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In Vitro Biological Activities of Kombucha Drink and Evaluation of Microbiota for Bioethanol Production

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ABSTRACT:
Kombucha is regarded as a functional food due to its potential to deliver various health benefits when incorporated into a balanced diet and consumed consistently.This study explores the potential of kombucha microbiota for sustainable bioethanol production.With its growing popularity, kombucha is extensively sold as a health-enhancing drink, claimed to provide multiple benefits to those who consume it. Organoleptic characterization revealed varying sensory properties, while pH analysis indicated an acidic nature. Anti-inflammatory properties were confirmed through protein denaturation assays. Four microbiota isolates (W2, Y1, Y2, Yellow) were characterized, with W2 and Y2 showing highest bioethanol production. BLAST analysis identified W2 as *Bacillus subtilis* and Y2 as *Saccharomycopsis fibuligera*. This research demonstrates the promise of kombucha microbiota for bioethanol production and highlights the characteristics of promising isolates for future research

Keywords **—Kombucha, Microbiota, Bioethanol production, Anti-inflammatory properties, Fermentation**

I.INTRODUCTION

A.*Probiotic products*

Probiotic products have been shown to benefit human health via various gastrointestinal routes. Recent developments in the preparation of probiotics have focused on the development of beneficial effects in humans. As a result, probiotic products have evolved to include double-coated or encapsulated cells, various strains of probiotics, and probiotic products with live cells (more than 1010 live cells per gram). To date, most probiotic products contain live cells but do not focus on other products, such as anti-inflammatory products or products containing bacteriocins, that may provide the benefits of cell products, including safety and security. If the product is sold with health claims, the control of these health claims will be more stringent [1]. Therefore, regulatory agencies will require significant scientific and clinical evidence supporting the safety and efficacy of probiotics to complete a health application. This demand may impact the future outlook of the probiotics market.

B.*Healthy lifestyle using probiotic products*

As people prioritize healthy living, probiotics have become increasingly important for overall well-being and longevity. Probiotics, beneficial live microorganisms, promote digestive health and immunity by balancing gut microbiota [2]. They support digestion, nutrient absorption, immune function, and mental health. You can find probiotics in foods like yogurt, kefir, sauerkraut, and kimchi, or take supplements.Fermented products, made from milk, grains, meat, fruits, and vegetables, are prized for their probiotic properties. While dairy products are common probiotic sources, non-dairy options like kombucha have gained global popularity, especially among vegans and those with lactose intolerance or high cholesterol.

Kombucha, a fermented tea drink, contains a diverse range of microorganisms, including lactic acid bacteria and yeasts. The traditional fermentation process involves a symbiotic colony of bacteria and yeasts (SCOBY) aerobically fermenting tea and sugar, resulting in a collaborative metabolic process between prokaryotic and eukaryotic microorganisms. This complex microbial community produces probiotic-rich drinks through a joint process [3].

C. *Kombucha*

Kombucha, a fizzy tea drink, originated in China around 5000 years ago and has since gained global popularity. Its flavor profile ranges from fruity to vinegar-like, depending on fermentation conditions. Regular consumption is associated with potential health benefits, including antioxidant, antimicrobial, and liver-protective effects.

Kombucha is fermented using a symbiotic culture of acetic acid bacteria and yeast (SCOBY) in a sugared tea base [4],[5]. Black tea is commonly used, but green tea, oolong tea, and medicinal herbs are also used. The fermentation process involves the conversion of ethanol to acetic acid and glucose to gluconic acid, contributing to Kombucha's acidity and potential health benefits [6].

Understanding Kombucha cultures is challenging due to the diversity and complexity of microbial communities involved. Variations in culture composition may arise from climatic and geographical factors [6],[7].Identifying and understanding microbial characteristics allows manufacturers to control the fermentation process, ensuring safer production.

Kombucha has proven anti-bacterial, antioxidant, anti-diabetic properties, and supports the immune system and liver detoxification. It contains minerals, vitamins, amino acids, and compounds formed during fermentation. Parameters like tea type, fermentation time, SCOBY colonies, and temperature influence Kombucha's properties and content [7].

