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COMPARATIVE STUDY AND CHARACTERIZATION OF NURUK (STARTER CULTURE) AND BAKERS YEAST USED IN THE PRODUCTION OF LOCAL RICE BEER

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ABSTRACT: Nuruk is a fermentation agent which has been used for the production of traditional alcoholic beverages in korea. The aim of the present study was isolation and molecular identification of microorganisms in nuruk (starter culture) and starter yeast for the production of rice beer. The isolation was done by diluting the sample in a 9-fold dilution. The colony forming units of the pour plate were 8x104cfu/ml. The identified organism were Sacchromyces cerevisiae YJM1592 (Accession ID-Cp006433.1), Rhizopus microspores vsoligosporous ATCC (Accession ID- KU729104.1), Rhizopus microsporus ATH64 (Accession ID-KF709990.1), Rhizopus microsporous (Accession ID-MN238825.1), Rhizopus oryzae strain (Accession ID-CBS126971.1), Sacchromyces cerevisiae IFM 40211 (Accession ID LC413772 and uncultured fungus clone CMH146 (Accession ID-ICF80000237.1) identified by phylogenetic inference based on an internal transcribed space 2 region sequence analysis. The alcohol content in rice beer containing nuruk was 5.0 % and rice beer containing nuruk-additional yeast was 5.5%, respectively. The major free sugar detected in all samples were glucose (2.5%.) The sensory analysis done determine that p-value <0.05 considered to be significant. The proximate analyses were taken, the highest value was increased by moisture content at 90.0000% in control and the lowest value was determined in fat content at 0.05% in rice beer containing nuruk.

Key word: Nuruk; Bakers yeast; local rice; fermentation; DNA extraction; sequence

I.INTRODUCTION

Nuruk is a traditional Korean fermented starter . Nigeria has been faced with a general economic breakdown of over dependence off the nation on foreign goods. This has led to the problem of economic recession, general inflation and high rate of contra bounds goods. An apicultrist stated that the value of the beer industry in Nigeria is 36.8 million naira and has a Iot of prospects to turn the industrial sector around. This research is designed to explore means by which rice drink can be produced in Nigeria by using a natural culture and bakers yeast which in turn will reduce over dependence on imported. It will also rebuke the cost of purchasing wines, stout at a high cost based on currency exchange rate. The current aim of this study was to produce rice beer from traditional local rice using Nuruk as a starter culture.

II.MATERIAL AND METHODS

The sample nuruk (starter culture) were collected from market seller at south korea in asia, while local rice was collected from abakpa and ogbete main market, Enugu state Nigeria. The starter culture which was collected from the market seller at south korea was directly brought to the laboratory for microbiological molecular characterization and fermentation of local rice, the rice been produced and its proximate and sensory evaluation. The research was done at microbiology laboratory in the department of applied microbiology and brewing science in faculty of natural science, Enugu State University of science and technology, enugu Nigeria.

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Preparation of media for serial dilution

The media broth were used for inoculation of nuruk (starter culture) in a serial dilution method which contains 1g of the sample nuruk was added into (10^{-1}) using micropipette, 1ml of the solution was transferred into a second test tube (10^{-2}) (Ezigbo et al., 2014).

While 250mls of SDA and PDA were also prepared for pour plate and sub culture. The media were sterilized using autoclave at 121°c for15 minutes. The serial dilution was been inoculated in the media by pour plating in triplicate (Harigan, 1998) at aseptic conditions. The plates were moved in figure eight shape in other to allow the media and nuruk mixed properly. The SDA plates were placed at 30°c 1-3 days while the PDA plates were placed at 28°c for 2-5 days.

Preparation of pure cultures

After good growth of fungi and yeast colonies, distinct colonies and different colors were purified on sabourand dextrose agar and potato dextrose agar by streaking and dot method. The plates for yeast growth were incubator at 37°c for 1-3 days while fungal plates were allow growing at 28°c for 2-5 days in the incubator

Slide Culture Technique for Fungal Identification

Culture preparation

The petri dish containing V-shaped rod slide and coverslips was sterilized

The sterile slide was then placed on top of the V-shaped glass rod using a sterile forcep.

A sterile scalpel blade was used to cut out SDA agar block about 1cm square and the agar block was placed on top of the slide using inoculating loop or sterile scalpel blade.

The fungus under investigation was inoculated at the four sides of the agar block.

Using a sterilized forceps, the sterile coverslip was picked and placed gently on the inoculated agar block. About two millileter of the sterile distilled water was poured aseptically into the plate to provide humid environment suitable for fungal growth.

The plate was incubated at 25-28°c for at least four days to allow growth and sporulation to occur undisturbed (Ogbo Frank, 2005).

Mounting of growth for slide culture preparation:

After four (4) days, the coverslip was gently removed with sterile forceps and the slide with growth was turned to face upwards. The agar block was gently removed with inoculating needle without disturbing the growth on the slide and the block discarded into a disfectant jar to avoid spreading the spores on the block in the lab.

Water bubbles on the slide and cover slip within the area of fungal growth were removed by dropping 70% ethanol to dry them up. A new clean slide and a new coverslip were used to mount the growth in the coverslip and slide respectively using lactophenol cotton blue.

