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Microbial Diversity and Fermentation Analysis of Kombucha: Isolation, Characterization, Consortium Development and Tolerance of Microorganisms to pH and Bile Salt

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ABSTRACT
The consumption of traditional non-alcoholic beverages has been overshadowed by the aggressive marketing of sugar-laden sodas from major companies such as Coca-Cola, Pepsi Co., and Red Bull. In contrast, healthy drinks like Kombucha, Kefir, Ayran, Kvass, and others have receded into the background. Kombucha, a fermented tea, has garnered interest due to its unique taste and health benefits. Produced through the fermentation of sugared tea using a symbiotic culture of bacteria and yeast (SCOBY), this effervescent beverage contains bioactive compounds linked to improved digestion, immune function, detoxification, and more. This study investigates kombucha preparation, fermentation, and analysis, focusing on the isolation and characterization of microorganisms involved in the process. The 10-day fermentation process was monitored for pH, acidity, alcohol content, and total carbohydrates. The SCOBY was characterized using Fourier Transform Infrared Spectroscopy (FTIR), and a microbial consortium was developed to study their combined fermentation capabilities. The research examines the relationship between pH, acidity, and bile salt tolerance in the isolated microorganisms, highlighting their adaptability. Analysis of carbohydrate consumption and alcohol production during fermentation provides insights into the role of SCOBY in the process. The FTIR analysis confirms the presence of bacterial cellulose in the SCOBY. Lastly, the study identifies specific bacteria and yeast responsible for SCOBY formation and kombucha fermentation through the preparation of a microbial consortium.
Keywords; *Kombucha, Fermented tea, SCOBY, Microbial consortium, Bacterial cellulose*

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INTRODUCTION

Beverages consumed by humans are broadly classified as alcoholic and non-alcoholic. When addressing non-alcoholic beverages, the companies like Coca-Cola, Pepsi Co., Red bull have overwhelmed the commercial markets where we have forgotten the non-alcoholic traditional beverages. The currently available beverages in form of soda drinks by large companies are loaded with sugars and are responsible for negatively influencing the human health. There are non-alcoholic drinks such as Kombucha, Kefir, Ayran, Kvass, Hardaliye, Gilaburu juice, Water kefir, Sima etc. are some traditional non-alcoholic extremely healthy drinks that are forced to be forgotten by aggressive marketing of pop culture sugar infused soda beverages by Coca-Cola, Pepsi Co., Red bull, etc.[1,2].

Kombucha, a traditional fermented tea beverage with an ever-growing global popularity, has garnered significant attention in both scientific and health communities. Kombucha has a long history and has been consumed for centuries in various parts of the world [3]. This effervescent drink is produced by fermenting

sugared tea using a symbiotic culture of bacteria and yeast (SCOBY). The intricate process of kombucha fermentation not only imparts unique flavours and effervescence to the beverage but also results in the formation of numerous bioactive compounds, which have been associated with various health benefits [4]. The mechanism of kombucha fermentation involves a complex series of biochemical reactions carried out by the SCOBY. The yeast in the SCOBY breaks down the sugar in the sweetened tea into glucose and fructose. The yeast then ferments the glucose into ethanol (alcohol) and carbon dioxide (CO_2) through a process called glycolysis [5]. The CO_2 is responsible for the fizziness in kombucha, while the ethanol serves as a substrate for the bacteria in the next stage. The bacteria in the SCOBY, mainly acetic acid, convert the ethanol produced by the yeast into organic acids, primarily acetic acid and gluconic acid. This process is called oxidative fermentation, and it occurs on the surface of the SCOBY where oxygen is available. The bacteria also produce cellulose, which forms the structure of the SCOBY and helps it grow in size [6]. During the fermentation process, various other compounds are produced, including vitamins, particularly B-vitamins and vitamin C; amino acids; and enzymes. These compounds contribute to the nutritional value of kombucha [7][8]. The specific types and amounts of these compounds depend on the composition of the SCOBY, the type of tea used, and the fermentation conditions. The kombucha fermentation process is a delicate balance between the yeast and bacteria in the SCOBY [9]. The yeast produces ethanol, which is then utilized by the bacteria to produce organic acids. These organic acids create an acidic environment (low pH) that inhibits the growth of harmful microorganisms and promotes the growth of beneficial microbes. This balance ensures the stability of the kombucha ecosystem and contributes to its unique taste profile [10]. Bacterial cellulose derived from SCOBY kombucha has various applications due to its unique properties. It is used in biomedical applications (e.g., wound dressings, tissue engineering), food industry (e.g., packaging, edible films), paper and textile industries, electronics (e.g., flexible electronics, sensors), acoustic materials, and environmental applications (e.g., water purification, oil spill clean-up) [11]. The kombucha market in India has shown promising growth and potential, driven by increasing health consciousness, urbanization, and the influence of international trends. While kombucha remains a niche product compared to more established beverages, its popularity is likely to continue to rise as more consumers become aware of its unique taste and health benefits such as improved digestion, enhanced immune function, detoxification, cardiovascular health, weight management, antioxidants and antimicrobial properties [12].

