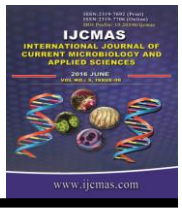
Int.J.Curr.Microbiol.App.Sci (2016) *5*(6): 32-41

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 6 (2016) pp. 32-41**
Journal homepage: http://www.ijcmas.com



Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.506.004

Isolation and Characterization of Bacteria and Yeast from Kombucha Tea

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Keywords

Acetobacter, Kombucha, lichen, yeast, Acetic acid bacteria.

Article Info

Accepted:
07 May 2016
Available Online:
10 June 2016

A B S T R A C T

Kombucha is a sugared tea fermented with flat, pancake like culture of yeast and acetic acid bacteria (AAB), which is consumed worldwide for its refreshing and beneficial effects. In this study Kombucha culture from a fermented tea was screened for the presence of AAB and yeast using selective media. Biochemical analysis for identification and confirmation of the genus of the bacteria and yeast were performed, followed by molecular characterization using partial sequencing targeting conserved sequences of both organisms. Biochemical analysis confirmed presence of organisms belonging to genus *Acetobacter*. Yeast was unidentified by biochemical analysis. Molecular characterization of the isolates identified acetic acid bacteria as *Komagataeibacter saccharivorans* and yeast as *Zygosaccharomysis bailli.*

Introduction

Kombucha a fermented tea beverage known worldwide by many, originated in China which later spread to Russia and rest of the world. Microbiologist earlier had classified Kombucha as lichen based on the fact that Kombucha is symbiotic association of fungi and AAB. However, typical lichen is a symbiosis of algae and fungi and required light to carry out photosynthesis, unlike Kombucha that grow in dark (Grow youthful health at any age, 2014; Velićanski *et al.*, 2014).

Kombucha culture appears as a white rubbery pancake and appearance changes during fermentation period. Initially it is creamy white colour but when brewed in black tea the culture darken due to presence of tannins in tea.

Mushroom culture is steeped in sugared tea for fermentation. During this process original mushroom floats in the tea and baby mushroom is produced on the surface of the original culture. This new mushroom can now be used as culture for further process of fermentation (Jayabalan *et al.*, 2014).

In various studies Kombucha is found to be a symbiotic association of bacteria such

Acetobacter xylinoides, A. pasteurianus, A.xylinum, A. aceti, and *Bacterium gluconicum* and yeast like *Schizo-saccharomyces pombe, Saccharomycodes ludwigii, Saccharomyces cerevisiae, Zygosccharomyces bailli, Brettanomyces bruttanomyces, B. bruxellensis, B.lambicus, B.custersii and pichia species* (Chen & Liu, 2000; Fu *et al.*, 2014). The exact microbial composition also depends on the source of the innocula of the tea fermentation. Bacteria and fungus in Kombucha present powerful symbiosis which are able to inhibit the growth of potential contaminating bacteria (Dufresne & Farnworth, 2000).

Due to the variability of Kombucha, the bacteria and yeast present in given Kombucha from a certain source can vary at large. Due to this variability the fermentation of tea and its properties tend to differ. Thus it is important to identify the organisms present in the Kombucha used from a certain source for tea fermentation.

In the present study, the objective thus remains to isolate and identify the organisms present in the Kombucha that has been procured from a source and is being used for tea fermentation.

Material and Methods

Tea Preparation

Branded tea was used for the preparation of Kombucha tea. 2% tea was added to boiling water and allowed to infuse for 5 minute. The infusion was filtered through a sterile sieve. 10% sucrose was dissolve in hot tea and preparation was left to cool. 10% previously fermented liquid tea broth was aseptically added in to the fresh tea. The tea was then poured in sterile glass bottles to which 3% freshly grown tea fungus that had been previously cultured in the same medium for 28 days was added. The bottles were covered with sterile muslin cloth and fastened tight.The fermentation was carried out at room temperature (RT) in static condition and in dark for 28 days (Jayabalan *et al.*, 2008).