Pure isolates were placed on SDA and PDA slants and stored at 4°c until needed.

After removing excess mounting fluid on the slides as well as on the coverslip was observed under the microscope to obtain two conventional slide mounts.

Quality Control

All samples were collected following standard operating procedures. The sterility of culture media was ensured by incubating some of the prepared media at 28°c and 30°c. Each batch of media preparation, performances of all prepared media were also checked by inoculating pure cultures properly.

Preparation of Rice for fermentation

10002.75g of local rice was weighed out (5 cups), sorted to remove unwanted ones, after which were dehulled, washed and need to go beyond standard rice washing, continuously filling up the bowl with water, stirring with your hand, and dumping out the dirty water for best result, do this up to 8times.

Steam the Rice

Steam until rice is slightly yellow. Stir the rice and cook it evenly. Sterilize or boil the cheese cloth or the bamboo basket in water to avoid contaminat. The rice was removed and allowed to cool, sieve and spread over a sterile foil and it was allowed to cool and air dry for 3-4 hours because of the internal temperature will be higher than the outer sections.

Fermentation of the local rice

The earthen pot was washed thoroughly, rinsed with purified water and then sterilized with bursen burner. After air –dry of the boiled rice, the use of sterile forcep to scrap out the rice was done carefully and it was been transfer into

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earthen pot filled with sterile 5cups of water. nuruk powdered which was weighed out, moisten with sterile water and create a paste with baker`s yeast all mixed together was added into the earthen pot containing prepared rice with sterile water. the nuruk (starter culture) serves to break down starch present in the ferment so it is accessible for fermentation via the added yeast. Sterile cheesecloth was placed over the mouth of the earthen pot and held in place with a rubber band. The ferment was stored at room temperature and it is essential to avoid dramatic temperature changes and drafts. The cheesecloth will prevent insects from flying and dying, but also allow for the mixture to breath. Exposure to light especially UV radiation was avoided. The ferment was stirred once a day with a sterilized spoon or turn garri. The pH and temperature of the fermentation was monitored over the next seven days.

pH range and temperature determination was carried out for local rice fermentation

Genomic DNA Isolation

Genomic DNA from the isolates was extracted using Zymo Research Quick-DNA Fungal/Bacteria Miniprep kit (cat. D6005). 1000µl of 2 (two) days fungal isolate broth culture of the samples was transferred into a well labelled 1.5ml microcentrifugetubes respectively and centrifuged at 14,000 rpm for 5 minutes to collect the fungal cells. 100 mg (wet weight) of the fungal cells resuspended in 200 µl of water and added to a ZR Bashing Bead lysis tube. 750 µl of BashingBead buffer was then added to the tubes. The tubes were vortexed for 20 minutes to disrupt the cells. After which the tubes were placed in a microcentrifuge and centrifuge at 10,000 rpm for 1 minute. 400 µl of the supernatant was transferred into a Zymo-Spin III-F filter in collection tubes and centrifuged at 8,000 rpm for 1 minute. 1,200 µl of genomic lysis buffer was added to the filtrate in the collection tubes. (for optimal performance, beta mercaptoethanol was added to the genomic lysis buffer to a final dilution of 0.5 % v/v). 800 µl of the mixture was transferred to a Zymo-Spin IIC columns in collection tubes and centrifuged at 10,000 rpm for 1 minute. The flow through was discarded from the collection tubes and then the step was repeated. 200 µl of DNA Pre-Wash buffer was added to the Zymo-Spin IIC columns in new collection tubes and centrifuged at 10,000 rpm for 1 minute. 500 µl g-DNA Wash buffer was then added to the Zymo-Spin IIC columns and centrifuged at 10,000 rpm for 1 minute. The Zymo-Spin IIC columns were transferred to a clean well labelled 1.5ml microcentrifugetubes and 100 µl of DNA elution buffer was added directly to the columns' matrix and centrifuged at 10,000 rpm for 30 seconds to elute the DNA.

Polymerase Chain Reaction for fungal DNA (ITS)

The primer was prepared with loading dye from the company, so it doesn't need loading dye anymore. Twenty-three microliter of the primer in PCR bead and then 2 microliter of the DNA template were amplified in the thermal cycler (PCR) set at the program .All amplification reactions were performed in a GeneAmp® PCR System 9700, Applied Biosystems for 35cycles as follow: Initial denaturation step of 95°C for 2 minutes and 30 seconds, denaturation step of 30 seconds at 95 °C, annealing step of 56 °C for 30 seconds, extension step of 72 °C for 30 seconds and initial extension step of 72 °C for 10 minutes, final extension step 4-12°C forever.

Primer	Sequence
ITS1	TGTAAAACGACGGCCAGTCCGTAGGTGAACCTGCGG fungi cocktail
ITS4	CAGGAAACAGCTATGACTCCTCCGCTTATTGATATGC fungi cocktail

Sequencing was performed using Genetic Analyser.