This study focuses on the preparation, fermentation, and analysis of kombucha, a fermented tea beverage. The kombucha SCOBY was acquired, and the sweetened tea infusion was prepared for fermentation. After 10 days of fermentation, microorganisms were isolated, and their characteristics were analysed. Chemical characterizations, such as pH, acidity, alcohol content, and total carbohydrates, were determined throughout the fermentation process. Additionally, microbial isolates were tested for pH and bile salt tolerance. SCOBY was further characterized using Fourier Transform Infrared Spectroscopy (FTIR). Lastly, a consortium development was conducted using selected bacteria and yeast to study their combined fermentation capabilities.

This study explores the process of kombucha fermentation, isolation and characterization of microorganisms involved, and their tolerance to pH and bile salts. The batch fermentation process is closely observed, noting the growth of new SCOBY layers and effervescence as indicators of active fermentation. A total of 7 microbial colonies are isolated and characterized based on their colony and morphological characteristics. The relationship between pH, acidity, and bile salt tolerance is analysed to understand the adaptability of these microorganisms to various conditions. Total carbohydrate concentration and alcohol content during the fermentation process are evaluated, revealing the consumption of carbohydrates by SCOBY and the production of organic acids, carbon dioxide, and trace amounts of alcohol. The pH and bile salt tolerance of isolated bacteria and yeast cultures are studied, with some showing high tolerance, potentially playing an active role in kombucha fermentation. The SCOBY is further characterized using Fourier Transform Infrared Spectroscopy (FTIR), confirming the presence of bacterial cellulose. Finally, a consortium of bacteria and yeast is prepared to identify specific microorganisms responsible for SCOBY formation and kombucha fermentation.

MATERIAL AND METHODS

Materials and inoculum

Primary inoculum, Kombucha SCOBY was procured from Latambarcem brewers, Goa. Long leaf black tea was used as substrate for fermentation. Granulated white sugar was used as carbon source which was obtained from local market.

Preparation of tea infusion for fermentation

Sweetened tea infusion was prepared with the following protocol: 2.0 L of RO water was boiled before adding 100 g of sugar (5% w/v). After complete dissolution of sugar 10 g of black tea (0.5% w/v) were added and steeped for 10 min at 95 °C. After that, the tea was filtered and poured into a glass jar. The sweetened tea was cooled at room temperature, then, the pH was adjusted to 4.5 using a liquid broth of 10% (v/v) of a previously fermented kombucha. At the last, 7% (w/v on a wet weight basis) SCOBY was added for inoculation to start the fermentation. The glass vessel was closed with loosely covered cap for the aeration purpose as kombucha fermentation take place in aerobic condition. Glass vessel was placed in clean and dry place without direct exposure of sunlight at 25 ± 5 °C for 10 days in static condition [7][9].

Isolation of microorganisms from kombucha

After 10 days of fermentation, Conventional spread plate method was applied for the isolation of microorganism from kombucha with the help of Nutrient Agar (NA) as solid medium. pH 4.5 of media was set by using glacial acetic acid to prevent contamination of common moulds and other microorganisms in medium. 1 mL of sample was put into 9 mL of 0.9% (w/v) normal saline solution. Serial dilution was made up to 10^{-6} and 0.1 mL inoculum from each tube was spreaded on NA medium. The incubation was done at the temperature of 30 ± 2 °C for 72 h in the incubator. Isolates with different cultural characteristics were picked and streaked on freshly prepared NA media as well as alphanumeric numbers were given to each microbial isolate. Isolated colonies of different microorganisms were maintained on NA slants as well as plates and stored at 4 °C for further microbial study purpose.

Microbial characterization of kombucha isolates

The selected, microbial isolates were subjected to a set of cultural and morphological characterization for the purpose of tentative identification.

Colony characteristics

Activated culture of different isolates were streaked by four-flame method on NA plates. To note the colony characteristics, the plate was observed under 40 X magnification (4 X objective and 10 X ocular lens) after 48 h of incubation time. The colony characteristics were thus, noted.

Morphological characteristics (Gram's staining)

Activated bacterial culture was smeared on a clean glass slide and heat fixed. The smear was covered with crystal violet for 1 minute. Excess stain was then washed off with tap water and mordant iodine was poured on the slide and allowed to stand for 30 seconds. Then the slide was washed with decolourizer (acetone) for another 10-20 seconds. The slide was washed with distilled water and drained. Safranin was applied on smear for 1 minute as counter stain, washed with distilled water and blot dried. The slide was observed under microscope for gram reaction. For the yeast isolates monochrome staining was used to observe their morphological characters for that, activated cultures were smeared on a clean grease free glass slides and were heat fixed. The prepared smears were further covered with crystal violet and were kept for 1 minute, followed by washing with tap water and blot drying. The slides were then observed under oil immersion lens of compound light microscope.

Chemical Characterization of kombucha

Sample collection

Fermentation medium broth was carefully withdrawn using tap of glass jar without disturbing the fermentation process. During sample collection aseptic technique was applied as before and after sampling, 70% (v/v) isopropyl alcohol solution was sprayed on to tap of glass jar and wiped with clean, dry and lint free cloth.

Sample preparation

Collected sample was centrifuged at 10,000 rpm for 10 min to sediment microbial load. Supernatant was further filtered using Whatman no.1 filter paper to minimize the interference of other particles during experiments of chemical characterization of kombucha.

Determination of pH

An electronic pH meter was used to measure pH of fermented medium broth. The determination of pH was measured in triplicate up to 10 days of fermentation constant.