Isolation of Bacteria and Yeast

Enrichment and Isolation techniques were employed for determining the bacteria and yeast present in Kombucha that was used for fermentation of tea. The enrichment media used was Glucose yeast extract broth (GY). Inoculated broth was incubated at room temperature for 48 hours. Isolation was carried out on two different selective media for isolation of Acetic acid bacteria, Glucose-ethanol medium (GEM) and Glucose yeast extract calcium carbonate medium (GYC). Isolation of yeast was carried out using Sabouraud Dextrose agar (Hi-Media Laboratories Pvt. Ltd., India). All Plates were incubated for 7 days at room temperature (RT) (Hanmoungjai *et al.*, 2007; Soheir & El-Salam, 2012)

Biochemical Characterization of Acetic Acid Bacteria

The morphology and Gram nature of acetic acid bacteria isolated on the selective media was determined. Its biochemical characterization involved catalase, oxidase, over oxidation of ethanol by use of Carr medium, oxidation of acetate and oxidation of lactate (Amoa-Awua *et al.,* 2007; Asai *et al.*, 1964; Kadere *et al.,* 2008; Maal & Shafiee, 2010)

Biochemical Characterization of Yeast

The morphology and microscopic analysis of yeast isolated on Sabouraud Dextrose agar (HiMedia Laboratories Pvt. Ltd., Mumbai) was determined. Biochemical characterization was carried out involving

Isolation on HiCrome Candida Differential Agar (HiMedia Laboratories Pvt. Ltd., Mumbai), Germ tube test and urease production test (Marinho *et al.,* 2010; Salviya *et al.,* 2015). Identification of yeast other than *Candida* was performed by ethanol tolerance test (Guimarães *et al.,* 2006). Acetic acid tolerance test was also performed using acidified media (Kurtzman *et al.,* 2001).

Molecular Characterization

Identification of organisms by phenotypic and biochemical analysis is intricate and time consuming, thereby making molecular characterization an easy, precise and a reliable technique. The molecular method employed was partial sequencing using 16S rRNA gene and 18S rRNA gene as targets for the identification of bacteria an yeast respectively and the sequence was analysed using BLAST (Nithya & Bhaskar, 2013; Soheir & El-Salam, 2012). The amplification and sequencing of the bacteria and yeast was outsourced to GeneOmbio Technology Pvt. Ltd.

Results and Discussion

Fermentation of Tea

Fermentation of tea gets initiated after adding inoculums of Kombucha (10% v/v) along with piece of pan cake (3% w/v) in prepared black tea**.** As growth proceeds the new growing culture starts to form its own pancake layer. During this period, visible changes in the tea observed are typical fermentation odour and formation of gas bubbles as result of CO_2 generation. Over a period of 28 days the culture darkens due to presence of tannins in black tea and Kombucha cake grows up to cover the entire circumference of the glass bottle.

Isolation of Bacteria & Yeast

The enriched broth after incubation of 48 hrs showed turbidity indicating presence of organisms in the sample. Isolated colonies with zone of clearance around them after incubation at room temperature on GYC and GEM selective media confirmed the growth of acetic acid bacteria (Fig. 1). Similarly white colonies with yeast like morphology on Sabouraud Dextrose agar at room temperature were confirmed as Yeast.

Biochemical Characterization of Acetic Acid Bacteria

The isolate was Gram negative short rod, catalase positive and oxidase negative. Complete oxidation of acetate and lactate was observed with no brown pigmentation on GYC medium. An initial colour change from green to yellow and reversion to green colour on further incubation was observed on the Carr medium due to the growth of the isolate (Fig. 2) (Table 1).

Biochemical Characterization of Yeast

Isolate obtained from Sabouraud Dextrose agar was Gram positive oval budding yeast. It showed pink coloured colonies on HiCrome Candida Differential Agar while urease production and germ tube test were negative. The organism was found to tolerate ethanol and acetic acid in respective media (Table 2).