Statistical analysis

Data was analyzed using SPSS version 20 (SPSS Inc., Chicago, IL). Student t- test was used to ascertain the significance of differences between mean value of two continuous variables. To determine the differences between the aroma, tatse, texture and mouth feel. It was performed to determine the proximate analysis of the food component of the local rice beer produced.

III.Result

The samples collected were inoculated in the culture plates containing 2 different media. the isolate from nuruk (starter culture) on different media yielded several organisms and were counted. In table 1 showed the microbial mean count

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of the isolate from nuruk (starter culture) counting the colonies identified using the colony forming unit/ml (cfu/ml). table 2 showed the macroscopic, microscopic features of the isolate and its identification using the slide culture techniques listed in table 2. The isolates that were identified are ; *Rhizopus oryzae, Rhizopus azygosporus, Rhizopus microspoues* and *Sacchromyces cerevisiae, uncultured clone.*The proximate analysis of rice beer containing :(local rice/nuruk/starter yeast and local rice /nuruk with its control local rice) were showed in figure 1. The alcohol content and sugar level were also determined showed in figure 1. Sensory analysis of the rice beer produced were evaluated to determine the aroma, mouth feel, taste and appearance showed in figure 2. Different fermentation variables such as pH and temperature were taken and showed in figure 3 and 4 respectively.sequence of the amplified DNA were showed in figure 5 which identified the isolated organism in its nucleotide that make up the DNA.

Table no 1 Microbial mean count of the isolate

SAMPLE ISOLATE	NO OF ISOLATES	CFU/ML (COLONY COUNT)
SDA 1	80	$8x\ 10^4$

Table no 1 showed the microbial mean count of the isolate 80 in the counted colonies by calculation of $8x10^4$ cfu/ml (colony formimg unit per milliliters. It was only one plate that showed positive of the colony counted.

Table no 2 Shows colonial charcteristics of the isolates

SAMPLE ISOLATE	COLOR	TEXTURE	SHAPE	ELEVATION	LACTOPHENOL STAIN	**PROBABLE IDENTITY OF ISOLATESS**
SDA 1	Whitish / cream	Smooth	Circular	Raised	Blast conidia, budding sac	***Sacchromyes Sp.***
SDA 2	Whitish / brown grey	Rough	Spread / covered	Raised	Collumela, rhizoid, umbrella, like structure	***Aspergillus Sp.***
SDA 3	Whitish brown	Hairly	Irregular	Flat	Sporangium, sporangia, rhizoid	***Rhizopus oryzae***
PDA 1	White cottony / brown	Fairly floss	Irregular	Flat	Smooth walled, collumela, and rhizoid singly from nodes	***Rhizopus. Sp.***
PDA 2	Dark brown	Hairly	Irregular	Flat	Simple rhizoid, collumella,	***Rhizopus. Sp***

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					sporangiosphore, sporangium	
PDA 3	White cottony to grey black	Fairly floss	spread	Flat	Sporangiospore, smooth walled and ovoid	***Rhizopus. Sp***
PDA 4	Cream	Smooth	Circular	Raised	Blast conidia , sac	***Sacchromyces. Sp.***

Table no 2 showed the result of the colony morphology of the isolates, its color characterization, texture, shape and probable organisms in all the samples was determine after dot method preparation to achieved a pure culture.

Table no 3 Sequence result

Sequence Accession ID	Identities in Percentage (%)	Sequence Identified
KF709990	87%	*Rhizopus microsporus ATH58*
KU729104	97%	*Rhizopus microsporous vs oligosporous ATCC*
MH864361	97%	*Rhizopus oryzae CBS 126971*
CP006433	99%	*Sacchromyces cerevisiae YJM1592*
LC413772	97%	*Sacchromyces cerevisaie IFM 40211*
KF800237	99%	*Uncultured fungus clone CMH146*
MN2388251	78%	*Rhizopus microsporous*

Table no 3 showed the sequence identification of the isolates with its accession number and percentage. CP006433, 99% and *Sacchromyces cerevisiae* YJMI592, KF709990, 87% and *Rhizopus microspores* ATH58, KU729104, 97%, *Rhizopus microsporous vs oligosporous* ATCC, MH864361 97%, *Rhizopus oryzae* CBS 126971, LC413772 97%, *Sacchromyces cerevisaie* IFM 40211, KF800237 99%, uncultured fungus clone CMH146, MN2388251 78%, *Rhizopus microspores* respectively shows the identification of the identified and its character which was compared against those sequence available in the genbank database using the blast program.

