Fermentation Potentials of Citrus Limon and *Hibiscus Sabdariffa* Juice Extracts

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ABSTRACT

This study aimed to determine the fermentation potentials of yeast isolates from Citrus limon and Hibiscus sabdariffa for the fermentation of juice extracts of C. limon and H. sabdariffa. Isolation and morphological studies of yeast cells were carried out by standard protocols. Fourier Transform Infra-red (FT-IR) spectrophotometer was used to characterize the fermentation products. The result of yeasts morphology showed cylindrical, slender and spherical yeast cells. The standardized fermentation showed significant change in pH (α=0.05>p 0.00). The FT-IR profiles suggest the presence of alcohol in the fermented plant juices thus suggesting C. limon and H. sabdariffa juices as good sources for environment friendly biofuel.

INTRODUCTION

Fermentation refers to the process by which complex organic compounds, such as glucose, are broken down by the action of enzymes into simpler compounds without the use of oxygen. Fermentation results in the production of energy in the form of two ATP molecules, and produces less energy than the aerobic process of cellular respiration[1]. The other end products of fermentation differ depending on the organism. In many bacteria, fungi, protists, and animal cells (notably muscle cells in the body), fermentation produces lactic acid and lactate, carbon dioxide, and water. In yeast and most plant cells fermentation produces ethyl alcohol, carbon dioxide, and water. Alcoholic fermentation is a process that was known to antiquity. Before 2000 B.C the Egyptian apparently knew that crushed fruits stored in a warm place would produce a substance with a pleasant intoxicating power[2]. Alcoholic fermentation occurs when yeast cells convert carbohydrate sources to ethanol and carbon dioxide. Fermentation reactions are common in muscle cells, yeast, some bacteria, and plants[3].

Citrus limon (aka lemon) belongs to the flowering plants family *Rutaceae*. *Citrus limon* is believed to have originated in the part of Southeast Asia. *Citrus limon* has been cultivated in an ever widening areas since ancient times. The plant is large shrub

or small tree, reaching 5 – 15 tall, with spiny shoots and alternately arranged evergreen leaves with an entire margin. *C. limon* is notable for its fragrance, partly due to flavonoid and limonoids (which in turn are terpenes) contained in the rind, and mostly juice laden. The juice contained a high quality of citric acid giving it characteristic sharp flavor. They are also good sources of vitamin C and flavonoid. The flavonoid include various flavanones and flavones[4].

Hibiscus sabdariffa has more than 300 species which are distributed in tropical and subtropical regions around the world. *H. sabdariffa* belongs to *Malvaceae* family. It is an erect mostly branched, annual shrub. Stems are reddish in color and up to 3.5m tall. Leaves are dark green to red, alternate, glabrous long – petiolate, palmately divided into 3 – 7 lobes with serrate margins. Flowers are red to yellow with a dark center containing short peduncles. The flowers have both male and female organs. Seedpods are enclosed in their red, fleshy calyces which are commonly used for making food and tea[5]. Medicinally, *H. sabdariffa* tea is used to suppress high blood pressure. The leaves are a source of mucilage used in pharmacy and cosmetics. Extracts are often used to treat colds, toothache, urinary tract infections and hangovers. Leaf juice is used to treat conjunctivitis. Leaves are applied as a poultice to treat sores and ulcers[4].The aim of the current study is to determine the fermentation potentials of yeast isolates from *Citrus limon* and *Hibiscus sabdariffa* for the fermentation of juice extracts *of C. limon* and *H. sabdariffa*.

MATERIALS AND METHODS

Sample Collection and Handling

The plant materials namely: *Citrus limon* and *Hibiscus sabdariffa* were collected in clean polythene bags in May, 2012, from Kafur local government, Katsina State, Nigeria. The samples were transported to the Department of Biological Science, Nigerian Defence Academy, Kaduna, Nigeria for authentication and confirmation of taxonomic identity. Press of plant materials were prepared and voucher specimens *(C.limon* :201001and *H. sabdariffa:201003)* were deposited in the Department's herbarium.

