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OPTIMIZATION OF PROCESS PARAMETERS IN THE PRODUCTION OF KOMBUCHA TEA AND FLAVOURS INFUSION TO ENHANCE PALATABILITY

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ABSTRACT
Kombucha is a fermented beverage of bacteria and yeast. It is a slightly acetic acid flavored beverage. Kombucha is produced during 6 to 10 days of fermentation period under aerobic conditions and it is two step fermentation. In brief the black tea is sweetened with 15% sugar, the yeast ferments sugar into ethanol, which is oxidized by acetic acid bacteria that produce acetic acid which reduces the pH of the medium. As consumer demand for kombucha grows in North America, there is a need to optimize, predict and control the fermentation process. This study aims to infuse the flavours in kombucha for better taste and antioxidant activity of bacteria. The experiment measured the pH, color intensity and acidity of the tea over the fermentation period (4-14) days.16S rRNA also has been done to identify the specific organism in the kombucha. The kombucha has been claimed is to be beneficial human health.

KEY WORDS: Kombucha, tea fungus, physical, chemical parameter, flavour infusion, antioxidant, 16S rRNA.

INTRODUCTION

Kombucha is a beverage obtained from the fermentation of tea, mostly black tea added with a sugar (sucrose) as a substrate for fermentation process. Kombucha tea is slightly sweet, slightly acidic refreshing beverage consumed worldwide. It is obtained from infusion of tea leaves by the fermentation of SCOBY (symbiotic culture of bacteria and yeast) forming "tea fungus" which is a rubbery raft that floats on surface of kombucha though green tea can be used for kombucha preparation, black tea and white sugar are considered as the finest substrates[7]. Kombucha is the internationally used germanize form of the Japanese name for a slightly fermented tea beverage. It was first used in East Asia for its healing benefits. In 414 A.D, the physician brought the tea fungus to cure the digestive problems of the Emperor Inkyo. The main acetic acid bacteria species found in kombucha were *acetobacteria*. It has been reported that the main compounds in kombucha beverage are acetic, lactic, gluconic, glucoronicacids, ethanol, glycerol and B-complex vitamins. Kombucha, rich in antioxidants when made black tea and green tea the kombucha broth exhibited strong DPPH (1, 1- diphenyl-2-picrylhydrazyl). Eight different flavours of kombucha were prepared by fermentation process and biochemical characterization of bacteria was studied. Kombucha improves the intestinal nicrobiota, regulates nutrient absorption and reduce the risk of chronic non-communicable diseases[8]. According to fractional knowledge, kombucha is supposed to improve hair growth and restore gut flora (R.A. Batista, 2009). Kombucha contains large amount of probiotics and acetic acid. It has been proven to aid weight loss. Consumers still use kombucha for the benefits. It helps in hypertension, anemia, pulmonary disease and has antioxidant capabilities (J. Peterson *et al*,). The sequencing of 16S rRNA genes has been done to identify kombucha bacterial isolates[21]. After sequencing of the samples, the data is analysed to see which organisms are common to the culture, to identify the organism. The objective of this study is to enhance palatability in kombucha by infusing flavours from fresh fruits. There was no significant difference in the pH between the various flavours. The bacteria was isolated from the fermented brew and identified by 16s rRNA sequencing method. Antioxidant and biochemical characteristics for bacteria were also studied.

MATERIALS AND METHODS

Sample collection

Assam black tea samples:
Assam black tea samples were collected from Matheson Bosanquet Enterprise. Pvt. Ltd, Kochi, Kerala, India.

Fruit samples:
A total number of 8 fruit samples weighing from 150g – 200g each were collected from local market, Bengaluru. The fruit samples collected were Watermelon (150g), Apple (150g), Lemon (2.4g) & Ginger (5g), Strawberry (200g), Pomegranate (150g), Pineapple (200g), Guava (200g) and Orange (150g).

