biochemical and growth tests as mentioned previously.

After 7days of incubation *kombucha* was centrifuged in a cooling centrifuge at 5,000 rpm for 10 min. The aqueous contents were used for all the biochemical analyses and growth parameters.

DPPH assay

Antioxidant activity of *kombucha* was determined through DPPH free radical scavenging assay carried out by the method of De Ancos *et al.*, (2002) [7]. The procedure, in brief, is as follows: 10 µL of centrifuged sample mixed with 90 µL of methanol and 3.9 ml of 0.1 mmol methanolic DPPH solutions. The mixture was thoroughly vortexed, kept in the dark for 30 min and absorbance read at 515 nm. Percentage inhibition of DPPH was calculated using the following formula.

$$\% \text{ inhibition of DPPH} = \frac{\text{Absorption of control} - \text{Absorption of sample}}{\text{Absorption of control}} \times 100$$

Carotenoid estimation

The sample after incubation period was centrifuged at 5000 rpm for 10 min at 4 °C. The samples were kept away from light, and their absorbance was read at 450 nm [8].

Estimation of strength of acetic acid

The strength of an acetic acid in *kombucha* was determined by titrimetric method [9]. The method, in brief, is as follows: 1.5 ml of *kombucha* sample was made up to 20 ml with distilled water, to which 3 drops of phenolphthalein indicator was added and titrated against 0.1M NaOH until the colour changed to pale pink as an end point. Acetic acid strength was determined by the formula:

$$\text{Concentration of acetic acid} = \frac{1.5 \times \text{Vol of NaOH}}{\text{Vol of reaction mixture (20 ml)}} = X \text{ mol/l}$$

$$\text{Strength of acetic acid} = X \times 60 = Y \text{ g/l}$$

Estimation of ascorbic acid

Ascorbic acid content in *kombucha* was determined by titrimetric method [10]. The method, in brief, is as follows: 5 ml of the sample was transferred into 250 ml Erlenmeyer flask to which 35.6 ml distilled water and 0.3 ml starch indicator was added. The mixture was titrated against 5 mmol Iodine solution. The end point was identified as a permanent trace of dark blue colour. Percentage of ascorbic acid was determined by the following formula:

$$\text{Normality of ascorbic acid} = \frac{N \times V}{\text{Volume of sample}}$$

$$\% \text{ ascorbic acid} = \frac{\text{Normality of ascorbic acid} \times \text{Equivalent weight of ascorbic acid}}{\text{Volume of sample (5 ml)}} \times 100$$

Antimicrobial activity of Kombucha

Antimicrobial activity of *kombucha* was determined by agar well diffusion method [11]. The method is as follows: Nutrient agar medium plates were prepared and known bacteria to be tested for susceptibility were swabbed and agar wells were made using a 6 mm sterile cork borer, into which 30 µl of *kombucha* was transferred and the plates were incubated at 37 °C for 24 h. The plates were observed for clear zones around the wells.

Biomass determination

After 7 d of incubation, the thick pellicle of SCOBY formed on the surface of tea decoction was removed, an excess of moisture was removed by blotting and its wet weight was recorded as gram wet weight/litre (gww/l).

The dry weight was determined by drying the culture pellet at 100°C in a hot air oven for overnight or until getting constant weight and was recorded as gram dry weight/litre (gdw/l).

Statistical analysis

Data, expressed as mean±SD was statistically analyzed using one-way ANOVA. Duncan's multiple tests were used to compare means and significance was accepted at $P<0.05$ [12].

RESULTS AND DISCUSSION

Biochemical characterization of SCOBY

The bacteria and yeast in *kombucha* were characterized based on various biochemical parameters such as Gram staining, motility test, endospore formation, catalase test, urease production, H_2S production, indole, methyl red, Voges-Proskauer, citrate utilization test, gelatin and starch hydrolysis, glucose, lactose and sucrose fermentation test. The results are summarised in table 1.