Estimation of acidity by acid-base titrimetric method

Acidity of Kombucha was measured using acid-base titration method till 10 days of fermentation. A 5ml sample of Kombucha broth was taken in a 100ml conical flask and diluted the sample by adding 20ml distilled water. To this, 2 to 3 drops of phenolphthalein indicator was added, and the system was titrated against 0.5N NaOH solution [13]. The determination was measured in triplicate and the result was expressed as g/L. The acidity due to acetic acid was calculated by following formula [13].

Acetic acid (g/ml) = Volume of NaOH × 0.03 × 20

Alcohol estimation by hydrometer

The hydrometer, after being thoroughly sanitized, was positioned in its tube. The hydrometer tube was filled until the hydrometer floated unobstructed (around 200mL). The hydrometer was then placed on a, flat surface and gently swirled back and forth to dislodge any bubbles that might have adhered to its surface. Ensuring that the hydrometer was floating without contacting the sides of the tube, its measurement was read at eye level from the base of the meniscus. The formula for estimation of alcohol by volume in percentage (ABV (%)) is given below [14]. Alcohol estimation was performed every three alternative days till last day of fermentation.

ABV (%) = (Initial Gravity - Final Gravity) × 131.25

Estimation of total carbohydrates by phenol sulphuric method

Estimation of total carbohydrate of fermentation medium was performed throughout the 10 days. A 0.2, 0.4, 0.6, 0.8 and 1ml of working standard (with 1mg/ml conc.) of glucose was taken in boiling tubes and the final volumes of each tube was made 1 ml by adding distilled water. 1ml of 5% Phenol and 5ml of 96% Sulphuric acid was added one by one in each tube and shook well so that the Phenol and Sulphuric acid get mixed thoroughly with working standard. After 10 minutes all the tubes were placed in water bath at 25-30 °C for 15 minutes. Blank was set with 1ml of distilled water and O.D. of each tube was taken at 490 nm with the help of spectrophotometer. Then the whole process following Phenol and Sulphuric acid method was repeated with 1.0 ml of different samples of kombucha tea and the O.D.s of sample solutions were taken [15].

Study of pH and bile salt tolerance

Loopful of activated cultures were inoculated into Nutrient broth having different pH (pH 2,4,6) and nutrient broth medium supplemented with different bile salt concentrations of 0.15 and 0.5 g%. which were then incubated at 30 ± 2 °C for 24 h to 48 h. After incubation, turbidity was observed for the evaluation of the microbial growth [16]. pH was set using commercially available glacial acetic acid.

Characterization of SCOBY by FTIR analysis

The presence of different functional groups in SCOBY was studied by FTIR analysis. The air-dried raw SCOBY was directly analysed. The infrared spectra of the samples were recorded in the wave number range from 400 to 4000 cm−1 using a Bruker (Model alpha II) FTIR spectrophotometer. Data interpretation is in the form of stretching and bending of the peaks for the concerning compound [17].

Fermentation by consortium development

One individual bacteria and yeast (BK1 and YK1) were selected for consortium development based on their phenotypic characteristics and tolerance towards pH and bile salts. For the development of microbial consortium, loopful of both isolates were inoculated into sugared tea infusion (same as the kombucha production medium) and incubated at room temperature (25 ± 5 °C) in shaking condition at 180 rpm for 48 h. After incubation 5 ml of inoculum was inoculated in same medium (300 mL) and was incubated under dark condition at room temperature (25 ± 5 °C) for 15 days.

RESULTS AND DISCUSSION

Production of Kombucha

The batch fermentation was used to study kombucha fermentation. The SCOBY sinks to the bottom of the glass jar after being added to the fresh fermentation media. The effervescence and gas production were observed at the end of the day 2 that was the sign of initiation of fermentation. Simultaneously, SCOBY was lift to upside at the liquid-air surface during active fermentation stage. After the completion of the 10 days fermentation period the batch was having a strong acidic aroma and flavour. After 4th day of fermentation, slight layer of new daughter SCOBY was observed over the old mother SCOBY and gradually became thicker as fermentation have been carried. After 10 days of fermentation, new SCOBY layer grew in uniform layers as multiple layers of one pellicle and evenly covered upper surface of the old SCOBY. The comparative photographs of day 0 and day10 of fermentation process of kombucha are shown in **Fig. 1.**

Isolation of microorganisms from kombucha

A total of 07 different microbial colonies were isolated from a kombucha, collected from Latambarcem brewers, Goa. Among 07 microbial colonies, 5 bacterial and 2 yeasts colonies were identified on the basis of their cultural and morphological characteristics. These representative indigenous colonies were picked up and maintained as pure cultures on NA plates until further use. Fig.2 shows the representative indigenous colonies isolated from kombucha

Microbial characterization of kombucha isolates

Colony characteristics

The colony characteristics, on NA medium are mentioned in **Table 1.** The representative indigenous colonies isolated from kombucha on NA plates are shown in **Fig. 2.**

Morphological characteristics

Morphological characteristics were evaluated using Gram staining. Among 5 bacteria, 4 bacterial colonies were found as gram positive bacteria, simultaneously 1 bacterial colony was found as gram negative. To understand morphological characteristics of two yest colonies, Monochrome staining was applied to suspensions of yeast colonies. Results of a morphological characters of various colony suspensions are mentioned in **Table 2**.