Preparation of medium for primary fermentation:
1000 ml of tap water was boiled until it reaches 100˚C, 4g of black tea and 2g of sucrose were added into it and steeped for 10 minutes. The infusion was filtered through a sterile sieve of pore size 0.297 mm and the filtrate was served as a medium for tea brew. A layer of SCOBY was inoculated into the tea brew under aseptic conditions.

Preparation of medium for secondary fermentation:
After 14 days of primary fermentation, the fermented brew (Kombucha) was used as medium for secondary fermentation.

Primary fermentation
Submerged fermentation was performed for the cultivation of microorganisms grown in liquid media, with high content of free water. It has the advantages such as instrumentation and control like monitoring pH and concentration of water soluble molecules. In this fermentation microorganisms break down the nutrients and release the desired enzymes into solutions and the microbes rapidly set to work on initial raw ingredients like tea leaves. The fermentation may involve growing carefully selected microorganisms (bacteria and yeast) in a closed vessel containing a rich broth medium with high concentration of oxygen. During fermentation, the yeast present in SCOBY converts sucrose to alcohols/acids under aerobic condition. The *acetobacter* present in SCOBY ferments alcohols by the yeast into acetic and other acids, increasing the acidity and limiting ethanol content. The prepared tea brew (sample solution) was allowed to cool at room temperature (28˚C) and was then poured into 2 lit sterile bottles, with lid protected from sun light, and has been inoculated with a layer of SCOBY and incubated for 14 days of fermentation at (28˚C). The fermented brew is called kombucha. The sample solutions were collected regularly and filtered through the sterile sieve of pore size (0.297mm), then centrifuged (5 minutes at 10,000 rpm) and the supernant was stored for further analysis. The pH of the sample was measured every day. The absorbance was observed at 460nm and 620 nm by using digital calorimeter (Infra Digitm)[11].

Parameters
The physical and chemical parameters of tea brew were analysed regularly for primary fermentation.

Estimation of acidity
Titration was carried out for 50 ml of fermented brew with 0.02N NaOH and methyl red indicator. The appearance of pale pink colour indicates the presence of acid. The acidity (Glauber, 1658) was calculated using the equation (1),

$$\text{Acidity} = \frac{V1\ X\ 100}{sample} \quad (1)$$

Where, V_1 = Titration value

Determination of pH
To control and predict the fermentation process, pH was analysed by using auto digital pH meter (Com Sys Technologies) ABNT (*Associaco Brasileria* de Normas Technique), 1989; ASTM (American Society for Testing and Materials), 2002.

Determination of colour intensity
Colour intensity of fermentation brew was observed at 460 nm and 620 nm by using digital photo calorimeter (Infra digitm)[11] to read the colour difference in the prepared fermented brew.

Processing of the sample
The isolation of *bacteria* from the fermented tea brew, serial dilution method was carried out. 10^{-5} dilution sample were added to 100 ml of nutrient agar medium containing (0.5g of peptone, 0.5g of sodium chloride, 0.3g of yeast extract) and autoclaved. The pH of the solution was maintained at 7.4.

Characterization
The individual colonies isolated from Yeast extract Calcium Carbonate glucose Agar and gram staining has been carried out.

Biochemical characterization
Biochemical properties were tested using starch hydrolysis test, casein hydrolysis test, catalase test, oxidase test, indole test, methyl red test, citrate test, urease test, nitrate test, gelatine liquefaction test and triple sugar iron test were analysed for isolated grown positive bacteria[12].

Isolation and Screening of *Kombucha bacteria*
The culturing of *Kombucha bacteria* 200µl of the culture were taken from the test tube of fifth dilution (10^{-5}) and were spread into the Nutrient Agar (NA) plates by spread plate method. The inoculated plates were incubated at 36° C for about 24hours. Bismuth Sulphite Agar (BSA) was used as a selective culture medium for the isolation of bacteria. Yeast extract calcium carbonate glucose Agar was taken and dissolved in 50 ml distilled water. The yeast extract plates were prepared. The nutrient agar plates containing individual colonies of *Kombucha bacteria* was were isolated and streaked into the Yeast extract Calcium Carbonate glucose Agar plates and incubated at 36°C for 24 hours.