The global Kombucha market has seen substantial growth, valued at USD 1.5 billion in 2018 and projected to reach USD 5 billion by 2025. The COVID-19 pandemic boosted sales, driven by increased consumer interest in healthful beverages. Companies are launching Kombucha products in major supermarkets and online channels, and demand is rising in on-trade channels like bars and restaurants.

D. *Health benefits of Kombucha*

Kombucha, a fermented tea drink, offers numerous health benefits due to its probiotic and synbiotic properties. Regular consumption as part of a balanced diet can positively impact gastrointestinal microbiota, boost the immune system, and improve overall health [8]. Kombucha has been found to alleviate menstrual disorders, menopausal symptoms, and eye problems, while enhancing cellular regeneration and skin health. Additionally, it has anti-hyperglycaemic, hepatoprotective, and cholesterol-lowering effects, protecting against vascular disease and coronary heart disease [9]. Kombucha also benefits immunological, endocrinological, cardiovascular, gastrointestinal, and urogenital health. Other benefits include improved hair, skin, and nail health, reduced stress and nervous disturbances, and prevention of kidney calcification. Overall, kombucha is a functional food with a range of health benefits, making it a great addition to a healthy lifestyle [9], [10].

E. *Consortium organisms in kombucha*

Kombucha's unique composition and potential health benefits stem from its complex fermentation process, involving a diverse consortium of microorganisms. This symbiotic association of bacteria and yeast produces various compounds, contributing to its characteristic taste, texture, and health benefits. Gluconobacter and Acetobacter produce gluconic acid and acetic acid, respectively, which contribute to kombucha's sour taste and potential health benefits [11]. Yeast, such as Brettanomyces, convert sugars into ethanol, influencing the growth and metabolism of other microorganisms [3][12].

The kombucha consortium's synergistic relationship is essential for producing its unique composition and potential health benefits. Understanding the roles of these microorganisms can uncover kombucha's full potential and applications in the food and beverage industry. Additionally, the consortium's metabolic versatility makes it an attractive candidate for bioethanol production [11]. By harnessing the collective potential of these microorganisms, novel fermentation strategies for bioethanol production could be developed, contributing to a more sustainable energy future. Further research into the kombucha consortium's metabolic dynamics and ethanol production capabilities could pave the way for innovative fermentation technologies and a greener tomorrow.

F. *Ethanol Production*

Kombucha's fermentation process involves yeast and bacteria in the SCOBY feeding on tea sugars, producing ethanol and carbon dioxide. Ethanol content varies depending on factors like yeast and bacteria type, sugar content, and fermentation time, ranging from 0.05% to 3.5% ABV, with an average of 0.5% ABV. Although generally considered safe, kombucha's ethanol content can pose risks to individuals with certain health conditions or those avoiding alcohol. The industry's lack of regulation leads to inconsistent ethanol levels in products [13].

Ethanol production in kombucha is a natural process, influenced by yeast strain, sugar content, fermentation time, and temperature. The yeast converts sugars into glucose and fructose, consumed by bacteria to produce ethanol, which is trapped in the fermentation vessel. Ethanol content varies significantly between products and manufacturers, posing concerns for consumers who need to avoid alcohol. Regulation is necessary to ensure consistent ethanol levels and protect consumer safety. Understanding the factors influencing ethanol production in kombucha is crucial for manufacturers and consumers alike.

II. Materials And Methods

A.*Collection of Sample*

The Kombucha(ginger), a probiotic drink was collected from nearby supermarket and brought to the lab. The sample was stored at 4°C for further studies.

B. *Physical characteristics of the drink*

1)*Organoleptic characterization*: The smell, colour and taste of the drink were observed and noted.

2) *pH*: The pH of the kombucha drink was measured to study the pH of the the sample.