Isolation and morphological Identification of Wild Yeasts associated with C. limon and H. sabdariffa

The C. *limon* (fruit) and *H. sabdariffa* (calyces) were sprinkled with sterile distilled water in sterile Petri-dishes and allowed to decomposed for 7 days. The decomposed plant materials were separately mixed with sterile distilled water and poured in sterile measuring cylinders (10ml).

Potatoes dextrose agar (PDA)

Potatoes dextrose agar (PDA) was prepared according to manufacturer's instruction. Therefore, fifteen grammes (15g) of PDA was weighed using electronic weighing balance (Sartorius 1401, Germany) and put

into 250ml distilled water in conical flasks (500ml). The preparation was properly homogenized and autoclaved at 121^0C for 15 minutes. The sterile medium was allowed to cool at 55^0C, subsequently poured in sterile labeled Petri-dishes (60x15mm) and bijou bottles for slants. The poured plates and bijou bottles were allowed to gel. Similar procedure was employed to prepare 7 grammes of nutrient agar.

Culturation of yeasts

The previously prepared inocula were radially inoculated unto the separate labeled PDA and nutrient agar plates using sterile wire loops. The inoculated plates were inculcated at 30^oC for 24hours. The PDA plate cultures were culturally and microscopically examined for yeasts. Subsequently, the yeast cells were sub cultured on slants and incubated at 30^0C for 24hours. The slant yeast cultures were stored in refrigerator prior to morphological identification.

Morphological identification of the wild yeast isolates

Smears of the yeast cultures were prepared for morphological identification using microscopy. Therefore, small drop of lacto phenol (LP) was placed on a clean microscopic slide. Thereafter small portion of the yeast colony was removed and placed into the drop of lacto phenol to suspend the cells. A clean cover slip was placed and the preparation was viewed under the compound microscope using x 10 and x 40 objective lenses. Digital eyepiece (model 5821 oplenic optronics, kina) was used to capture yeasts images with the help of minisee software.

Determination of fermentation potentials of the wild yeast isolates on T. Indica Juice extracts.

The wild yeast isolates of *C. limon* and *H. sabdariffa* plants were physiologically studied to evaluate their fermentation potentials on *C. limon* and *H. sabdariffa* juice extracts respectively.

Preparation of C. limon and H. sabdariffa Juice Extracts

Thirty percent (30% $^{v}/_{v}$) of *H. sabdariffa* juice extract was prepared. Therefore, six hundred grammes (600g) of *H. sabdariffa* (calyces) was weighed using weighing balance. The weighed sample was placed into aluminium pot with 2 litres of distilled water. The preparation was boiled using Bunsen burner. It was allowed to cool and subsequently sieved using muslin cloth. The *C. limon* juice (2 liters) was extracted aseptically, by squeezing the fruits manually. Specific gravity, sugar brix and pH of the extracts were measured using hydrometer, sugar refractor meter (made in England) and Hanna microprocessor pH meter (model 3305) respectively. The samples were stored in refrigerator prior to fermentation process.

Propagation of C. limon and H. sabdariffa Yeast Isolates

Potatoes dextrose agar (PDA) was prepared according to manufacturer's instruction and autoclaved at 121^0C for 15 minutes. The sterile medium was allowed to cool at 55^0C and subsequently poured into sterile plates. The medium was allowed to gel and the PDA plates were labeled accordingly.

i. ***Culturation of C. limon and H. sabdariffa Yeasts:***

Each of the yeast colonies from the stored *C. limon* and *H. sabdariffa* yeast cultures were inoculated separately unto the labeled PDA plates using radial streaking method with the help of sterile wire loop. The inoculated PDA plates were inverted and incubated at 30^0C for 24 hours.

ii. Preparation of 0.5 McFarland Turbidity Standard

A 1% $^V/_V$ solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of water in a volumetric flask. The preparation was properly mixed. A 1% $^V/_V$ solution of barium chloride was prepared by dissolving 0.5g of dehydrated barium chloride ($Bacl_2.2H_2O$) in 50ml of distilled water in a volumetric flask. A 0.6ml of the barium chloride solution was added into 99.4ml of the sulphuric acid solution and subsequently mixed properly. A small volume of the turbid solution was transferred into a screw cap bottle. The standard was kept at room temperature (20-28^0C) prior to inocula standardization[6].