DNA Extraction and Quantification
1.5 ml of bacterial suspension was centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and 560µl of Tris EDTA buffer was added to the pellet 40µl of 10% SDS and 3µl of proteinase were added and mixed. The tubes were incubated at 56°C for 2h. 100µl of 5M NaCl and 80µl of CTAB (Cetyl Trimethyl Ammonium Bromide) were added, and incubated at 65°C for 10 min. 400µl of 24:1 ratio of chloroform and isoamyl alcohol were added and centrifuged at 12000 rpm for 10min. The upper aqueous layer was added to 500µl of Phenol, Chloroform and isoamyl alcohol on the ratio of 25:24:1 and centrifuged at 12,000 rpm for 10 min. Again, the following steps were carried out by adding 500µl of chloroform and isoamyl alcohol at the ratio of 24:1 and centrifuged at 12,000 rpm for 10 min. The upper aqueous layer was again added to 300µl of chilled isopropyl alcohol. The tubes were incubated at -20°C for 1 h and centrifuged at 12,000 rpm for 10 min. The pellet was washed with cooled 70 % of ethanol and centrifuged at 10,000 rpm for 10 min. After discarding the supernatant, the pellets were air dried and resuspended in 30 µl of TE buffer (Jara *et al.*, 2008). After the DNA isolation, 10 µl of sample and 10µl of Bromophenol Blue (loading dye) were mixed together. 0.8 g of agarose in 80 ml of distilled water was taken and 0.5 µl of ethidium bromide was added. The electrophoresis was performed for 1 hour[13].

Amplification of DNA
Random Amplification of Polymorphic DNA (RAPD) amplification has been carried. The procedure with initial denaturation of DNA template of (20mg per 25µl reaction mix) at 94°C for 4 min, followed by 40 cycles at 94°C for 1 min, 37°C for 1 min, 72°C for 2 min and 72°C for 5 min in thermocycler (Biorad). Amplification was finished with incubation at 72°C for 13 min. The sequences of primers are Forward Primer: 5'-TCAAG TCCT CATGGCCC TTA TG -3"

Reverse Primer: 5'-TACACACGTGCTACAATGGGCG-3'

PCR amplification products were analyzed in 0.8% agarose gel in 1X TBE buffer. 25µl per sample of PCR products were mixed with 3-5µl gel loading dye Solution and loaded onto the agarose gel, containing 0.5 mg/ml ethidium bromide. In each agarose gel 100bp of Gene ruler DNA ladder was used as a marker. Electrophoresis was carried out and visualized under Gel doc.

TABLE 1: PCR Amplification

Cycle	Steps	Process	Temperature	Time
1	I	Initial denaturation	94°C	4 minutes
40	II	Denaturation	94°C	1 minutes
1	III	Annealing	37°C	1 minutes
1	IV	Primer extension	72°C	2 minutes
Repeat the step 2 for 39 cycles				
1	V	Extension	72°C	5 minutes

Nucleotide Sequencing:

PCR product was sequenced. The edited sequences were then used for similarity check using programme in the NCBI gene bank DNA database for identifying the sample.

Antioxidant activity

DPPH Radical Scavenging activity

The Antioxidant activity of Kombucha tea was estimated using the radical scavenging effect of the stable 1, 1-diphenyl-2-picyrlhydrazyl. Different concentration of Kombucha Sample ranging from (10-60) µg/ml. Then 1ml of methanol and 1ml of 0.01% DPPH was added to various concentration of Kombucha sample. Then, the mixture incubated at room temperature for 30°C minutes. 1ml of methanol and 1ml of 0.01% DPPH were used as a control. The absorbance was recorded at 517nm (UV-1700 Shimadzu). DPPH radical scavenging activity was calculated by using the following equation (2),

$$\text{Percentage of inhibition} = \frac{\text{Control OD} - \text{Sample OD X 100}}{\text{Control OD}} (2)$$