Figure no 1: PCR result on gel electrophoresis

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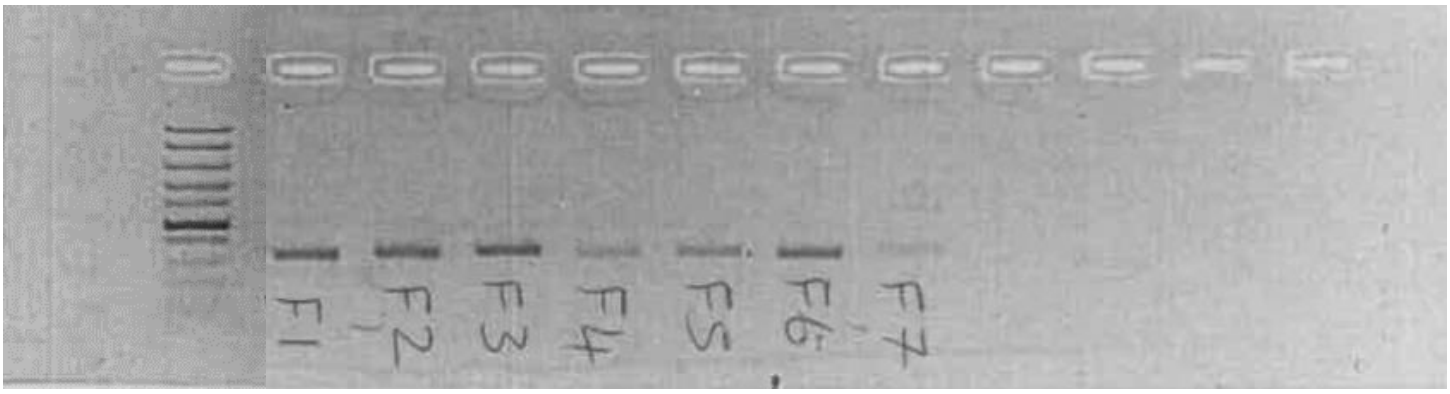

figure no 1 showed the PCR result on gel electrophoresis of DNA application. The amplified PCR product were electrophorizied using agarols gel and the DNA band that showed the estimated size was eluted from gel and purified. The first band showed the molecular marker (ladder) while the 1-7 bands in the second lane to the end shows the amplified band of each sample of DNA extracted. The bands produced are from range 400-650base pair.

Figure no 2: Proximate content of local rice beer

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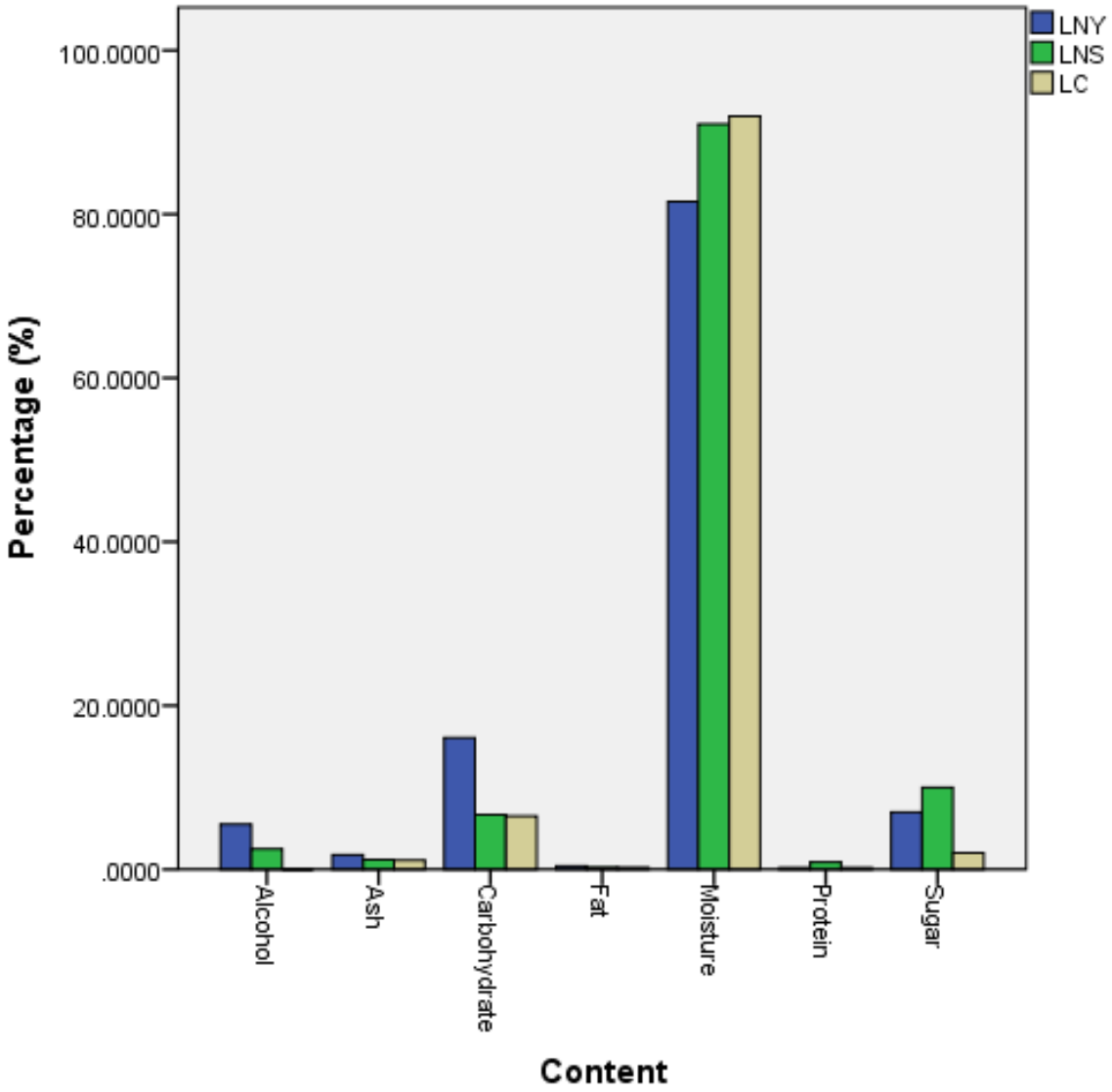