iii. Standardization of the Yeast Inocula

Sterile distilled water was used to wash the propagated yeast cells separately into sterile conical flasks (250ml). The yeast suspensions were adjusted to 0.5 McFarland turbidity standard using WPA spectrophotometer.

iv. Preparation of Yeast Starter Cultures

The standardized yeast inocula were separately mixed with 50ml of each juice extract in sterile conical flasks (100ml). The preparations were aseptically plugged with cotton wool and covered with aluminium foil. These were incubated at 30^0C for 24 hours, to obtain the starter cultures.

v. Natural and Standardized Fermentations of C. limon and H sabdariffa Juice Extracts using the Yeast Isolates.

The previously prepared starter cultures of: tamarind yeast isolates: *C. limon yeast* isolates: Cly1, Cly2, Cly3 and Hsy1, Hsy2 yeast isolates were used for both natural and standardized fermentations. The natural fermentations were carried out using the juice extracts without reconstitution. The standardized fermentations were reconstituted through pH and brix adjustment. The pH values were adjusted to pH = 5.5 using 150ml 5M NaOH solution. The varied sugar brix were adjusted to 20% using 200g sucrose. A total of 150ml of each juice extract was put into clean labeled plastic container (2.5 litres).The preparations were pasteurized at 71^0C for 15

seconds (High Temperature, Short Time, HTST)[7]. These were allowed to cool and subsequently the starter cultures were added. They were incubated at room temperature (25 ± 2^0C) (natural fermentations) and at 30^0C (standardized fermentations), for twelve days. Sugar brix and pH were measured on daily basis using sugar refractor meter and Hanna microprocessor pH meter (model 3305) respectively. After the twelve days fermentation period, the fermentation process was arrested by pasteurizing the fermented juices at 71^0C for 15 minutes and refrigerating prior to Infra Red analysis.

Determination of fermentative principles

The fermentative principles were identified by FT-IR spectroscopy. *C. limon* and *H. sabdariffa* fermented juice extracts were pelleted using KBr discs.

Recovery of Ethanol from the Fermented juice extracts

Distillation of ethanol from the fermented juice extracts was carried out using distillation setup. This was achieved through a threefold distillation process. The distillation was done at 78 – 79^0C. The first distillates were further redistilled at 78 - 79^0C and the second distillates were equally redistilled at 78 – 79^0C [8].

Test for Alcohol

A total of $1cm^3$ pure acetone and 1 drop of Jones reagent (chromic and sulphuric acids 1:1 $^V/_V$) were added in each distillate. Presence of green coloration indicates positive test for alcohol [9].

RESULTS AND DISCUSSION

The FT-IR profiles of *C. limon* and *H. sabdariffa* fermented juice extracts are presented in Figures 1-5. The results have shown consistency in broad band (3500 – $3000cm^{-1}$) of alcohol in both natural and standardized fermentations.

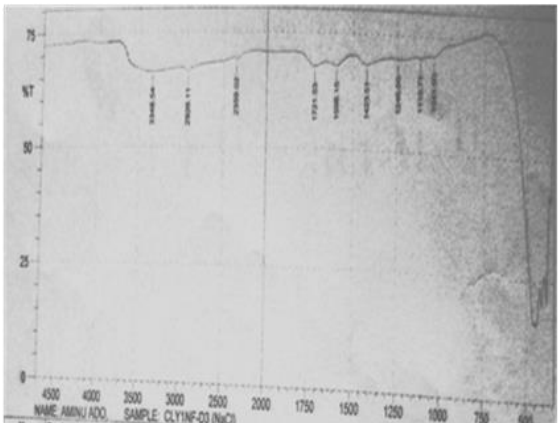


Figure 1: FT –IR Spectra for Citrus limon Yeast 1 Natural Fermentation Day 3 (CLY1NFD3)

