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YEAST IN SOUTHWEST MONSOON RAINWATER

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ABSTRACT

Strong evidence of the presence of bacteria and fungi in the tropospheric boundary layer is available in the literature. We report successful isolation of unique morphotypes of wild ascomycetous yeasts from rainwater samples collected directly in sterile containers, taking extreme care to avoid ambient contamination. Direct and quick visualization of fresh rainwater samples under a phase contrast microscope indicated the sporadic presence of yeast cells. Further confirmation of the presence of yeast was obtained by plating of rainwater on a medium with antibiotics to generate pure colonies. We described their characteristics while molecular identification revealed it as *Candida tropicalis*. Yeast species could contribute valuable knowledge about yeast transportation in the atmosphere. However, knowledge is insufficient about the yeast deposited from the atmosphere and its transportation across the atmosphere. We report and discuss these interesting and exciting results which are useful in understanding the microbiological dimension of meteorology and the southwest monsoon rainfall in the light of present discourse on global warming and climate change. We offer a tentative model for a possible source, role, and fate of the yeasts in rainwater.

Keywords: *Candida tropicalis*, molecular identification, PCR, sequencing, rainwater microbial

INTRODUCTION

The period June to September in India is considered the southwest monsoon period. It is the main rainy season of the Indian subcontinent. The whole country receives 75% of its rain in this season. The southwest monsoon originates in the general circulation of the atmosphere which is caused by a region of high pressure over the south Indian ocean and a region of low pressure which extends over the whole of central Asia (**Simpson. *et al*., 1921**). A controversial theory exists about the origin of the southwest monsoon. Many geologists believe that the monsoon first arose around 8 million years ago based on records from the Arabian sea and the record of wind-blown dust in the loess plateau of China (**Tambe**). The southwest monsoon first hit Kerala from the Arabian sea, which is a branch of the southwest monsoon and moves towards the northern region of India along the its western coast.

Nucleation of cloud

Aerosols are an important factor of climate, directly scattering light, seeding warm clouds, and seeding ice-clouds. (**Brooks, 2019**). A marine aerosol population transits into cloud droplets and ice crystals. Aerosol rises from the marine environment and from continents, are formedby the gas phase (e.g.. DMS) emissionand from primary particles.

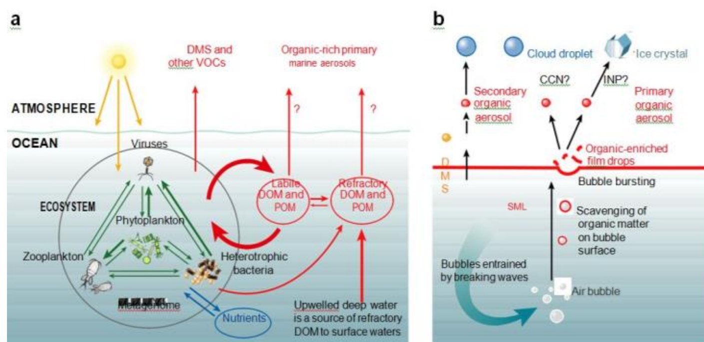


Figure 1 Formation of marine aerosol and cloud nucleation. (Adapted from **Brooks et al., 2018**)

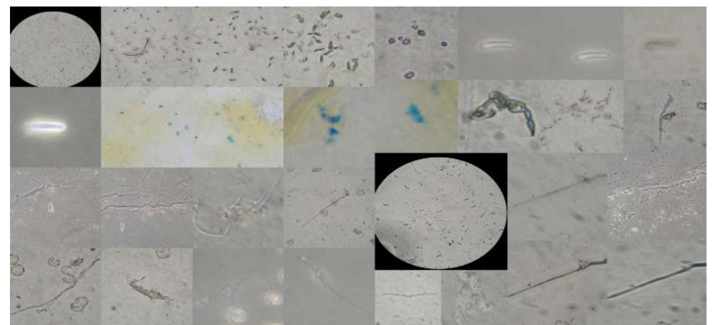


Figure 2 Microbial forms recovered from rainwater samples showed diverse cell morphologies- Coccoid, Bacilloid bacterial cells; cyanobacteria and numerous fungal spores and hyphae- all these are getting a piggy-back ride in monsoon clouds and landing with rainfall. (**Kamat. N. *et al*, 2014**)

The composition of micro-organisms in the atmosphere, in the clouds or rainwater is not well defined yet. Though many studies of bacterial communities have been performed. Bacterial strains were isolated from the cloud and their role in the forming the chemical composition of the atmosphere was studied (**Tina *et al*, 2015**). **Morris. *et al*., (2008**) suggested atmospheric dispersal may play an important role in bacterial biogeography patterns and later expressed bacteria in active form from an inactive state in a suitable environment. Fog and cloud provide protection to bacterial strains from ultraviolet rays and the provide nutrients for their survival ship (**Tina. *et al*, 2015**). Clouds help microbial transportation over a long distances (**Griffin., 2006**). **Baurer. *et al*, 2002** isolated bacterial and fungal spores from cloud water and tried to study the role of microbes in the contribution of carbon content. Strong evidence of the presence of bacteria and fungi in the tropospheric boundary layer, using dicarboxylic acid as a nutrient (**Amyot. *et al*, 2002**). The yeast biota in rain is less studied, so this study may help address an important gap in the study of yeast and its occurrence and ecological role in the atmosphere. An attempt has been made to collect a rainwater sample, isolation of microbeand to identify the isolates. The isolate is known to be *Candida tropicalis* after molecular identification.

C. tropicalis was considered yeast by **Kurtzman and Fell** (**2000**). Its habitat is quite broad and it can be isolated from various substrates and samples (bark, roots, leaves, gut, marine sediments, mud, waters, skin etc.). *C. tropicalis* is classed as fungi in the order of sachromycetales, and in the family of debaryomycetaceae. It is a common pathogen and can infect individuals with low immunity. Moreover this species is also reported in Kawasaki disease in Japan, which spreads from China as a windborne agent.Recently it has been found that *C. tropicalis* is of

biological and biotechnological importance (**Zhenming, 2010**). Numerous studies of *C. tropicalis* have been carried out, including substrate inhibition of phenol oxidation (**Stephenson, 1990**), a protoplast fusion technique (**Chang *et al*., 1995**) and degradation of phenol in the presence of other derivatives (**Komarkova *et al*., 2003**). Xylose as the sole carbon source can be produced by *C. tropicalis* . It has been employed to convert corncob hydrolysate to xylitol (**Cheng *et al*., 2009; Rao *et al*., 2006**). Strains of *C. tropicalis* have been used in the removal and recovery of zirconium (**Akhtar *et al*., 2008**). Differing *C. tropicalis* strains from a variety of marine environments have been proven for their potential applications in agriculture, in the fermentation industry and in the chemical industry. However, currently little is known about the distribution and diversity of *C. tropicalis* in various marine, soil, and air environments across the globe.
Zhenming (**2010**) studied the distribution and diversity of *C. tropicalis* strains from multiple marine environments and discussed the relevant potential applications in China. The marine yeast strains of *C. tropicalis* have many potential applications in biodiesel production (**Meng *et al*., 2009**), bioremediation (**Ukrit *et al*., 2009; Sonali 2008; Varma *et al* Komarkova *et al*., 2003** in the production of polyol sweeteners (**Rao *et al*., 2004; Cheng *et al*., 2009; Rao *et al*., 2006**), and in ethanol fermentation (**Ukrit *et al*., 2009**). **Chaves, *et al*** (**2017**) studied and reported that a strain of *C. tropicalis* isolated from the root of the mangrove tree *Rhizophora stylosa* in Zhanjiang, China could accumulate over 50% of oil during batch cultivation from glucose and hydrolysate of cassava starch. The authors concluded that the strain can be used in biodiesel production. C. *tropicalis* has been considered an osmotolerant microorganism and this ability to survive high salt concentrations may be important for fungal persistence in saline environments. This physiological characteristic makes this species suitable for use in biotechnology processes (**Chaves M. 2017**).