Figure no 2 LNY = Local rice /bakers yeast/nuruk/. LNS = Local rice / nuruk. LC= Local rice control. The proximate content of local rice beer in nuruk/bakers yeast in carbohydrate 16.065%, ash content 1.763%, fat content 0.412%, moisture 75.000%, protein content 0.003%, sugar content 7%, alcohol content 5.5%. the local rice beer with nuruk only showed carbohydrate content 7.0322%, ash content 1.184%, fat content 0.253%, protein content 1.10%, moisture content 95, 000%, sugar 9%, alcohol content 3.5%. the plain control local rice in water showed moisture 97.000%, ash content 1.133%, fat content 0.01%, carbohydrate 6.68%, sugar 2%. This proof proof that local rice produced with nuruk/ bakes yeasthas the highest value compared with local rice beer produce with nuruk only.

Figure no 3: Sensory analysis of local rice beer

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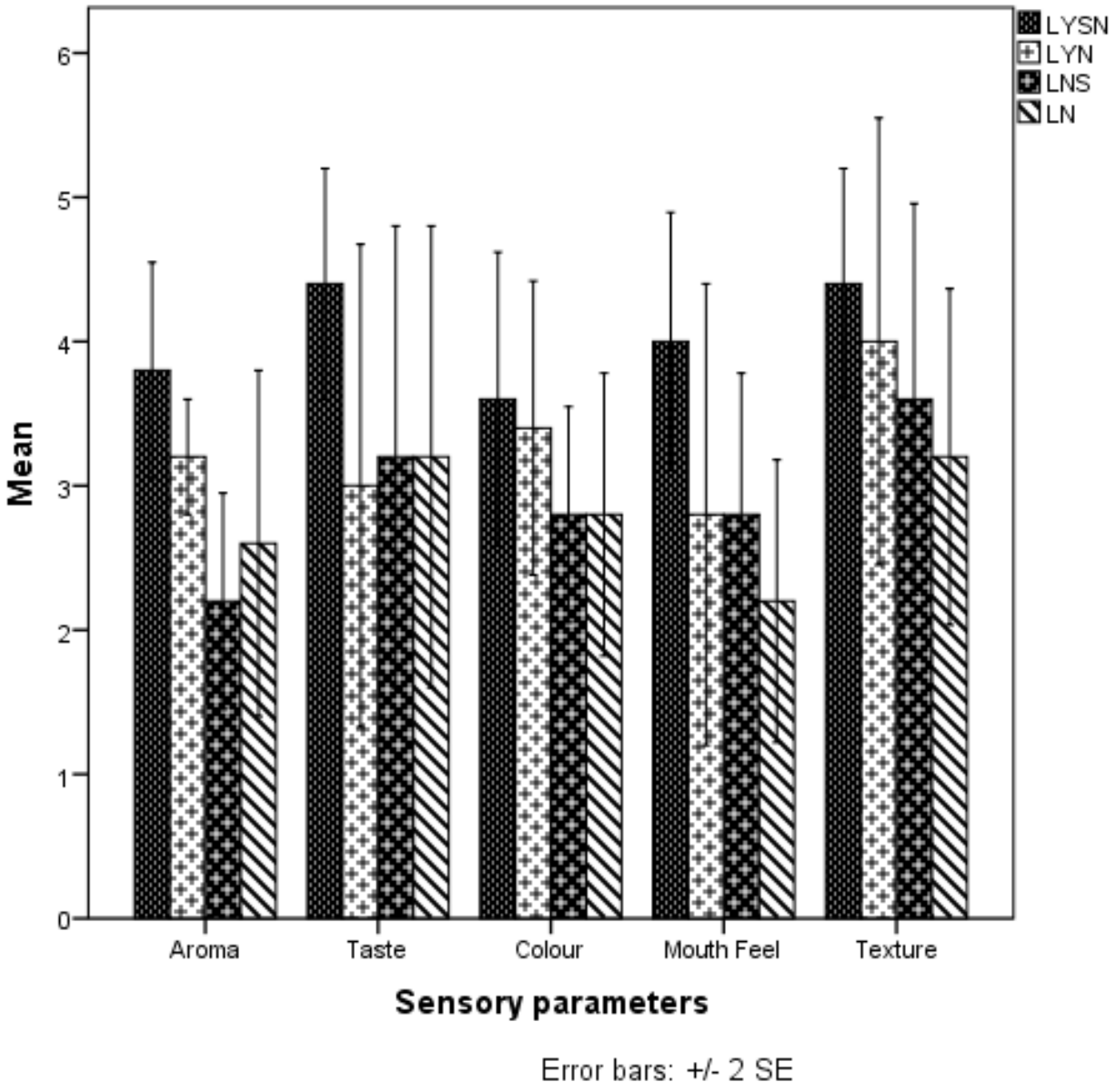