Determination of pH and acidity

The combined analysis of pH and bile salt tolerance provides insights into the behaviour and adaptability of kombucha bacteria and yeast cultures under varying conditions. Understanding these tolerance profiles can be helpful in selecting cultures for specific applications or predicting their behaviour and survival in environments such as the human gastrointestinal tract, where both pH and bile salts can have significant impacts on microbial communities.

The total acidity and pH of kombucha fermentation over a 10-day period reveal a strong relationship between these two parameters. As the total acidity increases, the pH decreases, indicating that the kombucha becomes more acidic as the fermentation progresses. At the beginning of the fermentation (Day 0), the total acidity is low (1.2 g/L), and the pH is relatively high (4.5). During the first two days, the pH remains constant at 4.5, while the total acidity increases slightly to 1.4 g/L on Day 1 and 1.8 g/L on Day 2. From Day 2 onwards, both the total acidity and pH show more noticeable changes. By Day 4, the total acidity reaches 2.4 g/L, and the pH declines to 4.0. The rate of change in total acidity and pH becomes more significant from Day 5. On Day 5, the total acidity jumps to 4.0 g/L, and the pH drops to 3.8. This trend continues until Day 10, with the total acidity reaching 20 g/L and the pH decreasing to 3.1**. Fig. 3 (a)** and **(b)** shows the graphical representation of pH and acidity of 10 days fermentation of kombucha.

Comparing the pH and bile salt tolerance data, it is evident that BK1, YK1, and YK2 demonstrate both high pH and bile salt tolerance, suggesting that these cultures may be more resilient to different environmental conditions. BK5 exhibits moderate tolerance to both pH and bile salts, while BK2, BK3, and BK4 display lower tolerance to these stressors, indicating that they may be more sensitive to environmental changes.

The increasing acidity and decreasing pH during kombucha fermentation can be attributed to the production of organic acids, such as acetic, lactic, and gluconic acids, by the symbiotic culture of bacteria and yeast (SCOBY) involved in the process. As the SCOBY metabolizes the sugar and other nutrients present in the kombucha, it releases these acids, leading to a drop in pH and an increase in total acidity. This rise in acidity contributes to the characteristic sour taste of kombucha and helps preserve the drink by inhibiting the growth of undesirable microorganisms.

Alcohol estimation using hydrometer

The specific gravity represents the density of a liquid relative to water. In the context of alcohol production, the initial specific gravity (prior to fermentation) is mainly influenced by the sugar content in the liquid. As fermentation occurs, yeast consumes sugar and transforms it into alcohol and carbon dioxide, leading to a decrease in specific gravity. Comparing the initial and final specific gravity measurements allows for the estimation of the alcohol content in the fermented product. This method provides approximate concentration of alcohol.

The alcohol estimation of kombucha fermentation by using a hydrometer at various time intervals is shown in **Table 3**. On Day 0, the initial specific gravity was measured at 1.024. Over the course of the fermentation process, the specific gravity changed, and the alcohol content (%ABV) was measured using formula given below. On Day 1, the final specific gravity remained the same as the initial value, at 1.024, indicating no alcohol production at this stage of the fermentation process. By Day 4, the final specific gravity had decreased to 1.016, resulting in an alcohol content of 1.05% ABV. This demonstrates that the fermentation process had started, and alcohol production was underway. Interestingly, on Day 7, the final specific gravity increased slightly to 1.018, resulting in a lower alcohol content of 0.79% ABV compared to the previous measurement Finally, on Day 10, the final specific gravity further increased to 1.020, yielding an alcohol content of 0.52% ABV. This suggests that the alcohol production had decreased over time, which is common in kombucha fermentation as the process shifts from alcohol production to the formation of organic acids. the kombucha fermentation process showed varying levels of alcohol production, with the highest alcohol content observed on Day 4.

Estimation of total carbohydrate

Standard graph of method was plotted and given in **Fig. 3 (c).** Based on standard graph, carbohydrate content of kombucha fermentation was calculated till 10 days. The total carbohydrate concentration during kombucha fermentation over a 10-day period shows a consistent decrease in carbohydrate content. This decline indicates that the carbohydrates are being consumed by the symbiotic culture of bacteria and yeast (SCOBY) involved in the fermentation process. At the beginning of fermentation (Day 0), the total carbohydrate concentration is relatively high at 58 g/L. The rate of carbohydrate consumption remains

steady in the second half of the fermentation process. On Day 6, the concentration drops to 32 g/L, followed by 28 g/L on Day 7, 25 g/L on Day 8, 23 g/L on Day 9, and finally, 22 g/L on Day 10. The graphic representation of the total carbohydrates produced during the kombucha fermentation process is shown in **Fig. 3(d)**.

This decrease in total carbohydrate concentration during kombucha fermentation can be attributed to the metabolic activities of the SCOBY. As the bacteria and yeast consume the carbohydrates, primarily in the form of sugar, they produce various organic acids, carbon dioxide, and trace amounts of alcohol. This metabolic activity leads to the characteristic sour taste and effervescence of kombucha. The decrease in carbohydrate concentration also reflects the gradual transformation of sugar into other compounds, such as organic acids and other metabolites, which contribute to the unique flavour profile and potential health benefits of kombucha.