Genetic characteristics

Butler *et al*. (**2009**) sequenced the genome of the diploid yeast *C. tropicalis*. **Doi *et al*.** (**1992**) reported 12 chromosomes per cell for *C. tropicalis* and stated it has a genomic size of 14.5 Mb, containing 6,258 genes encoding proteins and a guanine-cytosine content of 33.1%. The number of chromosomes is not known with precision. Prior to that *C. tropicalis* was considered as an asexual yeast, while some studies have reported that mating between diploid cells a and α, generating a/α tetraploid cells may occur (**Porman *et al*., 2011; Xie *et al*., 2012; Seervai *et al*., 2013**). Researchers have suggested that the cause of cells changing from white to opaque could be mating, which is regulated by colony phenotypic switching. **Seervai *et al*.** (**2013**) reported that strains of *C. tropicalis* can be induced to undergo a parasexual cycle without a meiotic reduction from a tetraploid state polyploidy, affecting cell gene expression and protein production (**Morrow, 2013**). *C. tropicalis* showed a reduction in ploidy and was considered a mechanism of adaptation; it may be associated with cell stress (**Bermanet *al*., 2012**). C. *tropicalis* has showed huge genetic similarity with *C. albicans* than other *Candida* species. (**Butler *et al*., 2009**).

MATERIAL AND METHODS

Assembly of sterile, dust- and contamination-free PVC containers

Pre-sterile plastic bottles were used to collect the rain water. Six times in every monsoon from 2016 to 2018.

Selection of clear vegetation-free open spaces permitting direct collection

Water was collected at Goa University campus at the same place during every sampling. The area was vegetation free and open in order to avoid contamination.

Microscopy and photomicrography

Each Raw water sample was analyzed under a light microscope with the help of a wet mount slide (bright field, dark field, phase contrast), followed by identification of sample components from morphology, optical properties, and published literature (Adapted from **Kamat, N. 2014**).

Isolation of yeast purification and maintance of pure culture on slant

Rain water samples were spread on MEA plates (containing 0.1mg/ml antibiotic) which were incubated for 48 hr at 25°C. The plates were observed under a microscope for the growth of yeast and colony morphology.

Control plates

Plates with nutrient agar and MEA (2%, with 0.1mg/ml antibiotics) were kept in the open air for one hour at the rain water collection site at the Goa university campus. Other plates were spread plated with water which was used for rinsingthe rainwater sampling apparatus. One plate with MEA (2%) was kept without spread plating rain water.

Molecular identification of strain

(By Triyat Scientific co., Nagpur,India)

DNA extraction

Cells grown in a monolayer were lysed by suspending 1-3 colonies aseptically and mixing with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and lysing the cells by repeated pipetting.
2. 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer was added.
3. The content was vortexed and the tubes were incubated for 30 minutes at 65˚C in a water bath. To minimize shearing the DNA molecules, DNA solutions were mixed by inversion.
4. The tubes were centrifuged for 20 minutes at 14,000 rpm at 10 ˚C.
5. Following centrifugation, the resulting viscous supernatant was transferred into a fresh 2 ml micro centrifuged tube without disturbing the pellet.
6. 600 µl of "B Cube" binding buffer was added to the content and mixed thoroughly by pipetting and the content was incubated at room temperature for 5 minutes.
7. 600 µl of the contents was transferred to a spin column placed in a 2 ml collection tube.
8. Contents were centrifuged for 2 minutes at 14,000 rpm and flow-through was discarded.
9. The spin column and the collection tube were reassembled then the remaining 600 µl of the lysate was transferred .
10. The contents were centrifuged for 2 minutes at 14,000 rpm and the flow-through was discarded.
11. 500 µL "B Cube" washing buffer I was added to the spin column and centrifuged at 14,000 rpm for 2 mins. The flow-through was discarded.
12. The spin column was reassembled; 500 µl "B Cube" washing buffer II was added and centrifuged at 14,000 rpm for 2 mins. The flow-through was discarded.
13. The spin column was transferred to a sterile 1.5-ml microcentrifuge tube
14. 100 µl of "B Cube" elution buffer was added to the middle of spin column.
15. The tubes were incubated for 5 minutes at room temperature and centrifuged at 6000 rpm for 1 min.
16. Steps 14 and 15 were repeated for complete elution. The buffer in the microcentrifuge tube contained the DNA.
17. DNA concentrations were measured by running aliquots on 1% agarose gel.
18. The DNA samples were stored at -20°C until further use.

PCR

This is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded.

Purification of PCR product

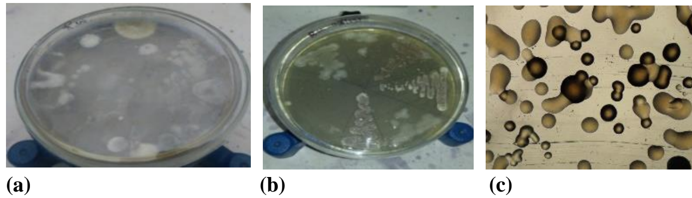
Unincorporated PCR primers were removed and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the primers. Sequencing reactions were performed using ABI PRISM®BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing (Bioinformatics)

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

A 10 to 200 ml rain water sample was collected (Fig.2), according to rain fall intensity. The rainwater was transparent and its pH temperature and TDS was recorded at 24°C, 6.92, and 9 ppm respectively. Six samples were taken from June to September. Isolation plates showed the same growth pattern of a wild colony during each sampling, on all triplicate plates. During plate observation it was noted that two dissimilar colonies had grown. The colonies were white and beige in color and their textures were rough and smooth. No growth was observed on the control plate, on which the rain water sample was not spreaded. Plates with nutrient agar kept at the sampling site showed a mixed growth of bacteria, mucor, penicilium, but yeast growth was not observed. While plates with MEA which were kept at the sampling site did not show bacterial and yeast growth, mucor and penicillium growth were observed. Molecular analysis proves isolate is *Candida tropicalis* yeast. So work which was undertaken to check that the southwest monsoon contains yeast has been proved. Further isolates were grown in the MEA media and modified agar media (prepared with agar and rainwater) and studied for their morphological change in nutrient rich (MEA) and nutrient poor media (agar prepared with rain water).Morphology variation was observed on the MEA media prepared with sterile distilled water and on the agar media prepared with sterile rain water. Colonies were grown on the agar media with rain water plate in a countable number and were very tiny, while on the MEA plates, the colony sizes were large and growth was observed. Colonies showed morphological variation grown on nutrient rich and nutrient poor media.