Figure no 3 shoewd LYNS = Local rice /bakers yeast/nuruk/sugar. LYN = Local rice /bakers l yeast/ nuruk. LNS = Local rice /nuruk/sugar. LN = Local rice control. The sensory parameters of 20 people in a panelist with scored analysis showed the presence of nuruk / bakers yeast with added sugar in the beer indicated the taste is 4.3, aroma 3.8, colour 3.6, mouth feel 4.00, texture 4.3 shows highly acceptable, while the one without sugar in fermented local rice beer with nuruk / bakers yeast showed aroma 3.2, taste 2.9, colour 3.3, mouth feel 2.5, texture 3.5 not acceptable. The fermented local rice produce with nuruk only added sugar after showed aroma 2.5, taste 3.2, colour 2.9, mouth feel 2.9, texture 3.3 highly acceptable, while the nuruk fermented local rice sugar –free showed

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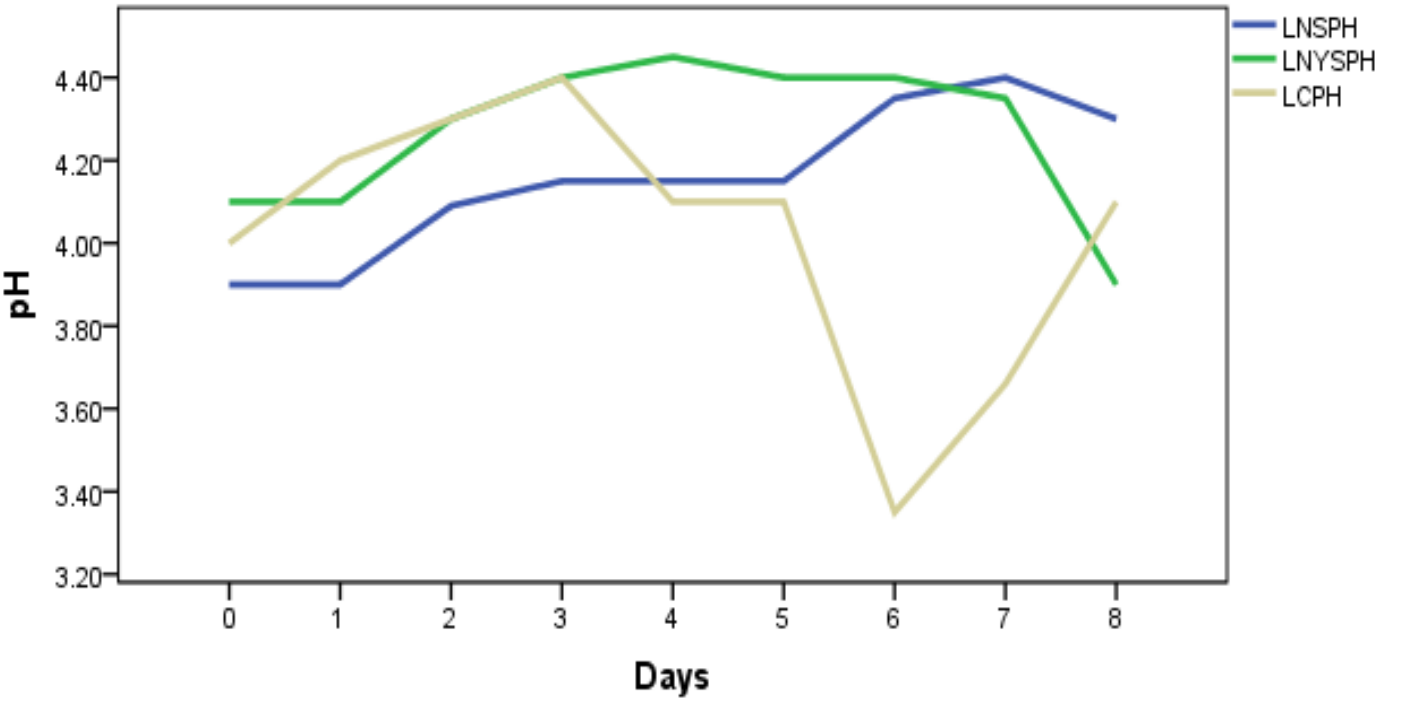
aroma 2.5, taste 3.3, colour 2.9, mouth feel 2.1, and texture 3.1 slightly acceptable.

Figure no 4: pH varia



tion of local rice beer during fermentation period

Table no 4 showed LNSPH = Local rice/ nuruks sample/ pH, LNYSPH =local rice / nuruk/ bakers yeast/pH, LCPH = local rice control pH. A sharp decrease in day 1 (one) was noted, after 24 hours, fermented local rice nuruk and bakers yeast increase to 4.10-3.80, while the fermented local rice with nuruk only increased at 4.40 Ph. The plain rice act as a control fall in range of 3.40-4.10.