Study of pH and bile salt tolerance

pH and bile salt tolerance for bacteria (BK1 to BK5) and yeast (YK1 and YK2) cultures from kombucha fermentation reveals the different degrees of growth and survival across various pH levels and bile salt concentrations. Comparing the two datasets allows us to analyse the overall tolerance of these cultures under different conditions, which can be important for understanding their behaviour in various environments, such as the human gastrointestinal tract.

In the pH tolerance study, BK1, YK1, and YK2 demonstrated better growth at higher pH levels, exhibiting optimal growth at pH 6. BK5 also showed optimal growth at pH 6, but with a moderate, rather than optimal, growth at pH 4. BK2 and BK4 had similar growth patterns with no growth at pH 2, moderate growth at pH 4, and optimal growth at pH 6. BK3 appeared to be the least tolerant to low pH levels, with no growth at pH 2 and pH 4 but thriving at pH 6. The growth turbidity of kombucha isolates observed after 48 h in various pH broths are shown in **Table 4.**

In the bile salt tolerance study, BK1, YK1, and YK2 showed high tolerance to bile salts at 0.15 g% concentration, with optimal growth. BK5 exhibited moderate growth at the same concentration, indicating a moderate level of tolerance. BK2, BK3, and BK4 were sensitive to bile salts at 0.15 g% concentration, with no growth observed. When the bile salt concentration increased to 0.5 g%, BK1 and YK1 maintained relatively high tolerance, while YK2 showed reduced tolerance and BK5 lost its ability to grow in the presence of bile salts. The growth turbidity of kombucha isolates observed after 48 h in different concentration of bile salts are shown in **Table 5.**

Study of pH and bile salt tolerance revealed that BK1 and YK1 microorganism might be potential bacterial, and yeast isolate that can tolerate pH and bile salt efficiently and may be active role in fermentation of kombucha.

Characterization of SCOBY by Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a powerful technique to examine the formation of inter- and intra- molecular hydrogen bonds in cellulose. The detailed database allows the establishment of strong correlation between the nature of hydrogen bonds and physical (e.g., solubility, hydroxyl reactivity, crystallinity) and mechanical properties of cellulose

The FTIR spectrum revealed the characteristic peaks associated with bacterial cellulose. Peaks at 3923.77, 3845.65, 3741.44, 3662.13, and 3271.09 cm^{-1} are related to O-H stretching vibrations in the cellulose structure, while peak at 2923.66 cm^{-1} correspond to C-H stretching vibrations of the CH and CH_2 groups in glucose units [18]. A peak at 1021.25 cm^{-1} represents the C-O-C stretching vibrations, characteristic of the glycosidic linkage between glucose units in cellulose [11]. The peak at 1411.34 cm^{-1} is related to C-H bending vibrations of CH_2 groups in cellulose. However, the FTIR data also reveals some peaks that suggest potential contamination or chemical modifications in the cellulose structure. The peak at 1726.84 cm^{-1} indicates the presence of a C=O stretching vibration, which is not typical for pure cellulose and could suggest contamination or chemical modification, such as ester or carboxylic acid groups. Peaks associated with 864.74, 814.38, and 773.55 cm^{-1} could be related to out-of-plane bending vibrations of C-H groups in cellulose or other contaminants. These obtained FTIR spectrum peaks correlated with previously reported data that confirm the polymer isolated from kombucha was bio cellulose. FTIR analysis of SCOBY derived from kombucha fermentation is shown in **Fig. 4**.

Fermentation by consortium development

As the microbial composition of SCOBY is not well defined there are chances that many of the microorganisms present in the SCOBY are not involved in the fermentation process required for Kombucha production. To have an idea of which specific microorganisms are responsible for SCOBY formation and Kombucha fermentation, consortium of a bacteria and a yeast obtained from microbial isolates was prepared. Based on the phenotypic characters, pH and bile salt tolerance study, among all isolates from kombucha fermentation process BK1 and YK1 were selected for the development of consortium. After 4

days of inoculation of microbial cultures to production medium very thin layer was observed same as routine SCOBY shows the characters. Further fermentation also revealed the strong acidic aroma and acidity after the 14 days of fermentation. Hence, further study is required to standardize the process of kombucha fermentation using BK1 and YK1. The development of thin layer (biofilm) during the kombucha fermentation process by consortium is shown in **Fig. 5**.