Molecular Characterization

Partial nucleotide sequences of Acetic acid bacteria and yeast have been represented in Fig. 3 and Fig. 4. Sequencing analysis obtained was subjected to BLAST. BLAST results illustrated that the test bacterium was similar to *Komagataeibacter saccharivorans* strain with 100% identity, query cover and

0.0 E value. The yeast exhibited 99% identity with 0.0 E value to *Zygosaccharomyces bailii*.

Kombucha is refreshing beverage well known for its health beneficial effects. Kombucha is a fermented tea which is obtained by inoculating sweetened tea with acetic acid bacteria and yeast (Blanc, 1996; Jayabalan *et al.,* 2010). After 10 to 14 days of inoculation a new tea fungus develops on the surface of tea. Kombucha tea consists of two portion cellulosic pellicle layer which floats at the top of broth and sour liquid broth. As fermentation proceeds characteristic odour of fermentation and gas bubbles formed due to carbolic acid produced are observed (Chen & Liu, 2000; Jayabalan *et al.,* 2014)**.** These observations are typical of growth of Kombucha culture and indicative of fermentation of tea (Jayabalan *et al.,* 2014).In present study, observation noted regarding signs of fermentation cycle is similar to study reported by Jayabalan *et al.,* thus it can be concluded that fermentation cycle carried out in present study was successful.

Isolated colonies on GYC and Sabouraud Dextrose agar was identified as AAB and yeast. Soheir *et al.,* Stated that Acetic acid bacteria shows clear halo after acidification on GYC medium (Soheir & El-Salam, 2012). Isolated strain exhibited similar result, thus confirmed as AAB.

According to Kadare *et al* isolate classified under Acetobacter genera are catalase positive, oxidase negative, oxidize lactate and acetate to CO2 and H2O. Acetobacter strains are able to overoxidise ethanol to acetic acid and finally to CO2 and H2O.As tricarboxylic acid cycle is functional in *Acetobacter* strain, genera is able to overoxidise organic acids. Ability to oxidise acetic acid to CO2 is a major distinguishing feature between genera *Acetobacter and Gluconobacter* (Kadere *et al.,* 2008; Ukwo & Ezeama, 2011). In the present study similar results were observed, therefore isolated strain in present study was confirmed Acetobacter.

As literature reports, in Kombucha various yeast cultures are present belonging to genus *Candida* and/or *Saccharomyces* (Jayabalan *et al.,* 2014). Thus isolated strain was subjected for germ tube test which was found to be negative. This indicates presence of non *Candida albicans* (NCA). To distinguish between *Candida Spps* HiChrome Candida Differential Agar was used (Marinho *et al.,* 2010)**.** Candida Differential Agar which, as per manufacturer's literature indicates presence of *Candida krusei*. For confirmation of *Candida krusei*, urease test was performed which was negative signifying possibility of presence of other genus of yeast in the culture (Deorukhkar & Saini, 2014). For further identification Acid and Ethanol tolerance test were performed. Isolated yeast was found to have Ethanol and Acid tolerance ability. Thus, indicating of presence of genus *Saccharomyces*.

Identification based on genotypic method is considered to be more accurate as compared to phenotypic assays. It is recognized that comparison of a stable part of genome can aid in determination of phylogenetic relationship and identification of organisms. Comparison of 16S rRNA and 18S rRNA is one of the preferred techniques for identification of bacteria and fungi respectively, especially in poorly described, rarely isolated and phenotypically inconclusive strains (Clarridge, 2004).

Table.1 Biochemical Characterization of AAB isolate from Kombucha

Biochemical test	**Result**
Gram nature	Game negative short rods
Carr medium	Isolates producing acid - characteristic change in colour of media observed (From Green to Yellow then Green again)
Catalase	+
Oxidase	-
Oxidation of acetate	+
Oxidation of lactate	+
Brown pigmentation on GYC	-

Key: + = positive, - = negative

Table.2 Biochemical Characterization of Yeast isolated from Kombucha

Biochemical test	**Result**
Gram nature	Gram positive oval budding yeast
Germ tube	-
Growth on Hichrome Agar	Pink colour colony
Urease test	-
Ethanol tolerance 10%, 13% & 15%	+ (for all concentrations)
Tolerance to acetic acid test	+