Figure no 5: Temperature variation of local rice beer during fermentation period

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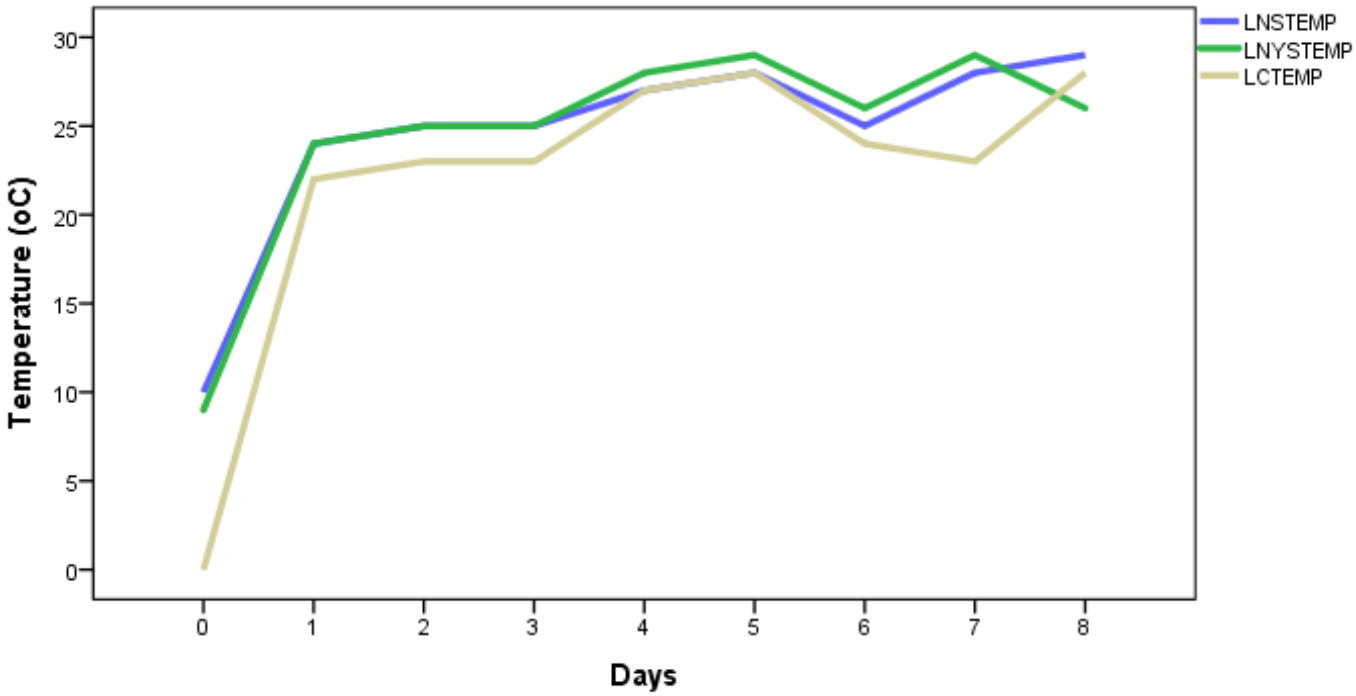

Figure no 5 showed LNSTEMP = local rice / nuruk/temperature, NYSTEMP = Local rice / bakers yeast/sample temperature , LCTEMP = local rice control temperature.the temperature during fermentation period and its variation ranged from 10-28°c. Local fermented rice nuruk/ bakers yeast has the highest at 28°c, while the local rice fermented with nuruk only has slightly different of 26°c. the temperature of the control does not chsnge much 18°c.

IV.Discussion

The nuruk (starter culture) is a wild fermentation starter used to brew rice and makegolli. It breaks down starches (amylase and amyglopectin) into simpler sugar then converts those sugars into alcohol;, and Co_2. It is accessible for fermentation via the added starter yeast. Fermentation produce a harmonious blend of tastes and color due to the sugar. It was steeped in water for eight (8) during which there was a change in (color turn from whitish creamy) of the physical appearance of the rice (Adam et al., 2018). The nuruk fermentation was complete with help of stirring every day to oxygenate the brew (aerobic fermentation) by encourage yeast colony to replicate after stirring for more than 3 (three) days, the brew will transit to an alcohol production phase (aerobic fermentation). It is also occured with the finding by Kim Jae et al; 2014 that fermentation is excellent thereby required oxygenation and replication process.

Various fungal species that were isolated from nuruk (starter culture) include; *Rhizopus species, uncultured fungus clone* and *Sacchromyces cerevisiae* as sh*own* in table 2. This organisms has be reported on the previous works done by early researcher like (Jin etal., 2011). The fermentation was carried out accordingly to the findings (Kim Jae et al., 2014), that nuruk produce enzyme know as amylase which break down to produce carbonation in addition of yeast for Co_2 and sweet taste, while the fat produced contain lipase which is amino acid that help to produce savory taste and protease helps to yield aroma. The rice beer consists of two steps fermentation which are the starch to sugar, sugar to enzymes plus sugar = alcohol via yeast (Kim Jae et al., 2014). The nuruk fermentation was complete with the help of stirring every day to oxygenate the brew (aerobic fermentation) by encourage yeast colony to replicate after stirring for more than three

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(3), the brew will transition to an alcohol production phase (anaerobic fermentation). It is also occurred with the finding by Kim Jae et al., 2014 that fermentation is exothermic thereby required oxygenation and replication process.