(a) **(b)** **(c)**

Figure 3 Wild colonies grown on isolation plate (a), Purified yeast culture (b) Magnified yeast colonies grown on MEA (c).

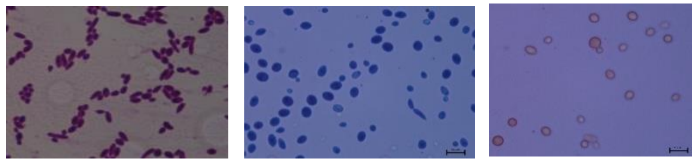


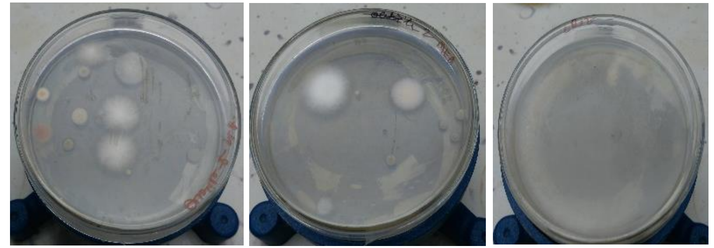
Figure 4 Microscopy images of isolate. (a) Darkly stained cells in crystal violet (b) Cells mount in lactophenol cotton blue (c) Cells stained in congo red.

Table 1 Characteristics of isolates

Strain designation	No of colony	Color	Texture
Rw1a	3	White	Rough shiny
Rw1b	2	Beige	Smooth
Rw2a	2	Beige	Smooth
Rw2b	1	White	Smooth shiny

Table 2 Rainwater isolate showed colony characteristics on MEA and modified media

	Growth Pattern	Colour	Texture	Size
Deionised water +MEA	Mat growth	White	Smooth	Large colony
Rain water+MEA	Mat growth	White	Smooth	Large colony
Rain water +Agar	Countable Colony	Gray	Slimy	Tiny colony



(a) **(b)** **(c)**

Figure 5 Control plates (a) Plate with nutrient agar kept outside for one hour, bacterial and fungal growth were observed, yeast was absent. (b) Plate with MEA (2% and antibiotics). No bacterial growth observed, mucor and pencilium were observed. No yeast growth was observed (c) Plate with MEA, spread plated withwater from sterile rinsed apparatus; no growthobserved.

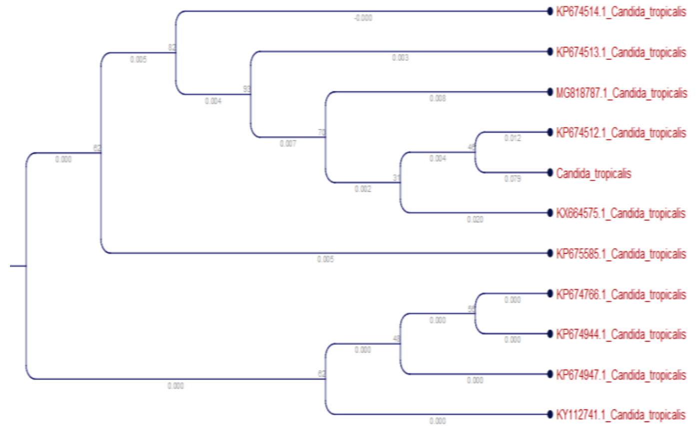


Figure 6 Phylogeny analysis of *Candida tropicalis.* Isolate showed 0.012 dissimilarity with type species *Candida tropicalis* (KP674512.1) obtained from gastric mucosa, China.

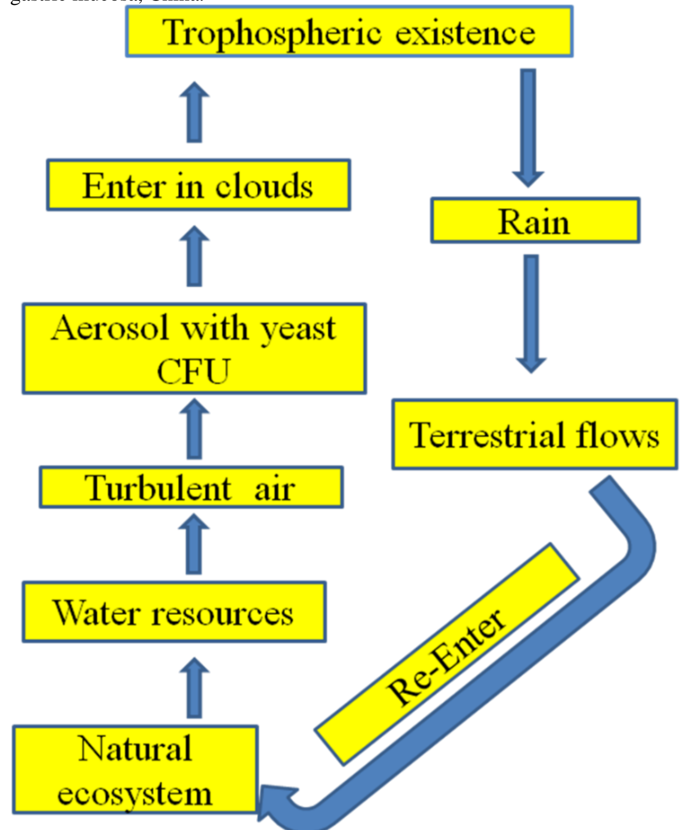


Figure 7 Postulated origin of hyper oligotrophic *Candida tropicalis* in southwest monsoon clouds

CONCLUSION

This is the first report in the world identifying yeast from southwest monsoon rainwater. *Candida tropicalis* has never been suspected as entering the southwest monsoon airborne cloud environment, therefore this is the first report of *Candida* sp. and *Candida tropicalis* specifically in rainwater. As shown in fig.7 it could enter the turbulent air from terrestrial disturbances. These properties of the strain to grow on rainwater agar without nutrients indicates that it may exist in the airborne environment as a hyper oligotrophic yeast. This has important ecological implications for the distribution of terrestrial and aquatic yeast species. Besides biometerologically, it needs to be seen whether *Candida tropicalis* contributes to cloud condensation nuclei (CCN). Moreover, it is required in the future to study southwest monsoon regions to confirm the presence of such species in rainwater. Studies are required from various southwest monsoon regions to confirm the presence of such species in rainwater. Work comprises three parts: First, the field sampling for rainwater collection, second, the microbiology for yeast isolation and purification, and third the molecular analysis for identification of species. Rainwater samples were collected in pre-sterile plastic bottles and tightly capped during the monsoon season from June to September (3 years) on the Goa University campus. During each sampling, rain water was analyzed under the microscope and showed diverse and numerous cells of bacteria, fungal spores, and yeast cells. In this study yeasts have been isolated from rain water as previous work in our laboratory has given the sign of microbes in rain water (**Kamat, N. *et al*, 2014**). The same morphological types of wild colonies were obtained on the isolation plates. A heavy concentration of antibiotics does not allow bacteria to grow on a plate. Isolates were studied for their morphological characteristics and after purification colonies were maintained on slant. DNA extraction, PCR followed by phylogeny analysis of pure isolate was performed. Molecular analysis revealed the species as *candida tropicalis*. Further study can cover the co-relation of the yeast concentration and weather parameters. The diversity of microbes in clouds can be studied at various sea levels as well as their role in ecology in ambient environments. Their role could be investigated in the formation of cloud nucleation as it has been suggested (**Sarah *et al*., 2017**) that cloud formation initiates CCN due to the presence of atmospheric particles and microbes e.g., bacteria, fungi, and phytoplankton.