Key: + = positive, - = negative

Fig.1 Growth on GYC media

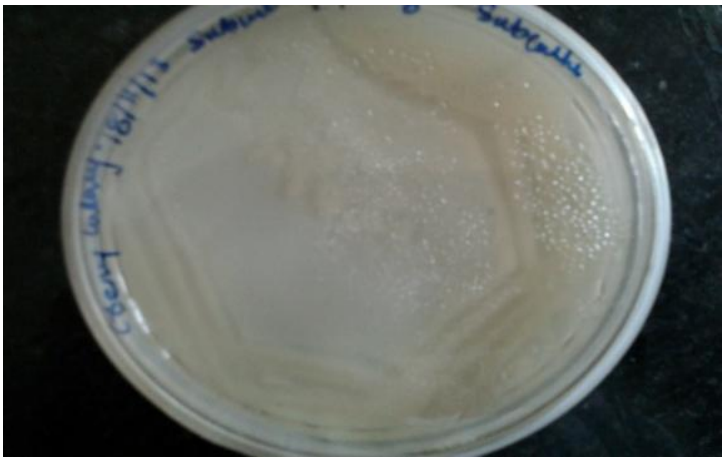

Fig.2 Change in Colour of Carr Media due to Growth of *Acetobacter*

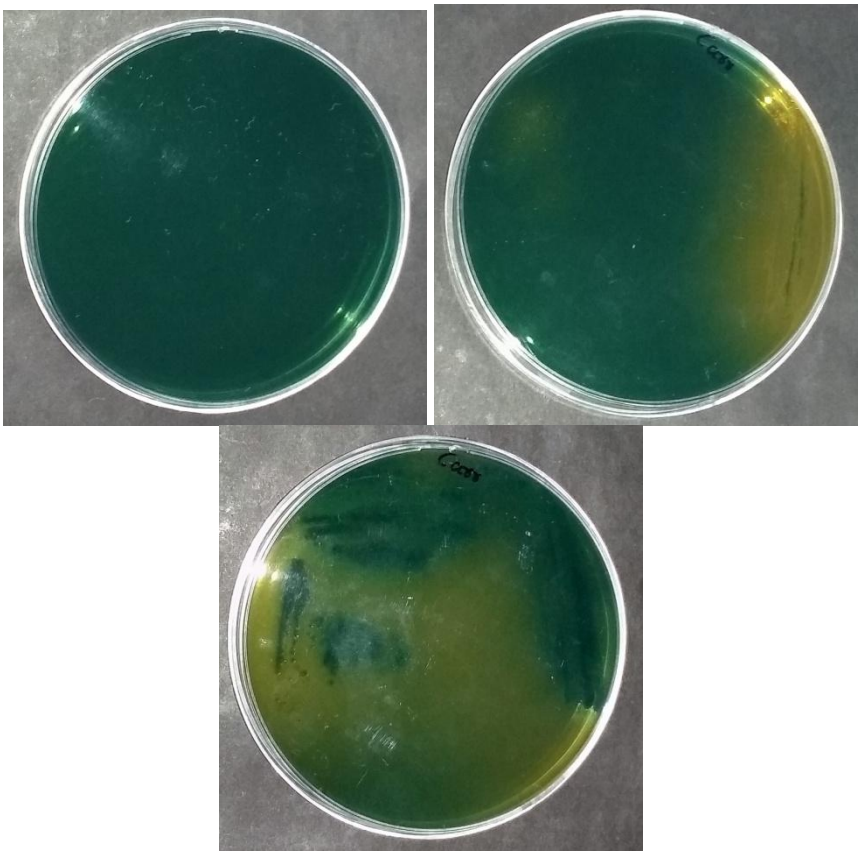

Fig.3 Nucleotide Sequence of AAB

>2221(16S2)

GCCAGGTTGCCGCCTTCGCCACCGGTGTTCTTCCCAATATCTACGAATTTCACCT
CTACACTGGGAATTCCACAACCCTCTCTCACACTCTAGTCTCAACGTATCAAATG
CAGCCCCCAGGTTAAGCCCAGGAATTTCACATCTGACTGTTAAAACCGCCTACGC
GCCCTTTACGCCCAGTCATTCCGAGCAACGCTTGCCCCCTTCGTATTACCGCGGC
TGCTGGCACGAAGTTAGCCGGGGCTTCTTCTGCGGGTACCGTCATCATCGTCCCC
GCTGAAAGTGCTTTACAATCCGAAAACCTTCTTCACACACGCGGCATTGCTGGAT
CAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGG
GCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTATCGATCAT
CGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCGAACGCAGGTTCCTCCAC
AGGCGACTTGCGCCTTTGACCCTCAGGTGTCATGCGGTATTAGCTTCAGTTTCCC
AAAGTTATCCCCCACCCATGGACAGATCCCTACGCGTTACTCACCCGTCCGCCAC
TAACCCCGAAAGGTTCGTGCGACTTGCATGTGTTAAGCATGCCGCCAGCGTTCGC
TCTGAGCCAGGA