3) *Determination of acidity*: The total acid content of the sample was estimated. In a clean glass beaker 10ml sample and 10ml distilled water and 5 drops of phenolphthalein indicator were added and titrated against 0.1N NAOH. The reaction was confirmed by a change in colour from light pink to magenta. The burette reading was noted and calculated. The total acidity percent and volatile acidity were calculated by using the following formula.

$$\text{Total acidity \%} = \frac{\text{ml of alkali} \times \text{normality} \times 7.5}{\text{Weight of the sample}}$$

$$\text{Volatile acidity} = \frac{\text{ml of alkali} \times \text{normality} \times 6}{\text{Weight of the sample}}$$

$$\text{Lactic acid\%} = \frac{0.1\text{M NAOH} \times \text{vol.of NAOH (in litre)} \times 90.08 \times 100}{\text{Weight of the sample}}$$

C. *Screening of anti-inflammatory activity*

1)*Protein denaturation assay using egg albumin*:The various concentrations of the extract & standard (100,200,300,400,500µg/ml) were prepared. Added 0.2 ml egg albumin and 2.8 ml PBS (6.4) to all tubes. Make up to 5ml using ethanol and kept for incubation at 37°C for 15 mins, followed by heating at 70°C for 5 mins. Then the solution was cooled at room temperature and measured the absorbance at 660nm. 5ml distilled water was taken as control & Aspirin was used as reference standard.

% of Protein denaturation= Absorbance of control-Absorbance of sample/Absorbance of control ×100

2)*Protein denaturation assay using Bovine Serum Albumin*: The various concentration of the extract & standard (100,200,300,400,500µg/ml) were prepared. About 0.45ml of BSA was added to all tubes. Make up to 2ml using ethanol. Then kept the reaction mixture for incubation at 37°C. After that heat the mixture at 57°C for 3mins. After cooling the mixture, added 2.5ml PBS to all tubes. Measured the absorbance at 660nm.

% of Protein denaturation= Absorbance of control-Absorbance of sample/Absorbance of control ×100

D. *Isolation of organism from Kombucha drink*

The kombucha sample was enriched in peptone broth and spread plated on three different plates.Glucose yeast calcium carbonate medium containing 2%D-glucose ,1%yeast ,2%Calcium carbonate and 2%agar which are mainly used for the isolation of Erwinia species. The modified glucose yeast extract peptone mannitol medium (GYMP) containing 0.2%D- glucose was used for propagating Acetic acid bacteria in kombucha samples [14]. GYMP may also contain 2.5% mannitol, 0..5%yeast, 0.3%peptone and 1.5%agar to inhibit the growth of yeast and lactic acid bacteria or acid tolerant bacteria respectively [15]. And Glucose Yeast extract agar (GYEA)containing 2%D-glucose, 1%yeast ,2%peptone and 2% agar used of the identification of yeast in food [16]. The GYEA plates are incubated in room temperature at 27°C for 48 hours and the GYMP and Glucose yeast calcium carbonate medium were incubated at 37°C for 48 hours. Further the isolated colonies from these plates are streaked into the newly prepared same media and subcultures are made on every 2 weeks to purify the strains.

E. Identification of organisms

The isolated cultures were subjected to colony morphology including shape, colour,
pigmentation, size, edges and elevation were determined and identified accordingly.

1) *Gram's staining*:The smear of the isolated (W2 and Y) cultures were prepared on a clean grease free side and was heat fixed. The crystal violet was added for 1 min. Then Grams iodine was added, which washes the crystal violet stain. Slide was rinsed in running water and the decolourizer was added. Again, the slide was rinsed and the smear was covered with the safranin for 1 min. Finally,safranin was washed on with water, slide was kept for air dry and was observe under the oil immersion lens [17].

2)*Methylene blue staining*:The morphological identification of the isolated yeasts was determined using staining methods.A smear was made from the colony on a clean grease free slide and allowed it to air dry. Heat fixed and flood the smear with methylene blue for 1 minute. Rinsed the slide with water then air dried and observed it under a microscope.

3) *Lactophenol cotton blue staining*: Placed a drop of stain on a clean glass slide and then gently teased a small portion of culture using a mounting needle. Place a coverslip on top avoiding formation of air bubble and remove excess stain around the edges of the coverslip. Examine under a microscope.