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REGULAR ARTICLE

PRODUCTION AND CHARACTERIZATION OF AN *Aspergillus niger* GS_1S_6 PHYTASE CULTIVATED IN PHYTIC ACID-RICH AGRICULTURAL SUBSTRATES

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ABSTRACT

In animal farming, improvement of growth performance through successful nutrient uptake and digestibility is critical. Phytases hydrolyze the anti-nutritive phytic acid present in grains to lesser derivatives and release the phosphorus trapped therein. Twenty-eight fungi obtained from poultry droppings, cereal-rich soils and garden soils were screened for phosphate solubilization and phytase production using Pikovskaya agar and phytase screening medium. Out of the fungi, 61% were positive for phytase producing abilities and the most proficient, isolate GS_1S_6 with a solubilization index of 121%, was identified as *Aspergillus niger* GS_1S_6. Optimum phytase production was achieved at a fermentation period of 120 h, pH 5.5, glucose, $(NH_4)_2SO_4$ using 72 hour old fungal inoculum. Mineral supplementation of natural agricultural substrates enhanced phytase production (1000U/L) the most in milled sesame seed medium. The ~31-38 KDa partially purified and characterized enzyme demonstrated optimum activities at 55ºC and pH 6.0, while cationic inclusions reduced phytase activities. The K_m and V_{max} were low (1.308 mM and 0.077 mM/mL/min) from Linear-weaver plot with increasing substrate concentration implying that *A. niger* GS_1S_6 phytase may efficiently mineralize phytic acid and therefore hold great prospect for its commercialization.

Keywords: Phytic acid; Phytase characterisation; *Aspergillus niger;* Submerged fermentation, Agro-based polymeric substrates

INTRODUCTION

Phytic acid is a major form of phosphorus storage in plants and a constituent of all plant seeds with about 1-5% occurring in many cereals and oilseeds. They account for 60-90% of the total phosphorus in seeds (**Afinah *et al*. 2010; Kalsi *et al.* 2016**). Traditionally, phytic acid is considered by as an anti-nutrient due to its ability to chelate and form protein-mineral-phytic acid complexes with divalent minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} (**Afinah *et al*. 2010; Aziz *et al*. 2015**). Thus, concern continues to trail the implication of phytic acid on certain vulnerable segment of monogastric and agaric population (fishes, poultry birds, pigs and humans) that lacks the required hydrolytic enzymes capable of disassembling these complexes in their gut since intestinal absorption requires that minerals remain in their ionic state in order to maintain normal mineral homeostasis (**Bae *et al*. 1999; Noureddini & Dang, 2008**). Hence, the continuous release of these faecal matter into soil and water bodies raise the amount of insoluble phosphorus in such environments, raising the pollution index therein (**Tariq *et al*. 2017**).
Phytases catalyse the sequential hydrolysis of phytic acid to inorganic phosphate and myo-inositol hexaphosphate derivatives (**Singh & Satyanarayana, 2011; Kumar *et al*. 2019**). In animal feed industry, especially for monogastrics, the eco-friendliness of phytases in the degradation of phytic acid has made them one of the most desired feed additives (**Bae *et al*. 1999; Wang *et al*. 2013**). Phytase supplementation in feed maximizes nutrients uptake in the animal thus minimizing the phatate ultimately released into environment through the undigested feed within faecal matter (**Robinson *et al*. 2002; Vashishth *et al*. 2017**). Despite the unique role that poultry farming holds in animal husbandry, the current high cost of commercial phytase continues to task nutrition, enzymology and microbiology researchers to find new microorganisms which can efficiently utilize cheaper agro-based substrates to produce phytases for application in animal feeding.
This work therefore isolated phytase producing fungi from cereal-rich soils and poultry waste. In addition we determined the production and enzyme characteristics of a fungal phytase produced in a submerged fermentation medium containing different underutilized agro-based 'waste' materials as fermentation substrates either devoid of, or supplemented with essential nutrients.

MATERIAL AND METHODS

Sample collection and isolation of fungi

Agro-based substrates (orange peel, sesame seed and watermelon seeds) were obtained from agro-produce vendors at Bodija market in Nigeria. The substrates were oven dried at 45ºC, milled and passed through a 60 mesh (250µm) sieve. Samples of poultry droppings and soils (from untilled farmland and cereal-storage/vending areas) were collected for use in microbial isolation. The soil samples were collected at a depth of 2-8cm into Ziplock bags while poultry droppings were collected from broiler, layer and turkey pens and appropriately labeled. Samples (10g) each was suspended in 90mL of sterile distilled water and then serially diluted (1:9) up to the 8th dilution. From each dilution, fungi were isolated by pour-plating 1mL of the different dilutions using Potato Dextrose Agar (**Harrigan & McCance, 1976**) amended with 100 µg mL^{-1} of streptomycin to inhibit bacterial growth. The plates were incubated at 27±2 °C for 5 days and selected fungi were sub-cultured onto agar slants and stored at 4ºC until further use.

Screening for phosphate solubilization and phytase producing microorganisms

Phosphate solubilization ability of 5 day old fungal isolates was determined by culturing 7mm fungal discs on Pikovskaya medium (PKV) and incubating for seven days at 27±2°C (**Pikovskaya, 1948**). The formation of clear, translucent halos around inoculated fungal cultures was indicative of phosphate solubilization by such isolates. Further screening for the ability of the PKV-positive fungi to solubilise phytic acid was achieved by inoculating them on a compounded Phytase Screening Medium (PSM) of **Nautiyal, (1999)** and incubating for 7days. Phytic acid solubilization was detected by the formation of zones around the inoculated positive phosphate-solubilising fungi and phytase activity was confirmed using the double counter-staining assay (**Bae *et al*., 1999**). Briefly, presumptive-positive fungi growing on PSM plates were flooded with 2% w/v cobalt chloride solution for 5 minutes and discarded, then counter-stained with equal volumes of a mixture of 6.25% ammonium molybdate solution and 0.42% ammonium vanadate solution for another 5 minutes. Thereafter, the Solubilization Index (SI) was calculated for the cultures which persistently exhibited distinct zones of clearance around the cultures after the double counter-staining (**Patki *et al*., 2015**):

$$SI = \frac{(\text{Colony diameter} + \text{Halo diameter})X\ 100}{\text{Colony diameter}}$$

Isolates which showed ≥50% solubilization efficacy were considered as good phytase producers and stored appropriately at 4°C on PDA. The fungus with the highest SI was selected for further work and was identified using cultural, morphological and microscopic characteristics.