Table 1: Biochemical characterization of bacteria and fungus in Kombucha optimization of temperature for biochemical parameters

Parameters	Results
Bacteria	
Gram reaction	Gram-ve, flexible rods
Motility	+
Endospore formation	+
Catalase	+
Citrate utilization	+
Urease production	+
H_2S production	+
Indole test	-
Methyl Red	+
Voges-Proskauer	-
Gelatin hydrolysis	+
Starch hydrolysis	+
Glucose fermentation	$acid^{+}gas^{+}$
Lactose fermentation	$acid^{-}gas^{-}$
Sucrose fermentation	$acid^{+}gas^{+}$
Fungus	
Carbohydrate tested	**Reaction**
Sucrose	$acid^{+}gas^{+}$
Glucose	$acid^{+}gas^{+}$
Lactose	$acid^{-}gas^{-}$

Abbreviation: '+' Positive test; '-'Negative test

The results obtained showed that the bacterium is a motile, Gram-negative, flexible rod, an endospore former, positive for catalase, citrate utilization, urease and H_2S production, methyl red test, gelatin and starch hydrolysis, while it was negative for indole production and Voges-Proskauer test. Fermentation of sugars such as glucose, lactose and sucrose showed acid and gas positive for glucose and sucrose while it was negative for lactose. Similar fermentation study was also carried out for yeast using same three sugars and the result obtained was similar to that for bacteria (table 1). From the results obtained through various biochemical parameters, it could be concluded that the bacterium found in scoby may be *Acetobacter* sps and yeast may be *Schizosaccharomyces* sps.

The SCOBY was cultivated at different temperatures ranging from 25-45°C with an interval of 5°C. The tea decoctions (5, 10, 15, 20 %) were inoculated with 1% inoculum and incubated at the above-mentioned temperatures and either under static or rotary conditions. The results shown in table 2 are only for the static condition since the data obtained for rotary conditions were insignificant. The results showed the highest biomass of 33.39 g/l at 25°C with 5% tea decoction followed by 10% showing 32.99 g/l which was not significantly different from that of 5%. At other temperatures such as 30, 35, 40 and 45°C, the biomass contents significantly reduced. The highest total carotenoid content of 92.5 µg/ml was found at 25°C with 5% tea decoction. This was followed by 20% tea concentration that showed 79 µg/ml of carotenoids (table 2). However, it was slightly significant than that of 5% tea concentration. Most of other samples showed significantly lower total carotenoid content, and some of them were even negligible.

Acetic acid strength was highest (2.25 g/l) at 25°C with 5% tea decoction and all other samples showed significantly lower acetic acid strength (table 2). The highest ascorbic acid of 31.6% was found at 25°C and 5% tea concentration. This was followed by 10% (30.2%), which was slightly lower. However, all other samples were significantly lower (table 2).

DPPH free radical scavenging activity was highest (76.95%) with an IC_{50} value of 3.25 µg/ml at 25 °C with 5% tea decoction followed by 10% tea that showed 73.22%. Other samples showed significantly lower inhibitory effect (table 2). The results indicate that there may be a direct correlation between biomass and other parameters showing higher values. *Kombucha* grows like a cellulose pancake or pellicle which floats on the surface of the tea broth when incubated in a static condition at room temperature for 9 d [13]. *Acetobacter* and *Gluconobacter* alike showed positive growth at 25, 30 and 40 °C while there was no growth at 45 °C [14]. In the present study, *kombucha* grew like a pellicle on the surface of tea decoction under a static condition at 25 °C.

Optimization of pH for biochemical parameters

In the present investigation, SCOBY was cultivated in 5% tea decoction which was set at different pH ranging from 3.5-7.5 with an interval of 0.5. After setting the pH using either 0.1 N HCl or 0.1 N NaOH, tea decoction was inoculated with 1% inoculum and incubated at 25 °C either under static or rotary conditions. The results were shown (table 3) are only for the static condition since the data obtained for the rotary condition were insignificant. The results showed highest biomass content of 34.1 g/l at pH 5.0 and that significantly lowered at other pH values. Kadare *et al.*, (2008) [14] have shown the growth of *Acetobacter* and *Gluconobacter* at pH 4.5 and 7.0 while no growth was shown at pH 2.5 and 8.5.

All other parameters such as total carotenoids (85 µg/ml), acetic acid strength (4.5 g/l), ascorbic acid (33.19%) and DPPH radical scavenging activity (76.75%) having IC_{50} of 3.26 µg/ml were highest in fermentation at pH 4.5. Other samples with pH either acidic or alkaline showed significantly lower values for all these parameters. From the result, it is clear that even with the slight reduction in growth rate at pH 4.5, all the biochemical parameters were higher when compared to pH 5.0.