Where, OD = optical density

Secondary fermentation

A batch continuous process was performed after 14 days of primary fermentation, the SCOBY was removed from the fermented brew (Kombucha).The brew was separated into 8 different sterile bottles of each 300 ml. To enhance the palatability eight fresh fruits such as Watermelon and pepper, Apple and cinnamon, Lemon and ginger, Strawberry, Pomegranate, Pineapple, Guava and Orange, cut into small pieces using sterile knife and transferred into 300ml of fermented brew each under aseptic conditions to infuse flavours and maintained at 25°C for 7 days. To prevent any fungal growth on surface of the fruits fermented brew was stirred for 1 hour using glass rod for 7 days. During secondary fermentation, the flavours in the fruits were infused in the fermented brew. The fermented brew was filtered using sieve of pore size 0.297mm to filter the fruit waste, and the collected sample was stored in refrigerator.

Parameters

The physical and chemical parameters of tea brew in secondary fermentation varied from the primary fermentation due to the infusion of flavours.

Estimation of acidity:

Titration was carried out for 50ml of flavoured kombucha with 0.02N NaOH and methyl red indicator. The appearance of pale pink colour indicates the presence of acid. The acidity was calculated using the equation (3)[4]

$$\text{Acidity} = \frac{V1\ X\ 100}{sample} \quad (3)$$

Where, V_1 = Titration value

Determination of pH

pH of flavoured kombucha were measured by using digital pH meter.

Determination of colour intensity

Colour intensity of flavoured kombucha was observed at 460nm and 620nm by using digital photo calorimeter (Infra Digitm)[11] and the colour difference were read.

RESULTS AND DISCUSSION

Primary fermentation

The samples were analysed for pH, acidity and colour intensity tests. It was concluded that sample-1 showed better results than compared to sample-2. This indicates that sample-1 has better pH, acidity and colour intensity than that of sample-2.

TABLE 2: Primary fermentation

Sl.no	Days	Sample-1				Sample-2			
		pH	Acidity	Colour intensity		pH	Acidity	Colour intensity	
				460 nm	620 nm			460 nm	620 nm
1.	4	4.80	10.4	0.25	0.11	4.48	8.0	0.22	0.10
2.	6	4.48	10.8	0.30	0.15	4.00	9.0	0.24	0.09
3.	7	4.25	12.4	0.25	0.10	4.28	9.4	0.24	0.07
4.	8	4.20	13.0	0.23	0.09	4.00	9.6	0.19	0.11
5.	10	4.15	13.4	0.25	0.10	4.00	10.2	0.23	0.10
6.	11	4.15	14.0	0.21	0.08	4.00	13.4	0.25	0.09
7.	12	4.00	14.5	0.24	0.09	4.50	14.0	0.21	0.09
8.	13	4.00	15.0	0.21	0.08	4.70	14.4	0.25	0.09

Estimation of acidity

Further, acidity is the important parameter in the fermentation process. During the process of primary fermentation, the acidity was found to be high which was increasing on a daily basis. This was due to the SCOBY, which releases certain acid (for example gluconic acid, acetic acid) during the process that helped in the formation of tea.

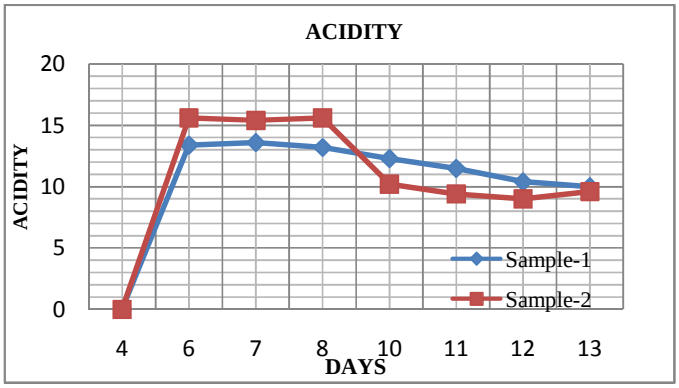

FIGURE 1: Estimation of acidity

Determination of pH:

pH is one of the most important environmental parameters affecting the fermentation of Kombucha, because some of the acids formed as acetic and gluconic, could be responsible of the biological activities of the resulting beverages it leads to increase the pH of the tea brew. It is also closely related to the microbial growth and the structural change of the compounds. The pH should be increased during the primary fermentation of the Kombucha tea.