F. *Biochemical Characterization*

The selected bacterial isolates were subjected to different biochemical tests. The test includes (IMViC) Indole test, Methyl Red test (MR test). Voges-Proskauer test (VP test), Citrate test, Oxidase test, Catalase test, Urease test and Triple Sugar Iron test (TSI). 24hrs grown fresh culture was used for the test and incubated samples were tested [18]. The yeasts were subjected to Catalase and Oxidase test

1)*Indole test*: Using this test, we can determine whether the bacterium is capable of producing the tryptophanase enzyme, which converts tryptophan into indole and other metabolites. In a tube with a tryptophan-containing media and a reagent called Kovac's reagent, a small inoculum of bacteria is added. If the bacterium produces tryptophanase, the tryptophan is converted to indole, which is subsequently identified by adding Kovac's reagent, which becomes pink or red. A loopful of 24hrs grown fresh culture was inoculated into tryptone broth and incubated at 37°C for 24hrs. After incubation, 0.5ml. of Kovac's reagent was added and agitated properly. Observed for cherry red colour at the top of the culture in the tube indicating a positive

result. Absence of cherry red ring indicates a negative result.

2) *Methyl red test (MR test)*: This test detects the production of acid by the fermentation of glucose so that pH of the medium falls and was maintained below 4. Inoculated the microorganism into prepared sterile buffered glucose broth medium. The medium was incubated at 37°C for 24 hours. After incubation 5 drops of methyl red were added. The medium turns to a bright red colour which indicates the positive result while the yellow colour indicates the negative results.

3) *Voges-Proskauer Test*:
The test was done to determine the capacity of some microorganisms to ferment carbohydrate with the production of non-acidic or natural end products such as acetyl-methyl carbinol or its reduction products i.e. 2,3-Butylene glycol. These products were produced from organic acids that result from glucose metabolism. Inoculated the microorganism into buffered glucose broth medium. The inoculated medium was incubated at 37° C for 24 hours. The pink colour that appeared after addition of Barritt's reagent 1 and 2 when the tubes were kept opened for 15 minutes that ultimately gets deepened either to magenta or crimson red in about 30 min duration.

4) *Citrate Test*: It is used to differentiate among enteric bacteria based on their ability to utilise citrate as the carbon source by citrate producing microorganisms.

Citric acid →oxaloacetic acid + acetic acid→ pyruvic acid

The citrate test was performed by inoculating the microorganism into an organic synthetic medium i.e. Simmon citrate agar, where sodium citrate is the only carbon source and bromothymol blue was used as an indicator when the citric acid was metabolized. The carbon dioxide was generated combined with sodium and water to form carbonate which changes the colour of the medium.

$CO2+2Na+H2O \rightarrow Na2CO3$ (Alkaline pH , blue colour).

The organisms were inoculated into the sterile Simmon citrate medium (green colour after sterilization). The inoculated tubes were incubated at 37° C for 24 hours. After incubation the tubes were examined for the colour change from green to Prussian blue which was considered positive.

5) *Catalase test*:
24 hours old culture was taken and added to the slide with the help of a toothpick. Poured 3% of hydrogen peroxide on it. Effervescence indicated positive results. During aerobic respiration,certain microorganisms produce H2O2 which is lethal to the cell. The enzyme catalase present in these microorganisms produces H2O and O2 which help them in their survival.

$2H2O2 \rightarrow catalase \rightarrow H2O+O2$

6) *Oxidase test*:
The ability of the microorganism to produce an oxidase enzyme or not was determined. Oxidase disc was placed on a clean slide and loopful of organism picked from a colony and smeared on the disk. After 20 seconds, the disc was examined. Formation of deep dark violet colour on the disc indicated positive result.

7) *Triple Sugar Iron Agar Test*:
TSI medium is composed of three sugar (lactose, sucrose and glucose), iron (FeSo4) and 1% phenol indicator. The medium was made with a little slant and with a deep butt and organism was inoculated by stab and streak method. The organism was differentiated based on its ability to ferment free sugars and H2S production. The tube was incubated at 37°C for 24 hours. The tube was observed for the color change in butt and slant along with formation of black precipitate that indicated the production of H2S gas the results were tabulated.H2S production was detected by blackening of slant or butt. Gas production can be detected by cracks in the medium. If the medium became yellow, there is the production of acid and if not, it was considered alkaline.