Phytase production

Phytase was produced using submerged fermentation by cultivating two 7mm agar plugs of the selected fungus (which exhibited the highest Solubilisation Index) in autoclaved and cooled 25 mL of Phytase Production Broth (PPB) containing (g/L): Glucose, 10; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; $MnSO_4$, 0.01; sodium phytic acid 0.5 with the pH adjusted to 5.5 (**Qasim *et al*. 2016**) and incubated at 27±2 °C for 7days. The production broth was centrifuged at 4000 *g* for 15 minutes and the phytase activity quantified as described under assay for phytase activity.

Effect of cultural parameters and medium components on phytase production

The selected fungus was cultivated in PPB over 7 days and assayed at 24 hour intervals to determine the optimum phytase production period. The effects of inoculum age (48, 72, 96, 120 and 144 hours old fungal cultures), pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) carbon (fructose, lactose, maltose, sucrose and starch), and nitrogen ($NaNO_3$, NH_4Cl, NH_4NO_3, peptone and yeast extract) were determined (**Wang *et al*., 2004; Singh & Satyanarayana 2012; Sareen, 2014**). Under the production conditions earlier described, each control carbon or nitrogen source variable (glucose or $(NH_4)_2SO_4$) were substituted for using the stated materials. Another control setup, devoid of the respective carbon and nitrogen source, was used to determine the effect of the absence of a carbon/nitrogen source on phytase production.

Milled sesame seed, watermelon seed and orange peel (1%w/v) were mixed in distilled water, autoclaved and used as sole substrates for phytase production in a 25ml flask culture cultivation. After cooling, each flask was inoculated with two 7mm fungal agar plug and incubated at 27±2°C. In another setup, the milled substrates were supplemented with all the medium components of PPB: (g/L) 0.1 $MgSO_4$, 0.2g KCl, 0.0001 $MnSO_4$, 0.0001 $FeSO_4$ and 0.5 $(NH_4)_2SO_4$ but devoid of glucose and sodium phytic acid. Inoculated phytase production broth was used as control. After incubation, the broths were centrifuged and assayed for phytase activity (**Singh & Satyanarayana 2012; Qasim *et al*., 2016**).

Assay for phytase activity

Phytase activity was determined using 0.5 mL enzyme mixed with 2 mL of 0.1% sodium phytic acid (in 0.1M of pH 5.5 acetate buffer) for 10min at 37ºC. The reaction was stopped with 2mL freshly prepared acetone-acid-molybdate stop solution. The orthophosphate released from the reaction setup was quantified by measuring the absorbance at 355nm and the values extrapolated from a standard curve prepared with K_2HPO_4. One unit of phytase activity was defined as that which liberated 1 µM phosphate per minute under the assay condition (**Choi *et al.* 2001**).

Partial purification of fungal phytase and enzyme characterization

Partial purification of produced phytase using the best production conditions was accomplished by the method of **Roy *et al.,* (2012)**. Cell free supernatants were subjected to ammonium sulfate precipitation (0-40% and 40-80% saturation) incubated overnight at 4°C, centrifuged afresh and the precipitate desalted using a dialysis bag suspended overnight at 4°C in a glass beaker containing sodium acetate buffer (pH 5.5) with continuous mixing using a magnetic stirrer. The molecular weight, phytase activities and total protein contents of the partially purified samples were determined (**Lowry *et al*. 1951; Laemmli, 1970**) .

Partially purified phytase enzymes were characterized by determining the effect of pH (3-9), temperature (25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C), and different metal ions inhibition of enzyme activity: Ca^{2+}, Co^{2+}, Cd^{2+}, Li^{+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+} and Fe^{2+} on phytase activity (**Roy *et al*. 2012; Sareen, 2014**). Effect of increasing substrate concentration by addition of fixed quantity of enzyme (0.5 mL) to 2 mL varied concentration of assay mixture 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6% of phytic acid in acetate buffer was also investigated to determine the maximum velocity (V_{max}) and Michaelis-Menten constant (Km) for the substrate (**Lineweaver & Burk, 1934**).

Statistical analyses

Studies were performed in triplicates and all data were analyzed using One-Way ANOVA ($p < 0.05$).

RESULTS

Screening for phytase and effect of cultivation conditions on phytase production

Twenty-eight fungi with phytase producing abilities were obtained in this work and the fungal count in the samples studied varied between 10^5 and 10^6 TFU/g sample (Table 1). Broiler poultry droppings (BPD) sample had the highest fungal load (1.6×10^6). Phytic acid solubilization was observed in 61% of the isolates (*n-28*) while the counterstaining assay confirmed fourteen fungi (50%) as phytase producers by eliminating the false-positive fungal producers initially observed before counterstaining. The solubilization index was highest (121%) in isolate GS_1S_6 and least in fungal isolate $TPDP_{10}$ (30%).

Isolate GS_1S_6 was therefore selected for phytase production studies. On PDA, the colonies of isolate GS_1S_6 presented as flat and compact mycelium with cream-brown basal mycelium which progressively became entirely dark brown with a cream/yellowish observe. By the fourth day, the culture was covered by dense layer of intense black conidial heads with powdery texture. Microscopically, the fungal morphology revealed hyphae bearing conidiphores on which strings of circular spores were suspended.

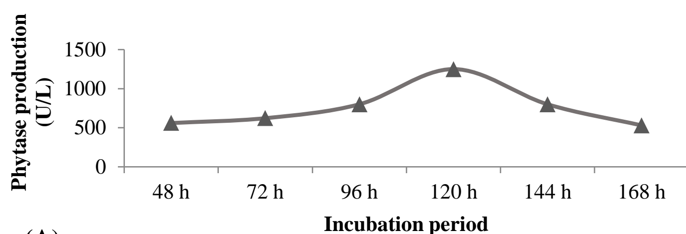
When the impact of the extent of incubation on phytase production was investigated, a significant amount of phytase was produced at 120 hours post inoculation (Figure 1a) beyond which the production dwindled. In Figure 1b, the inoculum age supporting the maximum production of phytase by *A. niger* GS_1S_6 (900U/L) was recorded when 72 hour old fungal discs were used for cultivation and significantly different from the numerically close value (800 U/L) recorded using a 96 hours old culture.

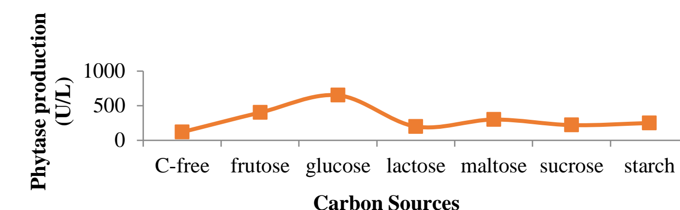
From the results of phytase production in adjusted pH media (Figure 1c), phytase production gradually was found to increase as the pH increased from pH 3 to 5 and then peaked at a pH of 5.5 (1100 U/L). After this, there was a steady decline in enzyme production. *Aspergillus niger* GS_1S_6 utilised simple sugars (glucose and fructose) with glucose producing highest phytase compared to other sugars used (Figure 1d). The use of more complex sugars resulted in lower quantities of phytase. *Aspergillus niger* GS_1S_6 produced phytase in medium devoid of carbon or nitrogen source and the least phytase (119.6 U/L) was produced in a medium devoid of a carbon source. When ammonium sulphate was used in the cultivation medium as a source of nitrogen, the highest phytase production (580 U/L) was observed (Figure 1e).