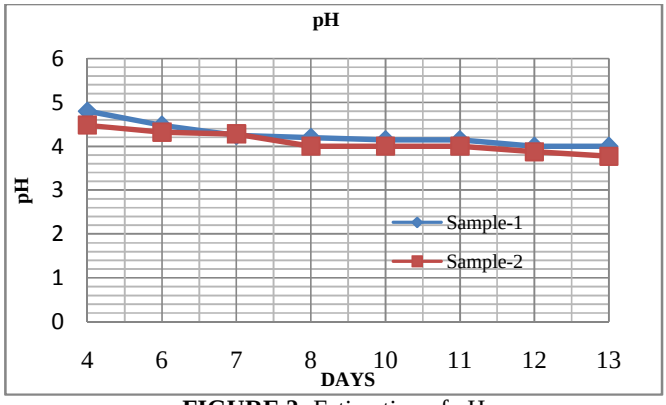

FIGURE 2: Estimation of pH

Determination of colour intensity:

The colour intensity of kombucha at 460nm

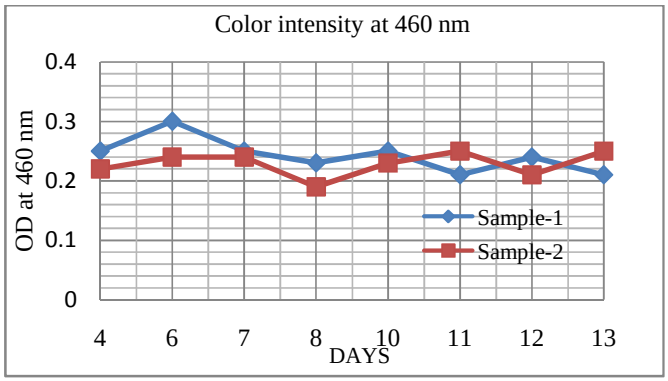

FIGURE 3: Estimation of colour intensity at 460nm

The colour intensity of kombucha at 580nm

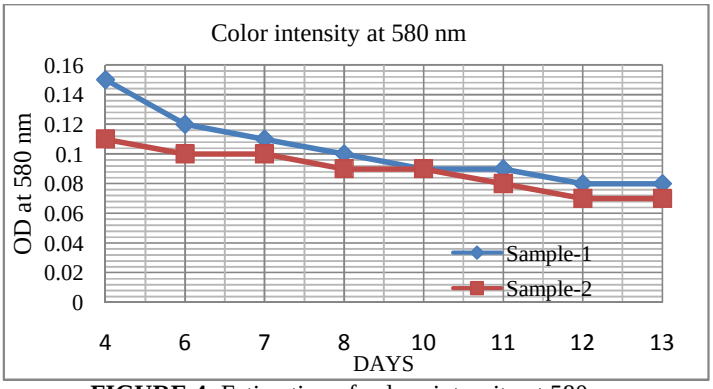

FIGURE 4: Estimation of colour intensity at 580nm

Sequencing

GTGGGTCTGATCACCTATTACGGTAAATGGGGCTACCTGTGGCGTGAATGGCTGACCTCGGTCGATCACA
AGCGCATTGGTGTCATGTATATCGTGGTTGCMCTGGTGGCGCTGTTCCGCGGTTTTGCCGACGCCATCAT
GATGCGTTCSCAGCTTGCGCTGGCCTATGCCGGGGATCCCGGCTATCTGCCGCCGCACCATTATGACCAG
ATCTTCTCCGCCCACGGCACGATCATGATCTTCTTCATGGCCATGGCGTTCATGCAGGGTCTGATGAACA
TTGTGGTGCCGCTGCAGATCGGTGCGCGCGACGTGGCCTTCCCGTTCGTGAACACRCTGAGCTTCTGGAT
GACGACCATCAGCTTCCTGCTGGTCAACGTCTCSCTGTTCATCGGTGAGTTCTCGCAGTGCGGCTGGCTT
GCTTATCCCCCCCTGTCCGAACAGCAGTTCAGCCCCGGKGTCGGTGTTGATTACTACATCTGGGCGGTGC
AGCTGTCCGGTGTCGGCACGCTGCTGACGGGTGTGAACTTCTTTGCGACCATCGTGAAGATGCGCGCGCC
TGGCATGAMCTACATGCGTATGCCGGTGTTCACCTGGACCATCTTCTGCACGACCGTG

The 16S rRNA sequencing has been carried out to identify the bacteria. The obtained sequence matches the same sequence which already exists for *Acetobacter xylinum* in NCBI.

Antioxidant test

The Antioxidant activity of Kombucha was determined by DPPH radical Scavenging. The percentage of DPPH Slowly increased at different concentration. Methanol was used the most effective DPPH radical Scavengers and their values between (1.77 ±0.33 to 30.62 ±0.87).The maximum $DPPH^{\cdot}$ radical scavenging activity observed was 30.62 ±0.87 % at 60 µg/mL concentration. The Kombucha tea showed high capacity for scavenging free radicals by reducing the stable DPPH (1,1-diphenyl-2- picrylhydrazyl) radical to yellow coloured 1,1-diphenyl-2-picrylhydrazine and the reducing capacity increased with increasing concentration of the tea.