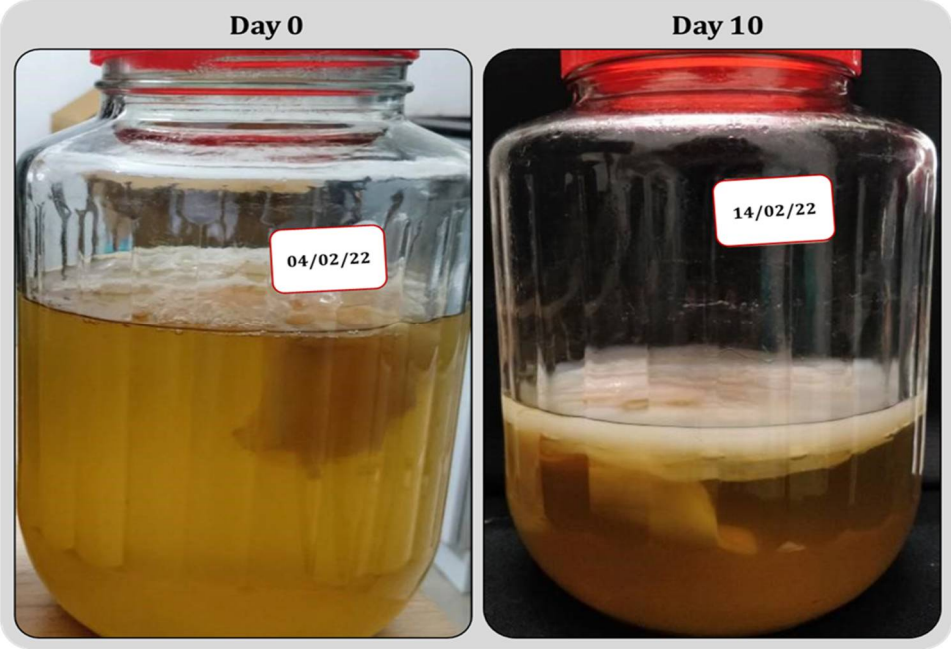

Fig. 1 Kombucha batch fermentation

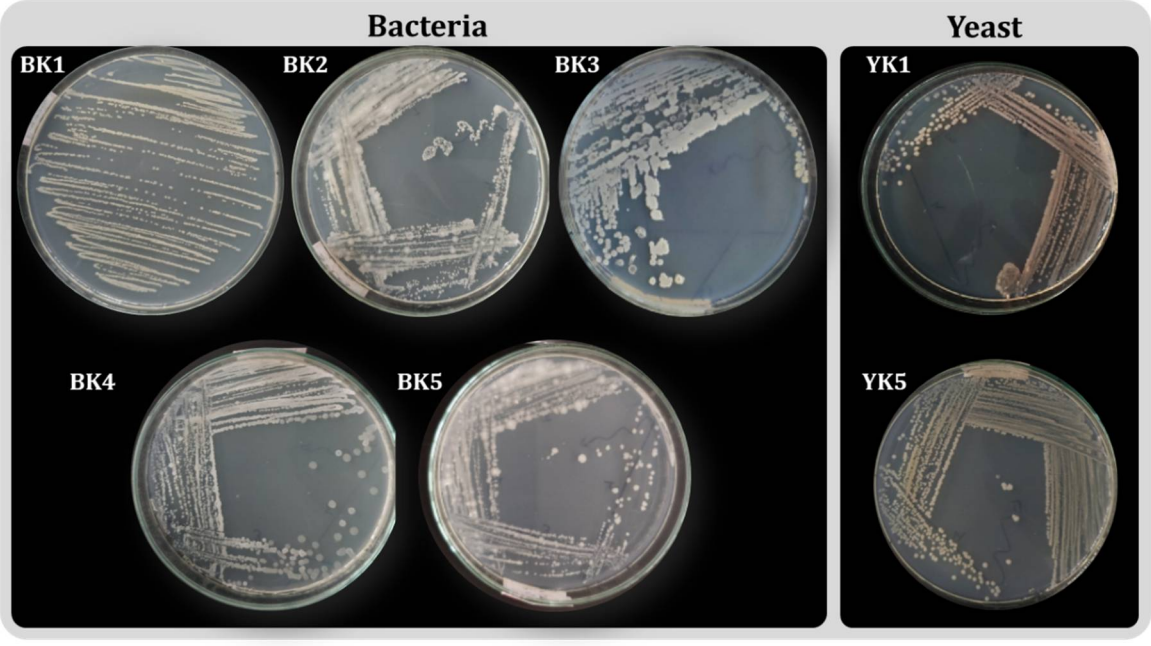

Fig. 2 Kombucha isolates on NA medium

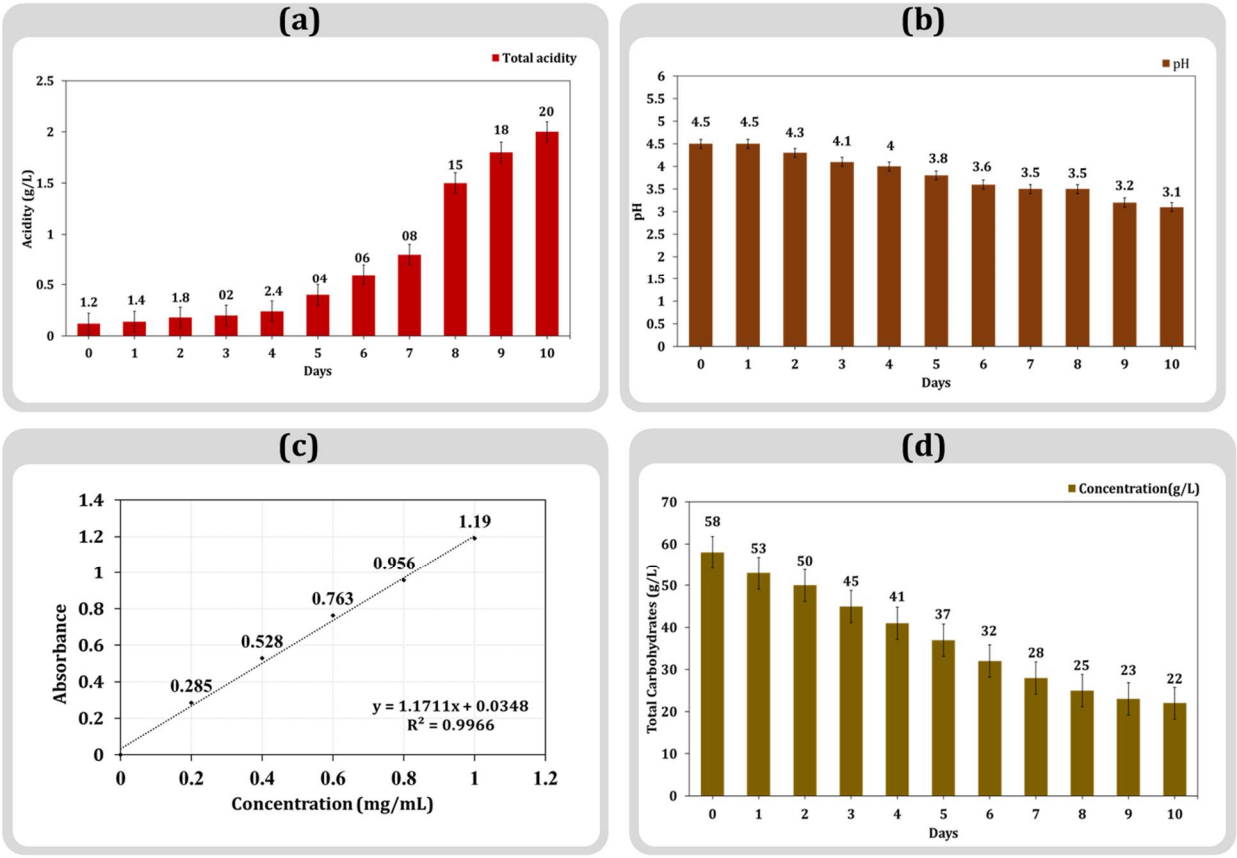

Fig. 3 Chemical characterization Kombucha fermentation process till 10 days, (a) Estimation of total acidity as acetic acid, (b) Estimation of pH, (C) Standard graph of phenol sulphuric acid method with glucose solution, (d) Estimation of total carbohydrates