The sensory evaluation was carried out by 20 (twenty) people in a panelist which showed in figure 2. The score sheet analysis showed the presence of nuruk with additional yeast; the taste is like sweet, partly alcohol while the aroma are like non –fermented palm wine, the color looks like milk (barley). The addition of sugar after brew indicate sweet taste while no additional of sugar indicate sour taste, slightly acceptable. The nuruk without additional yeast, no sugar, has mixture of sweetness and sourness, aroma slightly unacceptable. The nuruk without additional yeast, sugar present ; the taste is like best milk, aroma freshly taped palmwine, sweet from mouth down to throat. It occurred with the findings use back-sweetening as a choice- by (Lee et al., 2007). During the fermentation, some biochemical reaction change occured producing acids as show in figure 3. The pH values of the sample and control were similar throughout the 8 (eight) day fermentation period. A sharp decrease in pH on day 1 of the fermentation was noted in local rice beer- nuruk which increased at 4.40 followed by local rice- nuruk- additional yeast fall with the range of 4.10-3.80. The final pH value of local rice soaked in water as a control fall with the range of 3.4 – 4.10 which were showed in figure 2 similar early researches on (Lee, et al; 2010).

The alcohol content of the local rice beer on the final day of the fermentation period was 3.5% in local rice- nuruk and 5.5% in local rice beer- nuruk- additional yeast respectively in figure 4. This values fall within the range of 1 – 18% alcohol content given by Jin et al; (2008). It is one of the factors that affect the quality of nuruk and can be used to show the degree of fermentation throughout the fermentation period. Starch is converted to glucose by hydrolysis throughout the fermentation process therefore glucose is used by yeast to produce alcohol and Co_2. Thereby, formation of bubble can also be seen throughout the process.

The free sugar contents of the local rice beer- nuruk and local rice beer- nuruk- additional yeast with its control local rice- water are displayed in figure 3. The major free sugar detects in both the control and the samples were glucose. Glucose significantly decreased after the 8(right) day of fermentation period. Glucose plays an important role in fermentation as its production from starch allows for the production of alcohol in rice beer. Glucose is formed through the hydrolysis of starch by amylases in the nuruk. Therefore, through a-amylase, glucose increases in rice beer. Glucose was also found to be a major sugar in a study by Park et al; (2009). Fructose, glucose and maltose were found to be the major reducing sugars used by yeast to create alcohol and the reduction of these sugar was found to give rice beer its particular taste.

Variation in temperature during the fermentation period of local rice beer was also noted. The temperature variation ranged from 10^0c – 28^0c. the local fermented rice beer – nuruk- additional yeast has the highest temperature at 28^0c, while the local fermented rice beer- nuruk has the slight different of 26^0c as showed in figure 4. The rise in temperature is attributed to liberation of carbon dioxide by the fermenting organisms. This might be as a result of the exerted pressure on the substrate (Ojimelukwe et al; 2011). The rise in temperature indicates that fermentation reaction is exothermic which changes being due to metabolic activities of microorganisms (Achi, 2005).

The nutritional values (moisture, fats, proteins, ash, fibre and carbohydrates) were determined in this research work. The moisture content of local rice beer sample ranged from 80% - 97%. Local rice control- water had the highest value, followed by local rice fermented with nuruk and local rice fermented with nuruk- additional yeast had the least moisture content. Increment in temperature of the local rice beer and decomposition of the fermenting microorganisms on the product as product by Omefuvbe, et al; (2004).

The fat content of the rice beer sample, range from 0.001-0.003%. The local rice –nuruk-additional yeast had the least fat content as 0.412% followed by the local rice-nuruk at 0.253%.while the fresh local rice –water as a control had the least fat content. Reduction in fat content shows that starter culture is better and capable of breaking down fat contents in finding with (lee et al., 2005). The ash content of the samples were given as follows (local rice-nuruk=1.763% had the highest value followed by the local

rice-nuruk =1.184% and the control = 1.133% known as the least value. Increase in mineral components shows that mineral nutrients are essential and necessary health benefits.

Carbohydrate content of various rice beer was compared and values are following; the local rice beer- nuruk-additional yeast had the highest value as 16.065%, followed by local rice beer-nuruk 7.0322% while the control had the least value as 6.681%. This showed that carbohydrate content decrease with increase in fermentation period (Parkoudaet et al., 2009).

V.Conclusion

Traditional fermented foods and beverages have been an important part of our live as they have been utilized all over the world. In this study, local rice beer indicate a distinctive fermented rice product owing to the unique The metabolic substances produced during fermentation have proven nutraceatical activities. Local rice beer shown to have various physiological effects including anti- diabetics, Probiotic and anti- cancer activities such positive health properties lend credence to the view that local rice beer is a valuable food and warrants further laboratory in the production of the nuruk (starter culture) and purification multi species organisms and natural cereal grains used as the starter culture driving production.

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