Fig.4 Nucleotide Sequence of Yeast

>2222(ITS4)

TCCCTAAAGTCCCCTCTGTTCTTTCGGAGTCGGTAAAACCTAATACGACATTTGG
TTAGGAAAGAGGAGAGCAAGACGTTTCCGCCTAAAACTCACCCACACGCGTTCC
CCAAAAGGCTTGCAATTTCAAGTTAACCCAAATGAAAAACAGAGTATCACTCAC
TACCAAACACGAATGTTTGAGAAGGAAATGACGCTCAAACAGGCATGCCCCCTG
GAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACGGAATTCTG
CAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAG
AGATCCGTTGTTGAAAGTTTTGAATATTTTGTTTTTTAGTATTCGTTTTTGACTGT
AATATTGAAAAAAAAAAAAAAAAATTTGTTGGGTTTTTACCTTTGGGGAGGGG

The 16S rRNA and 18S rRNA sequences obtained, were compared with that of other bacterial and yeast sequences in the GenBank using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) to check its relationship and similarity. Based on sequence similarity, the AAB was identified as *Komagataeibacter saccharivorans* with 100% similarity. While the yeast was identified as *Zygosaccharomyces bailii* with 99% similarity.

Most commonly reported acetic acid bacteria include *Acetoacter aceti, Acetoacter pasteurianus, Acetoacter xylinoides* and *Gluconoacetobacter xylinus.* While the commonly identified yeast include *Brettanomyces bruxelensis, Brettanomyces intermedius, Candida famata, Pichia membranofaciens, Saccharomyces cervisiae, Sachharomycodes ludwigii, Schizosacharomyces pombe, Torulaspora delbrueckii, Zygosaccharomyces bailli and Zygosaccharomyces Rouxii* (Genette Belloso-Morales, 2003; Markov *et al.*, 2006; Sievers *et al.*, 1996).

The bacterial genera *Acetobacter and Gluconobacter* are predominant prokaryotes in Kombucha culture (Jayabalan *et al.*, 2014). Mayser *et al* reported *Acetobacter xylinum* as a primary bacterium and yeast composition is highly variable but *Saccharomyces, Brettanomyces, Zygosaccharomyces* occur most frequently observed to be part of Kombucha culture (Mayser *et al.*, 1995). *Komagataeibacter rhacticus* AF1(formerly known as *Gluconacetobacter rhaeticus*) was isolated from tea (Santos *et al.*, 2014). *Komagataeibacter* is decendent of *Acetobacter* (Lisdiyanti *et al.*, 2006; Yamada *et al.*, 2012). In the present study, *Komagataeibacter saccharivorans* was isolated which belongs to same genera.

In this study twin cultures of the Kombucha were successfully identified as *Komagaeibacter saccharivorans* and yeast *Zygosacchromyces bailli*.

In conclusion, the microbial composition of Kombucha differs depending upon the climatic, geographical area or source. Also use of different starting material (sugar and tea) and a different starter culture will lead to a variation in fermentation process and thereby formations of variable end products or metabolites which might show a difference in activity. Hence, it becomes necessary to identify the organism present in Kombucha. In this study twin cultures of the Kombucha were successfully identified in the study as *Komagaeibacter saccharivorans* and yeast *Zygosacchromyces bailli.* In future chemical characterization and assessment of

biological activity of fermented tea can be executed to understand the fermentation cycle and its beneficial effects on human health.

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How to cite this article:

Tehmeena Ashraf Mukadam, Kapil Punjabi, Sunita D Deshpande, Shashikant Prabhakar Vaidya and Abhay Shadashiv Chowdhary. 2016. Isolation and Characterization of Bacteria and Yeast from Kombucha Tea. *Int.J.Curr.Microbiol.App.Sci.* 5(6): 32-41.
doi: http://dx.doi.org/10.20546/ijcmas.2016.506.004