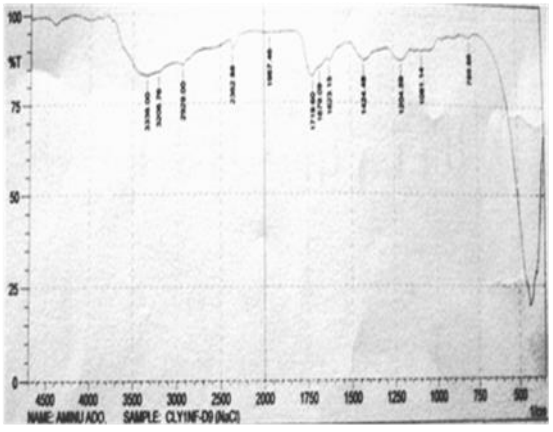


Figure 2: FT –IR Spectra for Citrus limon Yeast 1 Natural Fermentation Day 9 (CLY1NFD9)

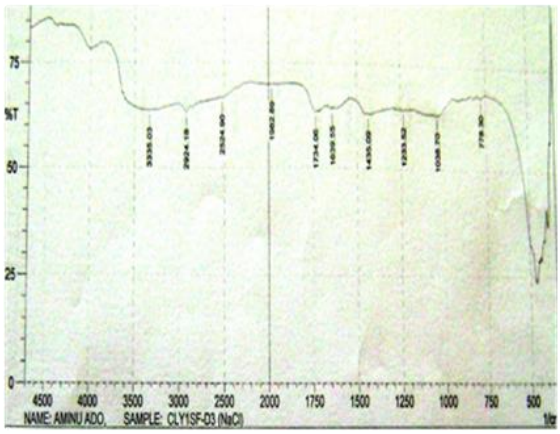


Figure 3: FT –IR Spectra for Citrus limon Yeast 1 Standardized Fermentation Day 3 (CLY1SFD3)

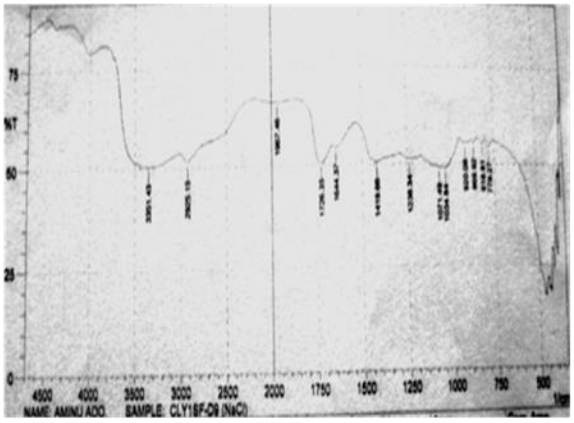
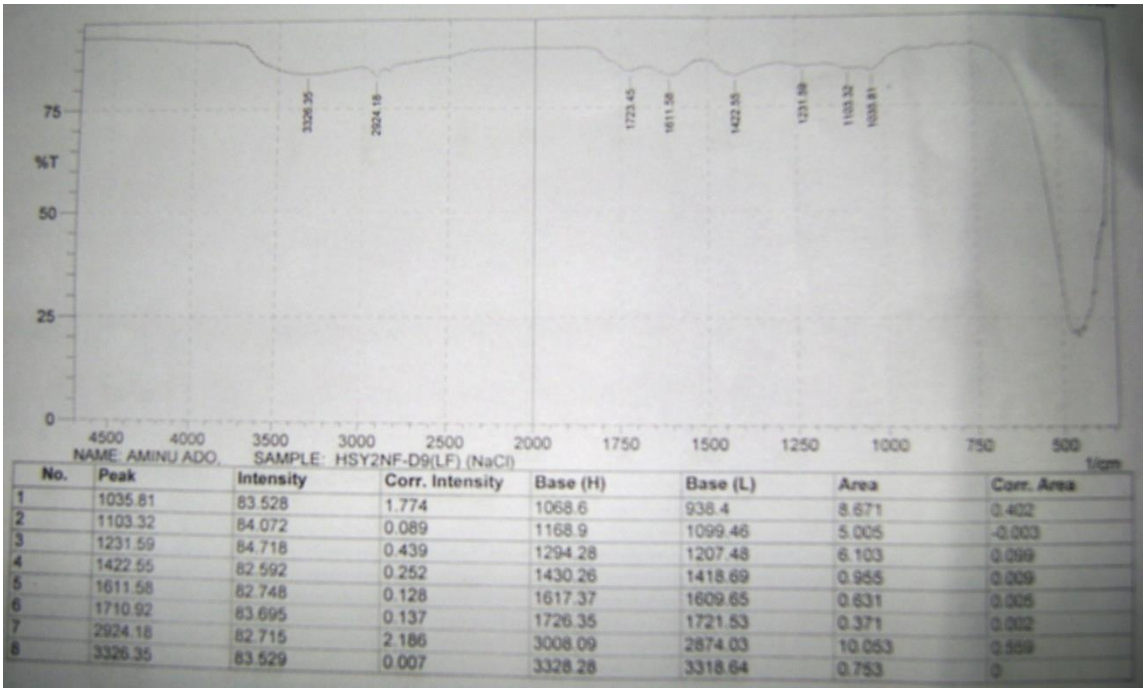