Table 2: Optimization of temperature on various parameters

Temp (°C)	Conc of tea (%)	Biomass (g/l)	Carotenoids (µg/ml)	Acetic acid (g/l)	Ascorbic acid (%)	DPPH Inhibition (%)
25	5	33.39±0.004^{a}	92.5±0.002^{a}	2.25±0.003^{a}	76.95±0.007^{a}	31.6±0.002^{a}
	10	32.99±0.003^{a}	44.5±0.004^{d}	1.35±0.004^{b}	30.2±0.004^{a}	73.22±0.008^{a}
	15	26.39±0.006^{b}	40.0±0.005^{d}	1.8±0.003^{a}	25.3±0.002^{a}	64.01±0.004^{b}
	20	22.50±0.005^{c}	79.0±0.003^{b}	1.0±0.002^{c}	15.4±0.003^{b}	49.39±0.002^{c}
30	5	21.48±0.004^{c}	59.5±0.005^{c}	0.9±0.002^{c}	12.6±0.002^{b}	8.00±0.003^{f}
	10	16.27±0.003^{d}	15.0±0.004^{g}	1.05±0.003^{b}	14.0±0.001^{b}	29.58±0.002^{e}
	15	18.23±0.007^{c}	25.0±0.004^{f}	0.9±0.005^{c}	13.3±0.001^{b}	20.05±0.004^{e}
	20	18.98±0.005^{c}	15.0±0.003^{g}	0.75±0.003^{d}	11.9±0.001^{b}	ND
35	5	17.56±0.006^{d}	59.5±0.002^{c}	0.75±0.003^{d}	14.0±0.002^{b}	34.57±0.006^{d}
	10	17.72±0.007^{d}	34.5±0.004^{e}	1.25±0.002^{b}	14.0±0.003^{b}	ND
	15	19.81±0.003^{c}	40.0±0.005^{d}	0.9±0.004^{c}	13.3±0.002^{b}	ND
	20	22.78±0.004^{c}	59.5±0.003^{c}	0.9±0.005^{c}	2.0±0.003^{f}	ND
40	5	17.78±0.005^{d}	0.021±0.004^{h}	0.6±0.006^{d}	2.0±0.004^{f}	37.69±0.003^{d}
	10	20.72±0.003^{c}	10.0±0.003^{g}	0.75±0.004^{d}	7.0±0.002^{d}	9.29±0.005^{f}
	15	18.32±0.006^{c}	0.014±0.004^{i}	0.75±0.002^{d}	8.4±0.003^{c}	13.59±0.006
	20	19.72±0.004^{c}	0.012±0.003^{i}	1.21±0.003^{b}	9.1±0.003^{c}	ND
45	5	11.71±0.008^{e}	20.0±0.004^{f}	0.9±0.007^{c}	5.6±0.002^{e}	9.86±0.003^{f}
	10	15.88±0.007^{d}	0.006±0.005^{i}	0.9±0.002^{c}	6.3±0.001^{d}	38.28±0.004^{d}
	15	16.31±0.004^{d}	0.002±0.006^{j}	0.6±0.004^{d}	5.6±0.002^{e}	22.52±0.005^{e}
	20	20.12±0.005^{c}	0.003±0.004^{j}	0.75±0.002^{d}	5.6±0.002^{e}	ND

All values are mean of triplicates with SEM and are significant at $p<0.05$ if the column not sharing same alphabets in the table, ND: the values were not determined

Table 3: Optimization of pH on various parameters in kombucha at 5% tea concentration