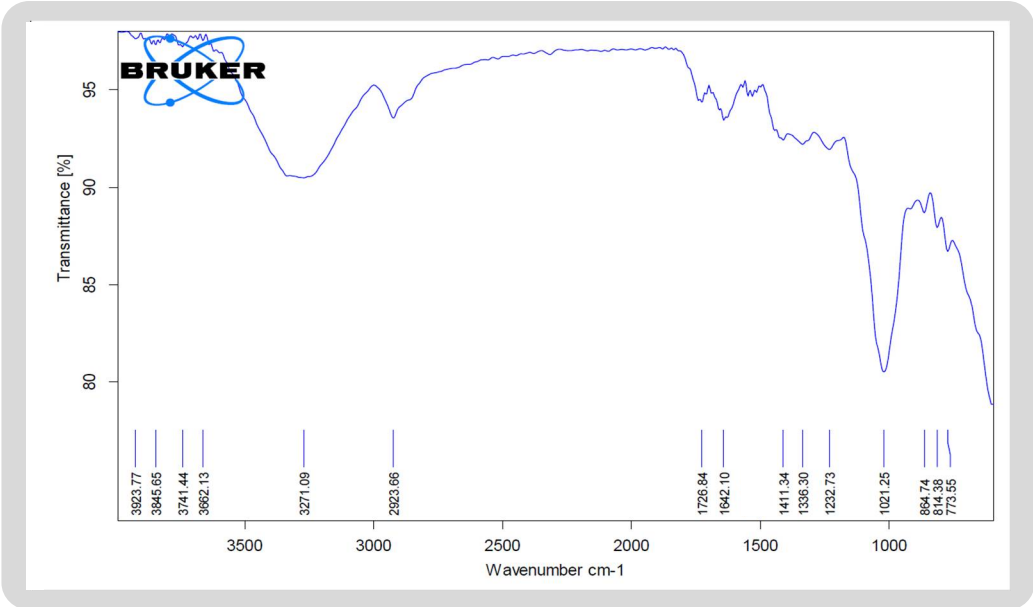

Fig. 4 FTIR analysis of SCOBY derived from kombucha fermentation process

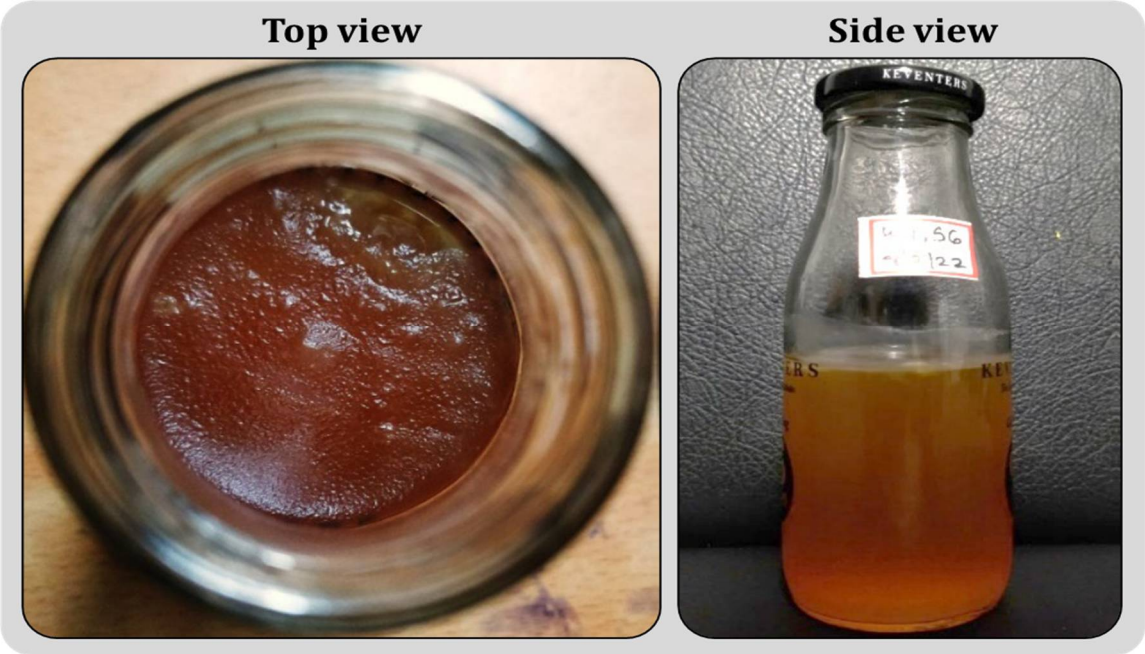

Fig. 5 Kombucha fermentation by using consortium development

Table 1 Colony characteristics of microorganisms isolated from kombucha

	Bacteria					Yeast	
Isolates	**BK1**	**BK2**	**BK3**	**BK4**	**BK5**	**YK1**	**YK2**
Size	Small	Small	Big	Big	Intermediate	Intermediate	Intermediate
Shape	Round	Uneven	Round	Round	Uneven	Round	Round
Margin	Entire	Wavy	Wavy	Entire	Wavy	Entire	Entire
Elevation	Raised	Flat	Raised	Flat	Flat	Flat	Raised
Consistency	Moist	Dry	Moist	Sticky	Sticky	Dry	Moist
Texture	Smooth	Rough	Smooth	Rough	Rough	Rough	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
pigment	Nil	Nil	Nil	Nil	Nil	Pink	Brown

Table 2 Morphological characteristics of microorganisms isolated from kombucha

	Bacteria					Yeast*	
Isolates	**BK1**	**BK2**	**BK3**	**BK4**	**BK5**	**YK1**	**YK2**
Size	Small	Big	Big	Big	Big	Big	Intermediate
Shape	Rod	Rod	Rod	Rod	Rod	Oval	Oval
Arrangement	Chain, single	Chain, single	Chain, single	Chain, single	Chain, single	Single	Single
Gram's reaction	Gram negative	Gram positive	Gram positive	Gram positive	Gram positive	-	-

*For the yeast, monochrome staining technique was used. Gram's staining can be only applied to prokaryotic organisms not for the eukaryotic organisms

Table 3: Alcohol estimation of kombucha fermentation

Days	**Initial specific gravity**	**Final specific gravity**	**%ABV (Initial specific gravity – Final specific gravity ×131.25)**
0	1.024	-	-
1		1.024	-
4		1.016	1.05
7		1.018	0.79
10		1.020	0.52

Table 4 pH tolerance study of kombucha isolates

Culture	pH 2	pH 4	pH 6
BK1	+	++	+++
BK2	-	+	+++
BK3	-	-	+++
BK4	-	+	+++
BK5	-	++	+++
YK1	-	++	+++
YK2	-	+	+++

Keys: '+': Low growth, '++': Moderate growth, '+++': Excellent growth, '-': No growth

Table 5 Bile salt tolerance study of kombucha isolates

Culture	0.15 g%	0.5 g%
BK1	+++	++
BK2	-	-
BK3	-	-
BK4	-	-
BK5	+	-
YK1	+++	++
YK2	+++	+

Keys: '+': Low growth, '++': Moderate growth, '+++': Excellent growth, '-': No growth

CONCLUSION

In conclusion, this research on kombucha production and its microbial composition provides valuable insights into the fermentation process, the role of specific microorganisms, and the physicochemical properties of the final product. The study identified 5 bacterial and 2 yeast colonies that were isolated from kombucha SCOBY and demonstrated varying levels of pH and bile salt tolerance. Among these, BK1 and YK1 were found to be potential candidates for efficient fermentation due to their high tolerance levels. The 10-day fermentation period showed a strong relationship between the increasing acidity and decreasing pH, which is attributed to the production of organic acids by the SCOBY. The gradual decrease in carbohydrate concentration during fermentation indicated the consumption of sugars by the bacteria and yeast, leading to the characteristic sour taste, effervescence, and unique flavour profile of kombucha. FTIR analysis confirmed the presence of bacterial cellulose in the SCOBY. Further research is required to elucidate the precise role of specific microorganisms in kombucha fermentation and SCOBY formation. The development of a bacterial and yeast consortium from the isolated microorganisms may help identify the key players responsible for SCOBY formation and kombucha fermentation. This understanding can contribute to the optimization of kombucha production and the development of novel probiotic strains with enhanced functional properties.

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