8) *Urea Hydrolysis Test*:
The ability of the microorganism to produce an enzyme that breaks down urea was determined.The test organisms were aseptically inoculated into the tubes containing sterile Christensen urea agar base with supplemented urea. The tube was incubated for 24 hours at 37° C after inoculation. Examined for color change of the medium

G. *Stress tolerance study*
1) *Alcohol tolerance*: The isolated strains are streaked onto the ethanol media containing 0.5%yeast, 2%agar and different concentrations of ethanol (2% ,4% ,6% & 8%). Ethanol is added after sterilization on various concentration and poured onto plates and streaked. Growth is observed after incubation at 37°C for 48 hours [19].

2)*Sugar tolerance*: The peptone liquid broth was used for detecting the ability of isolates to grow at different concentration of glucose. Various concentration of glucose 1%, 5%, 10%, 15% was added to the different sterilized YEPD broths [20]. Each test tube containing 5 ml of broth with different concentration of glucose. Then each was inoculated with selected isolates. Then kept for incubation at 28°C for 48 hours. After 48 hours cell density was measured at 600 nm for growth.

H. *Study of isolates sedimentation rate*
One mL of 24 h cultures on YPD broth was transferred to 1.5 mL Eppendorf tubes and centrifuged at 10000 rpm for 10 min and the pellets were resuspended in 1 mL NaCl (0.89%)solution for 2 h. The optical density was measured at 600 nm UV/Vis Spectrophotometer. The sedimentation rate was expressed according to
% of sedimentation= (1 - total drop in OD reading after 2 hour)/OD reading at 0 hour ×100

I.*Application of Isolates in Ethanol production*

1) *Estimation of bioethanol*: Bioethanol production was detected by Jones reagent prepared by potassium dichromate and sulphuric acid. About 1 mL of (2%) potassium dichromate, 5 ml, H2S04 and 3 ml, of sample was added after incubation for 20 minutes. It was reported that ethanol oxidized to acetic acid with an excess of potassium dichromate in the presence of sulfuric acid, giving off a blue-green colour [21]. The presence of a green colour indicates that the used carbon source was able to produce bioethanol after confirmation.

J. *Molecular characterization of isolates*

1) *Isolation of genomic DNA of isolate*: The strain was grown in 100 mL of Luria bertani broth (lb broth) for 24 h at 28°C. The cells were centrifuged at 10000 rpm/min and washed two times with Tris–EDTA buffer. Discarded the supernatant and collect pellets. Then resuspended in 400 µl of T.E buffer by repeating pipetting.Add 50 µl of 10% SDS and 50 µl of proteinase K. The contents were mixed and incubated the lysate for 1 hour at 37°C in a water bath. Added 500 µl of 1:1 mixture of phenol chloroform & closed the tube, gently mixed the contents by vortexing. Transferred the upper layer to a fresh tube and repeated the above step until no material is visible in the interphase. Extracted the aqueous phase with 500 µl of chloroform and then transferred the aqueous phase to a fresh tube. Added 25 µl of 5 M NaCl and 1 ml of 95% ethanol, vortex and centrifuged at 10000 rpm for 10min. Decanted the supernatant and carefully removed the residual ethanol. Wash the pellet with 70% ethanol. Decanted the supernatant and removed the excess alcohol with a pipette and the tubes were left open for 10-15 min. Dissolve the pellet in 100 µl of T.E buffer and mix by flicking the tube with your finger until the pellet dissolves.

2) *16S rRNA gene amplification and sequencing*:
The gene coding 16S rRNA of W2 was amplified by PCR from the isolated genomic DNA using the forward(5-TCACGGAGAGTTT-GATCCTG-3) and the reverse (5-GCGGCTGCTGGCACGTA GTT-3) primers. The gene coding 16S rRNA was amplified by PCR from the isolated genomic DNA Y2 using the ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) primers. For performing the PCR reactions ,about 2µl DNA sample, 1µl each Forward and reverse primer, 12.5 µl PCR mix (Red Taq® Ready mix PCR Reaction

Mix, Sigma Aldrich) and 8.5 µl sterile water were added and the reaction mixture was prepared to perform PCR. The PCR conditions consisted of initial denaturation at 95 °C for 3 min, 30 cycles at 94 for 30 s, primer annealing at 48°C for 30 s, elongation at 68 °C for 90 s and final extension at 68 °C for 5 min. The amplification products were detected by agarose gel electrophoresis and purified. The amplified products were sequenced and compared with similar sequences retrieved from Gene Bank database by using the standard nucleotide - nucleotide BLAST.