pH	Biomass (g/l)	Carotenoids (µg/ml)	Acetic acid (g/l)	Ascorbic acid (%)	DPPH Inhibition (%)
3.0	23.07±0.005^{b}	65.0±0.003^{c}	2.55±0.006^{d}	15.40±0.002^{c}	34.47±0.00^{d}
3.5	14.28±0.003^{d}	75.0±0.004^{b}	2.55±0.004^{d}	16.90±0.002^{c}	47.46±0.00^{c}
4.0	21.81±0.003^{b}	30.99±0.005^{a}	19.33±0.00^{f}	77.0±0.005^{b}	3.75±0.004^{b}
4.5	31.47±0.004^{a}	85.0±0.006^{a}	4.50±0.005^{a}	33.19±0.004^{a}	76.75±0.00^{a}
5.0	34.10±0.005^{a}	84.0±0.004^{a}	3.75±0.003^{b}	31.60±0.003^{a}	55.26±0.00^{b}
5.5	18.39±0.004^{c}	62.0±0.003^{c}	2.25±0.002^{d}	22.54±0.006^{b}	30.66±0.00^{e}
6.0	15.50±0.006^{d}	65.0±0.004^{c}	3.00±0.006^{c}	17.61±0.002^{c}	34.76±0.00^{d}
6.5	14.87±0.003^{d}	53.0±0.003^{d}	1.80±0.004^{d}	14.00±0.004^{d}	36.32±0.00^{d}
7.0	8.39±0.002^{e}	33.0±0.004^{e}	1.20±0.005^{e}	13.3±0.003^{d}	17.77±0.00^{f}
7.5	0.69±0.004^{f}	32.0±0.005^{e}	0.75±0.003^{f}	9.10±0.005^{e}	11.91±0.00^{g}

All values are mean of triplicates with SEM and are significant at $p<0.05$ if the column not sharing same alphabets in the table

Antibacterial activity of *kombucha*

Antibacterial activity of *kombucha* was checked against *Escherichia coli, Salmonella* spand *Staphylococcus aureus.* The result showed the largest zone of inhibition measuring 4 mm each with *kombucha* grown at 25 °C with 5 and 10% and 3 mm with 20% tea concentration against *Escherichia coli*.

There was an inhibition of 3 mm with *kombucha* grown at 25 °C with 15% and 2 mm each with 5 and 10% tea concentration against *Salmonella* sp. However, with other samples, there was no zone of inhibition and also none of the samples showed inhibition against *Staphylococcus aureus*. This indicates that *kombucha* grown at 25 °C with 5 and 10% was inhibitory for *Escherichia coli* and 15% of tea for *Salmonella* sp. The antibacterial property of *Kombucha* may be attributed to varied strengths of acetic acid produced at different concentrations of tea. Similar antimicrobial activity against *Escherichia coli* and *Salmonella* was also reported [15, 16].

Growth inhibition of *Shigella sonnei*, *Escherichia coli*, *Salmonella enteritidis* and *Salmonella typhimurium by the* presence of antimicrobial compounds other than organic acids or proteins (enzymes) produced during fermentation or the tannins originally present in the tea broth has been shown [17, 18].

Table 4: Antibacterial activity of *kombucha* at pH 4.5

Temperature (°C)	Conc. of tea (g/100ml)	Percentage inhibition		
		Escherichia coli	***Salmonella sp.***	***Staphylococcus aureus***
25	5	57.0	28.6	NZ
	10	57.0	28.6	NZ
	15	28.6	42.9	NZ
	20	42.9	14.3	NZ
30	5	NZ	NZ	NZ
	10	NZ	NZ	NZ
	15	NZ	NZ	NZ
	20	NZ	NZ	NZ
35	5	NZ	NZ	NZ
	10	NZ	NZ	NZ
	15	NZ	NZ	NZ
	20	NZ	NZ	NZ
40	5	NZ	NZ	NZ
	10	NZ	NZ	NZ
	15	NZ	NZ	NZ
	20	NZ	NZ	NZ
45	5	NZ	NZ	NZ
	10	NZ	NZ	NZ
	15	NZ	NZ	NZ
	20	NZ	NZ	NZ

NZ: No Inhibition zone

CONCLUSION

Kombucha is a miracle probiotic drink with tuft of health beneficial properties embedded. In the present study, it was found that different temperature and pH had an effect on growth and biochemical parameters such as biomass, total carotenoids, acetic acid strength, ascorbic acid, DPPH free radical scavenging activity and antimicrobial activity. The overall results indicated that *kombucha* grown at 25°C and pH 4.5-5.0 with 5 and 10% tea concentrations were optimum for maximum activities of the above-mentioned parameters.

However, there is a need to find whether it is the bacteria or yeast which is involved in the production of all the health beneficial bioactive in *kombucha*. It is also possible to obtain the desired quality of *kombucha* by controlling the fermentation conditions. Although this drink is known to people from ancient days, there is a need to create scientific awareness about its health benefits, convenient and hygienic cultural practices.

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CONFLICT OF INTERESTS

Declared none

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