Figure 4: FT –IR Spectra for Citrus limon Yeast 1 Standardized Fermentation Day 9 (CLY1SFD9)



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	1035.81	83.528	1.774	1068.6	938.4	8.671	0.402
2	1103.32	84.072	0.089	1168.9	1099.46	5.005	-0.003
3	1231.59	84.718	0.439	1294.28	1207.48	6.103	0.099
4	1422.55	82.592	0.252	1430.26	1418.69	0.955	0.009
5	1611.58	82.748	0.128	1617.37	1609.65	0.631	0.005
6	1710.92	83.695	0.137	1726.35	1721.53	0.371	0.002
7	2924.18	82.715	2.186	3008.09	2874.03	10.053	0.559
8	3326.35	83.529	0.007	3328.28	3318.64	0.753	0

Figure 5: FT-IR Spectra for Hisbiscus sabdariffa yeast 2 Natural fermentation Day 9.

Discussion

This study investigates fermentation potentials of *C. limon* and *H. sabdariffa* yeast isolates using standard protocols. The result of yeasts morphology demonstrated cylindrical, slender and spherical yeast cells. Fruits, vegetable, drinks and other agricultural products are very important microhabitats for different yeast species[10]. The presence of the different yeast isolates in the plant samples further supports the use of plants as source of yeast for fermentation process.[11] reported the occurrence of

Saccharomyces cerevsiae in *Cocos nucifera, Dimocarpus longan* spp, *Annona muricata, Bambusa vulgaris, Salacca zalacca,* and *Mangifera indica.* Yeasts of plants origin are found very useful industrially in a wide range of fermentation processes: medicinally as a source of B-complex vitamins and as a stage in the production of various antibiotics and steroid hormones and as feed and foodstuffs. Yeasts are very common in the environment, and are often isolated from sugar-rich material such as naturally occurring yeasts on the skins of fruits and berries (such as grapes, apples), and exudates from plants (such as plant saps or cacti)[12, 13].

The standardized fermentation showed significant change in pH (α=0.05>p 0.00).[8] reported that, pH and temperature have significant effect on the yield of ethanol with time at 5% level of significance. The FT-IR profiles suggest the presence of alcohol in the fermented plant juices. Alcoholic fermentation (ethanol fermentation) ensures the conversion of sugars such as glucose fructose and sucrose into cellular energy and thereby produces ethanol and carbon dioxide as metabolic waste products. Alcoholic fermentation occurs in the production of alcoholic beverages and ethanol fuel and in the rising of bread dough[11]. According to [10] all ethanol contained in alcoholic beverages (including ethanol produced by carbonic maceration) is produced by means of fermentation induced by yeast. Yeast fermentation of various carbohydrate products could be used to produce the ethanol that is added to gasoline.

CONCLUSION

This study reveals the presence of morphological wild yeasts in *C. limon* and *H. sabdariffa* plants. The wild yeast isolates have promising potentials in fermenting *C. limon* and *H. sabdariffa* juices to produce alcohol.

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turned pink that may persists for 10 – 30 seconds.

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