III. RESULTS

A.*Collection of Sample*

The Kombucha(ginger), a probiotic drink was collected and brought to the lab for the study. The sample was stored at 4°C for further studies

B. *Physical characteristics of the drink*

1)*Organoleptic characterization*: The kombucha ginger drink is a fermented product, it naturally has a unique, slightly vinegary taste and smell. The colour was pinkish in colour.

2) *pH*:The pH of the kombucha drink was measured to 5.5

3) *Determination of acidity*: Total acidity % = ml of alkali ×normality×7.5 x100/Weight of the sample

0.8 x 0.1 x 7.5/50 × 100 = 1.2 %

Volatile acidity = ml of alkali × normality ×6/ Weight of the sample

= 0.96 %

Percentage titratable acidity of acetic acid = Volume of NaOH × MNaOH × 8.9 /10

= 0.5×0.1×8.9/10

= 3.2%

C. *Screening of anti-inflammatory activity*

1)*Protein denaturation assay using egg albumin*:The protection of egg albumin against denaturation measured by the extract at 500 µl concentration were with inhibition percentage of 95.31% and 88.84% respectively for standard (diclofenac 1 mg/10 ml) as compared to sample. The protection percentage increase was dose dependent.

TABLE I
INHIBITION OF ACTIVITY BY EGG ALBUMIN METHOD

Concentra t-ion	Absorbanc e of sample (660nm)	Absorbanc e of standard (660nm)	Denaturatio n inhibition % of sample	Denaturatio n Inhibition % of standard
100	0.730	0.781	59.32	67.79
200	0.574	0.698	70.52	85.74
300	0.435	0.392	79.66	90.6
400	0.263	0.297	84.59	94.42
500	0.163	0.223	88.84	95.31
Control- 1.928				

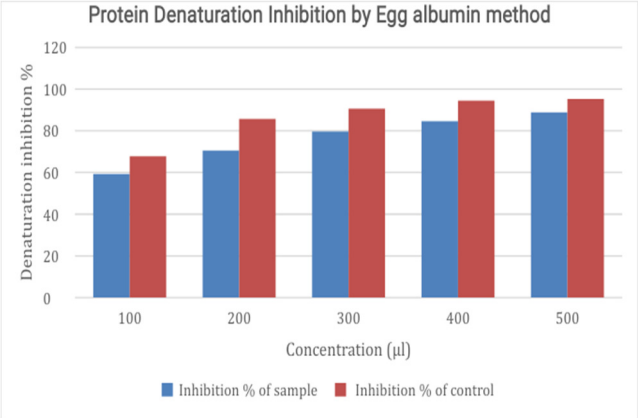

Fig.1 A Bar graph of Protein denaturation inhibition by Egg albumin method

2) *Protein Denaturation method using BSA*:The protection of BSA against denaturation measured by the extract at 500 µl concentration were with inhibition percentage of 95.78% and 90.82% respectively for standard (diclofenac 1 mg/10 ml) as compared to sample. The protection percentage increase was dose dependent.

TABLE II
INHIBITION OF ACTIVITY BY BSA METHOD

Concentra t-ion	Absorbanc e of sample (660nm)	Absorbanc e of standard (660nm)	Denaturatio n inhibition % of sample	Denaturatio n Inhibition % of standard
100	0.492	0.424	63.60	68.63
200	0.411	0.093	69.60	93.12
300	0.363	0.078	73.15	94.23
400	0.235	0.057	82.61	95.63
500	0.124	0.051	90.82	95.78
Control-1.352				