TABLE 3: Estimation of antioxidant activity

Sl. No.	Concentration (µg/mL)	%of inhibition
1	10	1.77±0.33
2	20	9.67±0.62
3	30	14.3±0.43
4	40	16.67±0.34
5	50	19.1±0.18
6	60	30.62±0.87

Secondary fermentation:
The flavours were infused with the Kombucha tea during the secondary fermentation. Once the flavours were added, the pH, acidity and the colour intensity of the tea were analysed on the 21st day.

TABLE 4: Secondary fermentation

SI. No	Flavours	pH	Acidity	Colour Intensity	
				460 nm	620 nm
1.	Strawberry	4.00	8	0.73	0.62
2.	Apple Cinnamon	4.30	10.2	0.60	0.53
3.	Orange	4.00	9	0.70	0.68
4.	Lemon & Ginger	4.00	10.7	0.54	0.47
5.	Pineapple	4.23	8.9	0.07	0.71
6.	Guava	4.16	8.4	0.66	0.63
7.	Pomegranate	4.00	9.2	0.52	1.27
8.	Watermelon & Pepper	4.00	11.2	0.85	0.87

Biochemical characterization of kombucha tea
Biochemical characterization tests were analyzed.

TABLE 5: Results of Biochemical characterization tests

Sl. no	Test	Acetic acid bacteria
1.	Indole	+
2.	Methyl red	-
3.	Oxidase	+
4.	Catalase	+
5.	Citrate	+
6.	Starch hydrolysis	+
7.	Urease	+
8.	Casein hydrolysis	+
9.	Nitrate	-
10.	Triple sugar iron	-
11.	Gelatin liquefaction	-

CONCLUSION

Kombucha drink is consumed as worldwide for its health benefits. Black tea and white sugar are the common substrate of kombucha but due to its acidic nature, it gives a sour taste while consuming kombucha. We infused flavours in the kombucha to enhance the palatability. The experiments suggested the impact that using different flavours on growth and metabolism of the SCOBY. It is observed that, there was no significant difference in the pH between the various flavours. A controlled fermentation process leads to reduce the risk of fermentation failure.

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