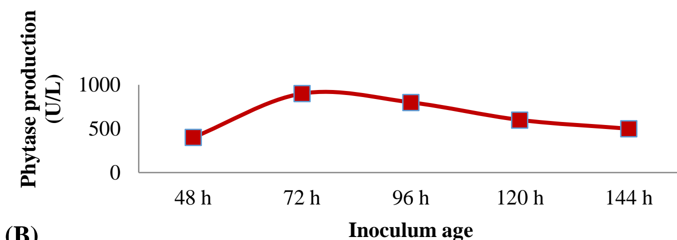
Table 1 Solubilization index and probable identity of phytase-positive fungal Isolates

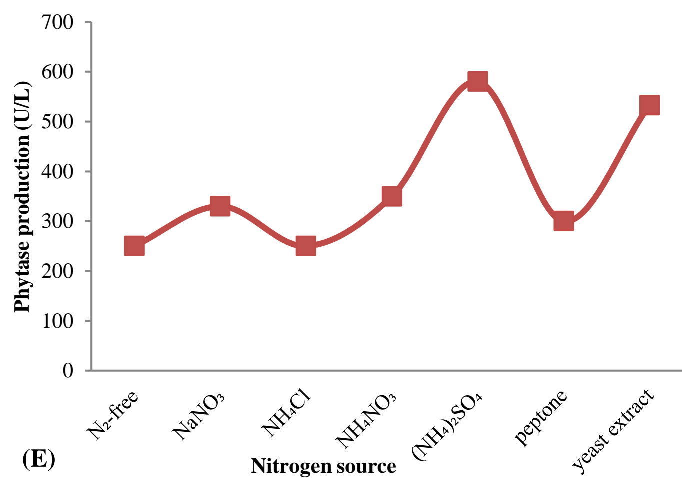
Sample Source	CFU/g Sample	Isolate code	% S.I	Probable identity
Cereal rich soil $(CRS)_1$	1.3 X 10^6	CRS_1C_1	58±0.08	*Aspergillus* sp.
		CRS_1C_2	ND	*Fusarium* sp.
		CRS_1C_3	77±0.05	*Mucor* sp.
		CRS_1C_4	55±0.07	*Penicillium* sp.
		CRS_1C_5	ND	*Fusarium* sp.
		CRS_1C_6	ND	*Fusarium* sp.
		CRS_1C_8	ND	*Penicillium* sp.
		CRS_1C_9	ND	*Mucor* sp.
Cereal rich soil $(CRS)_2$	9.0 X 10^5	CRS_2C_7	51±0.04	*Rhizopus* sp.
		CRS_2C_{10}	110±0.03	*Aspergillus* sp.
Broiler poultry droppings (BPD)	1.6 X 10^6	$BPDP_1$	ND	*Mucor* sp.
		$BPDP_2$	92±0.07	*Aspergillus* sp.
		$BPDP_3$	ND	*Penicillium* sp.
		$BPDP_4$	98±0.04	*Aspergillus* sp.
Turkey poultry droppings (TPD)	1.4 X 10^5	$TPDP_5$	ND	*Trichoderma* sp.
		$TPDP_6$	71±0.40	*Aspergillus* sp.
		$TPDP_7$	ND	*Trichoderma* sp.
		$TPDP_9$	ND	*Aspergillus* sp.
		$TPDP_{10}$	30±0.02	*Rhizopus* sp.
Layer poultry dropping (LPD)	1.0 X 10^6	$LPDP_8$	66±0.06	*Mucor* sp.
Garden Soil $(GS)_1$	1.0 X 10^6	GS_1S_1	ND	*Aspergillus* sp.
		GS_1S_2	55±0.02	*Mucor* sp.
		GS_1S_3	ND	*Penicillium* sp.
		GS_1S_6	121±0.06	*Aspergillus* sp.
		GS_1S_8	44±0.03	*Aspergillus* sp.
Garden Soil $(GS)_2$	8.0 X 10^5	GS_2S_4	ND	*Aspergillus* sp.
		GS_2S_5	68±0.01	*Penicillium* sp.
		GS_2S_7	ND	*Mucor* sp.

Key: BPD= Broiler poultry droppings, CRS= Cereal rich soil, GS= Garden Soil, LPD= Layer poultry dropping, TPD= Turkey poultry dropping, ND = Not Detected, S.I= Solubilization Index.









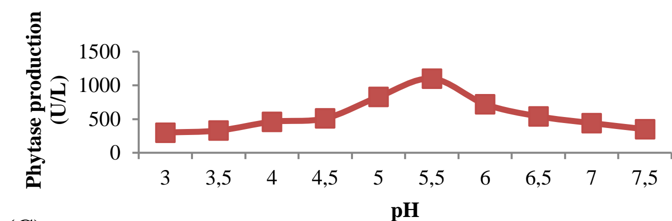


Figure 1 Time course of (A), effect of inoculum age (B), medium pH (C), carbon (D) and nitrogen sources (E) on phytase production by *Aspergillus niger* GS_1S_6

The sole use of agricultural substrates in sterile broth without additions of mineral components resulted in sesame seed supporting the highest phytase production (700 U/L) by *A. niger* GS_1S_6 while the least phytase production (310 U/L) was

recorded in the orange peel medium (Figure 2). The control phytase production broth (containing both glucose and sodium phytate) supported a phytase production of 980 U/L by the *A. niger* GS_1S_6. However, the inclusion of the mineral phytase production broth (devoid of both glucose and sodium phytate) to the agro-substrates medium boosted phytase production in all the media used. The mineral-supplemented sesame seed-containing medium demonstrated the overall highest phytase activity (1000 U/L) followed by the watermelon supplemented mineral medium which recorded 720U/L. Using the control phytase production broth void of both glucose and phytic acid, the *Aspergillus niger* GS_1S_6 still produced phytase (330 U/L).

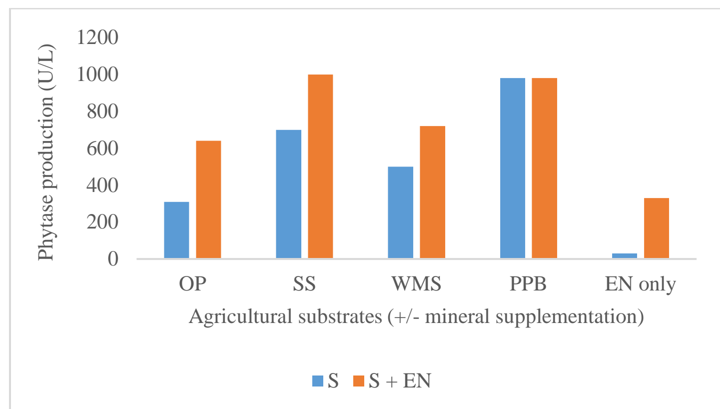


Figure 2 Phytase production by *Aspergillus niger* GS_1S_6 in broth media devoid of, and supplemented with essential nutrients using orange peel, sesame seed and watermelon seeds as sole substrates.