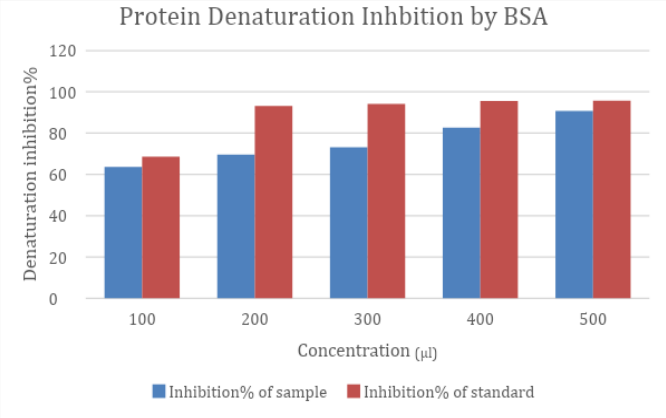

Fig.2 A bar graph of Protein denaturation inhibition by BSA method

D. *Isolation of organism from Kombucha drink*

The organisms (Y1 and Y2) were isolated in Glucose yeast agar. The organism (Yellow) were observed in Glucose calcium carbonate medium. The organisms (W2) were observed Modified glucose yeast extract peptone mannitol medium.

E. *Identification of Isolates*

1)*Gram staining*:The 2 organisms (W2, Y) whose growth were obtained on further maintenance were subjected to Gram staining. The organism W2 was purple coloured rod shaped bacteria and Y was purple coloured cocci in shape.

2) *Methylene blue staining*:The identification of morphology (cocci shaped cells) based on methylene blue staining.

3) *Lactophenol cotton blue staining*: The identification of morphology based on lactophenol cotton blue staining was done which showed cocci shaped cells.

F. *Biochemical Characterization*

The isolates organisms were subjected to biochemical characterization.

1)*Catalase test and Oxidase test*: In case of catalase test, all the isolates W2, Y, Y1, Y2 were positive since all of the produces effervescence on treating with hydrogen peroxide. The organisms W2,Y1 and Y2 were negative in case of oxidase test as they do not produce any colour in the disc whereas Y was positive as it produced a dark violet colour on the disc.

TABLE III
CATALASE AND OXIDASE

Test	W2	Yellow	Y1	Y2
Catalase	+ve	+ve	+ve	+ve
Oxidase	-ve	+ve	-ve	-ve

2) *IMViC, TSI and Urease test*: The IMViC, Urease and TSI of W2 and Y were performed and tabulated.

TABLE IV
IMViC, TSI AND UREASE TEST

Test	W2	Yellow
Indole	-ve	-ve
MR	-ve	-ve
VP	-ve	+ve
Citrate	+ve	+ve
TSI	K/K	K/K
Urease	-ve	+ve

G. *Stress tolerance study*

1) ***Optimization of Alcohol tolerance***: The isolated strains were plated on media at different concentrations of ethanol 2%,4%,6% and 8%. The isolates Yi and Y2 showed growth in all concentrations of ethanol -2%,4%,6% and 8%. The W2 showed growth in 2%, 4%, 6% whereas Y showed growth in2% only. The alcohol tolerance of Y was very less.

TABLE V
ALCOHOL TOLERANCE

Alcohol Concentration	W2	Yellow	Y1	Y2
2%	+ve	-ve	+ve	+ve
4%	+ve	-ve	+ve	+ve
6%	+ve	-ve	+ve	+ve
8%	-ve	-ve	+ve	+ve

2) ***Optimizations of Sugar Tolerance of the Isolates*****:** The yeast isolates showed growth and acid production till 15% of glucose concentration whereas W2 showed growth till 10% and Y in 1% and 5% only.

TABLE VI
SUGAR TOLERANCE

Sugar Concentration	W2	Yellow	Y1	Y2
1%	+ve	+ve	+ve	+ve
5%	+ve	+ve	+ve	+ve
10%	+ve	-ve	+ve	+ve
15%	-ve	-ve	+ve	+ve

H. *Study of isolates sedimentation rate*

The sedimentation rate of the isolates were calculated and tabulated. The sedimentation % was high in case of W2 and Y2 compared to other 2 isolates.