Phytase Characterisation

The phytase produced from a sesame seed-essential nutrient culture broth was used for enzyme characterization studies. With sequential purification, the phytase activity increased (Table 2) and the *A. niger* GS_1S_6 phytase had a specific activity of 0.211 U/mg after the dialysis of the ammonium sulphate precipitated enzyme. The protein content of the crude phytase was 5.476 mg/mL and this further reduced in the dialysed ammonium sulphate precipitated phytase (4.945 mg/mL).

The crude phytase (Lane 2) had two protein bands, both within the 31 and 38KDa molecular weight mark. However, one band (located between 31 and 38KDa) was observed in the partially purified *A. niger* GS_1S_6 phytase (Figure 3).

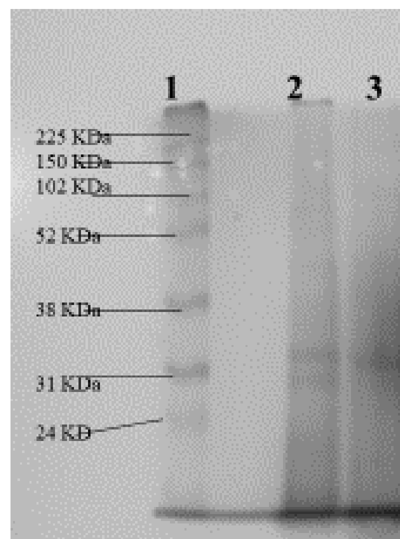
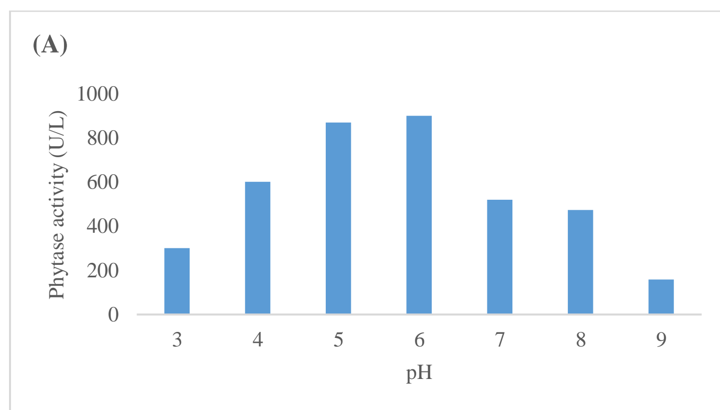


Figure 3 Electrophoretogram of the crude and partially purified *Aspergillus niger* GS_1S_6 phytase.
Key: *1~Molecular weight bands; 2~Crude phytase, 3~$(NH_4)_2SO_4$ precipitated and dialysed Aspergillus niger GS_1S_6 phytase*

Table 2 Partial purification of *Aspergillus niger* GS_1S_6 phytase

Purification steps	Total activity (U)	Total protein (mg/mL)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude phytase	81.826	547.595	0.1494	1.000	100.0
$(NH_4)_2SO_4$ precipitatation	95.555	530.731	0.180	1.205	116.8
Dialysed enzyme	104.118	494.503	0.211	1.409	127.2

A narrow pH range for phytase activity was observed between 5.0 and 6.0, having an optimum activity at pH 6 (899.82 U/L) beyond which a steady decline was observed from neutral to slightly alkaline regimes (Figure 4a). The optimum temperature for phytase activity was observed at 55ºC (980 U/L) and phytases subjected to higher temperatures beyond 55ºC had reduction in phytase activity (Figure 4b). At 65ºC, the residual activity was 51%. All metallic inclusions reduced the phytase activity recorded (Figure 4c). Enzyme activity was most inhibited by Cu^{2+}, which recorded a 17.11% activity and moderately inhibited by Mg^{2+} (80.5%).



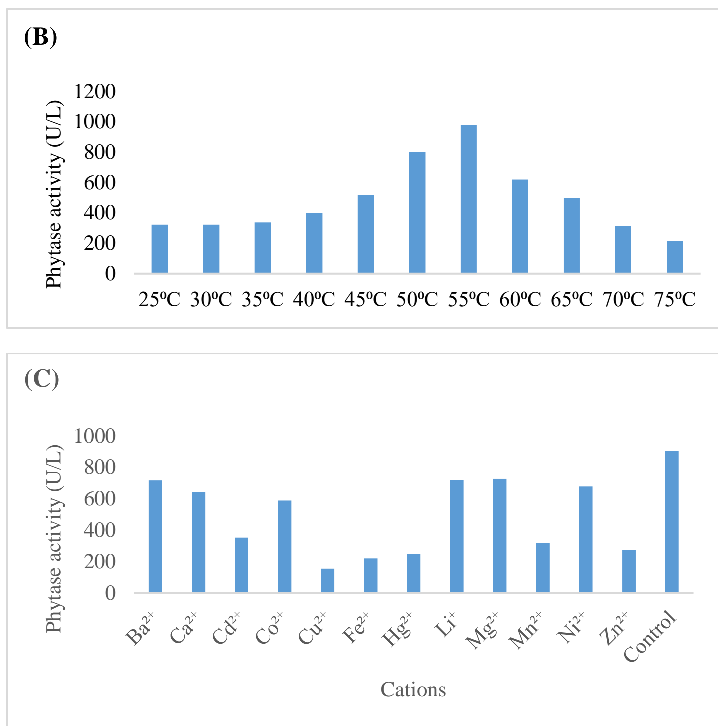


Figure 4 Effects of different (a) pH (b) temperature and (c) cations on the activity of phytase produced by *Aspergillus niger* GS_1S_6

The enzyme kinetics of *A. niger* GS_1S_6 phytase obtained when a fixed quantity of enzyme was used along with varied concentration of substrate (phytic acid) revealed that maximum velocity rate (V_{max}) of *A. niger* GS_1S_6 phytase was attained at 0.077 mM/mL/min while the Michaelis-Menten constant (Km) was 1.308 mM.

DISCUSSION

Fungal isolates with phytase producing abilities were obtained in this work from the samples studied and their load, which varied between 10^5 and 10^6 total fungal units (TFU)/g sample, were similar to other works. Other authors have also reported the isolation of phytase producing microorganisms from similar sources. **Awad *et al*. (2014)** isolated from root nodule soils while **Shivanna & Govindarajulu (2009)** isolated *Aspergillus niger* from soil samples around poultry livestock areas. The highest phytase activities as indicated by the solubilization index were recorded in four *Aspergillus* species (GS_1S_6, $BPDP_2$, CRS_2C_{10} and TPD_6), all of which had solubilization index ≥50%. While the selected isolate was identified as *Aspergillus niger* GS_1S_6 through its colony and microscopic descriptions, other authors also documented similar morphological descriptions for *Aspergillus niger* (**Lee *et al*, 2005; Tariq *et al.* 2017**). Similarly, **Tariq *et al.* (2017)**, also recorded a maximum solubilization index of 119% in their phytase studies. The counterstaining technique successfully eliminated false positive results caused by the presence of organic acid **(Bae *et al*., 1999; Qasim *et al*. 2016)** and the quantitative screening was an additional confirmatory test to complementary strain selection and development for possible strain commercialization process. Several authors have reported the isolation of phytase producing microorganisms from similar sources. **Awad *et al.* (2014)** isolated phytase producing *Penicillium purpurogenum* GE1 from root nodule soils while **Shivanna &Govindarajulu (2009)**, isolated a phytase producer from soil samples obtained from poultry livestock areas.