TABLE VII
SEDIMENTATION RATE

Organism	OD at 0th hours	OD after incubation	Sedimentation %
W2	0.555	0.589	74.1%
Yellow	0.572	0.577	73.3%
Y1	0.728	0.788	29.1%
Y2	0.525	0.528	89%

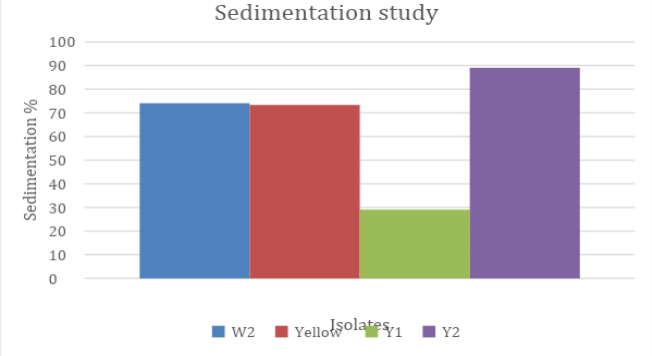

Fig.3 A bar graph of Sedimentation study

I.*Application of Isolates in Bioethanol production*

1)*Estimation of bioethanol*: The Jones reagent was added wherein green color was obtained indication the presence of bioethanol formation.

TABLE VIII
DETECTION OF ETHANOL

Isolates	OD at 500nm
W2	0.161
Yellow	0.123
Y1	0.101
Y2	0.17

J. *Molecular characterization*

The organism that showed more activity W2 and Y2 in sedimentation rate and bioethanol production was subjected to isolation of DNA and was subjected to gel electrophoresis. The DNA was found to be

greater than 1000 bp. The PCR for W2 and Y2 were performed and subsequently analyzed the result in BLAST (https://blast.ncbi.nlm.nih.gov) and found the sequence similarity to *Bacillus subtilis* 99.6% similarity and *Saccharomycopsis fibuligera* with 100% similarity.

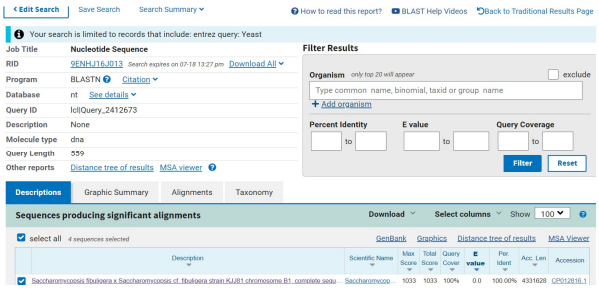

Fig.4 BLAST of *Saccharomycopsis fibuligera*

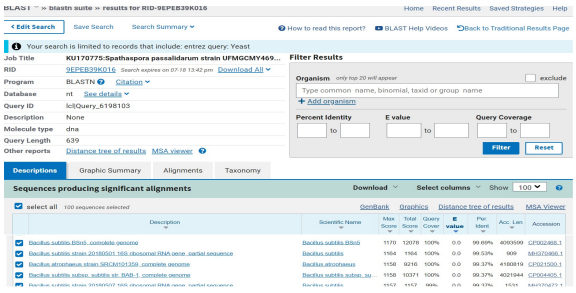

Fig.5 BLAST of *Bacillus subtilis*

IV. CONCLUSION

In this study, the isolated strains (*Bacillus subtilis* and *Saccharomycopsis fibuligera*) from kombucha drinks shows ethanol production.The successful application of these isolates for ethanol production marks a significant milestone in the pursuit of sustainable biofuels.These isolates indicates they have various aspects that applies in various industries such as food industry , agricultural industry and pharmaceutical industry. The further study will help to find out it's optimization activities ,(pH, temperature,sugar concentration etc.) carbon source ,active molecular characterization of ethanol etc. This study reveals that kombucha possesses a range of antioxidant and anti-inflammatory properties, making it a valuable functional beverage.The isolates derived from kombucha demonstrate promising ethanol production capabilities, without posing harm to human health. Notably, kombucha can be consumed as a natural, non-toxic alcoholic beverage, offering a unique alternative to synthetic alcoholic drinks.

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