In the first five of the seven days of cultivation, The phytase produced increased. The decline observed beyond 120 hours could possibly be attributed to an increase in fungal biomass which might have resulted in depletion of available nutrients in the medium, thus affecting the production of phytase **(Mittal *et al*., 2012)**. Inoculum age, being a function of microbial growth, is known to affect enzyme production. At 72 hours, fresh fungal cultures are at a rapid growth phase which might directly continue in the new medium into which the fungus is introduced for cultivation.

Phytase production was low at extremely acidic pH, peaked at pH 5.5 and reduced beyond pH 5.5. A similar observation was reported by **Gargova &Sariyska (2003)**, who documented the optimal phytase production at pH 5.0, while other authors reported an optimum pH of 5.5 for *Aspergillus* sp. L117 **(Lee *et al*, 2005)**. The ranges of carbon sources utilized for mycelial growth and enzyme production of different fungi vary widely. A diversity of sugars can be used as suitable carbon sources for the proliferation of fungi **(Odeniyi *et al*. 2009)**. Simple sugars (glucose and fructose) supported phytase production more than the disaccharide and polysaccharides used in the production medium, an advantage for the microorganism, since disaccharides and other complex carbohydrates would require an initial hydrolysis into monomeric units or their derivatives, before entering the various metabolic pathways (**Praveen & Arun, 2013**). In nature, both the organic and inorganic forms of nitrogen are available to fungi, but how these fungi utilize the various nitrogen sources is contingent on their specific response towards different nitrogenous substances. The high phytase production observed in this study when ammonium sulphate was used may owe to the fact that ammonium nitrogen needed no modification before entering the synthetic pathway **(Praveen & Arun, 2013)**.

Phytase production by *A. niger* GS_1S_6 was recorded in all the agro-material based fermentation medium (orange peel; sesame seed and watermelon seed), with or without mineral supplementation. The addition of mineral constituents to the substrates in the medium resulted in higher phytase activities. Studies have reported phytase production through submerged fermentation in medium containing wheat bran, a cheap agro-residue, which is however, also used as a component of animal feed **((Mittal *et al*., 2012; Sreedevi & Reddy, 2012)**. In many underdeveloped nations the preservation of perishable crops is almost non-existent, a condition which the Food and Agriculture Organization of the United Nations have researched extensively upon, worldwide. Some crop parts such as watermelon seeds and sesame seeds are considered underutilized **(Biswas *et al*. 2017)**. In Nigeria, watermelon, for example, is disposed of immediately fruit rot sets in and the seeds within are wasted. **Olasupo & Okorie (2019)**, however, reported that sesame seeds still find some use in traditional condiment production. Since watermelon seeds currently have no documented competing economic use in Nigeria, these may be considered as a cheap source for phytase production considering its 72 and 73.5% phytase production relative to that from sesame seed and phytase production broth, respectively. **Qasim *et al.* (2016)** reported similar findings for *Aspergillus tubingensis* SKA and suggested that enhancement in phytase production could be linked to improvement in the nutritional value of the fermentation medium as depicted in the mineral supplementation results. The ability of *Aspergillus niger* GS_1S_6 to produce phytase in medium devoid of both phytic acid and glucose, shows its constitutive nature. **Idriss *et al*. (2002)** reported a phytase constitutively secreted by a *Bacillus amyloliquefaciens* independent of the nutrient composition which had its highest phytase produced in a medium devoid of any form of phosphorus.

When the phytase produced using sesame seed-essential nutrient culture broth was subjected to precipitation and dialysis, the resulting enzyme had a higher activity and yield compared to the crude while its final protein content reduced compared to the crude. The molecular weight of the partially purified *A. niger* GS_1S_6 phytase was between 31 and 38KDa. **Tariq *et al.* (2017)**, documented phytases from *Aspergillus* species with molecular weights between 35 and 107.82 KDa and reported that the *A. niger* which produced the highest phytase recorded the least molecular weight. The optimum pH for *A. niger* GS_1S_6 phytase activity was between 5.0-6.0 beyond which the activity declined as it moved from neutral to slightly alkaline regimes. The pH optima of some microbial phytases have been reported in the ranges of 4.5-5.5 (**Roy *et al*. 2012**). The phytase of an *Aspergillus foetidus* was reported to have an optimum pH of 5.5, similar to what obtained in this work **(Ajith *et al*. 2019).** The optimum pH of phytase activity was similar to that at which the production medium of the organism also produced the highest yield. The phytase was optimally active within the temperature range 50ºC and 60ºC. **Vohra & Satyanarayana, (2003)**, reported that for most fungal and yeast phytases, the optimum temperature was usually 50-70°C. The phytase activity was inhibited by Cu^{2+}, Cd^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+}. Metal ions play an important role in enzymatic catalytic activities, however, many phytases do not require metal ions for maximum catalytic activity **(Fu *et al.* 2008)**. The inhibitory effect of mineral ions on phytase activity might be attributed to the strong chelating capacity of phytic acid, resulting in an insoluble metal-phytic acid complex that reduce the bioavailability of phytic acid for the enzyme due to the glycosylation folding of the enzyme structure **(Wang *et al*., 1980; Afinah *et al*., 2010)**. The

metal ion inhibition of the *A. niger* phytase was analogous to the observation of **Segueilha *et al*. (1992)**, who documented a reduction in phytase activity by 90% using 5 mM Fe^{3+} with *Pichia anomala*. The enzyme activity of a phytase from *Shigella* sp. CD2 was inhibited in the presence Fe^{2+}, Zn^{2+} and Cu^{2+} (**Roy *et al.* 2012**).
The K_m recorded was lower than those reported by **Elkhalil *et al.* (2011)** in an *Aspergillus* sp. A low phytase K_m allows the maintenance of sufficient substrate degradation leading to higher transformation **(Nagashima *et al*., 1999)**.

CONCLUSION

A phytase-producing *Aspergillus niger* GS_1S_6 was isolated from soil and glucose, $(NH_4)_2SO_4$, 5 days incubation, pH 5.5 and inoculum age of 72 hours were most suitable for phytase production. Phytase production was constitutive and the characteristics of the phytase obtained using different underutilized agro-based materials/'waste' as fermentation substrates in mineral replete/devoid systems revealed a partially purified enzyme which was most active at pH 5.5, 55°C and required no metal ion inducement. Alternative medium composition, especially in the form of oil-based seeds with and without mineral supplementation can be enlisted to economically produce phytase with commensurate activities instead of synthetic media. To the best of our knowledge this is one of the first works to utilize watermelon seed as a cost effective agro-material for phytase production thus solving the challenges of agro-waste management and reducing the cost of enzyme production of phytase which is held as a major drawback to phytase commercialization.

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REGULAR ARTICLE

THE POTENTIAL OF ENDOPHYTIC FUNGI AS BIOCONTROL AND PHOSPATE SOLUBILIZATION AGENT IN *Capsicum anuum*

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ABSTRACT

The productivity of chili (*Capsicum annuum*) in Indonesia is currently very low. Some factors that influenced it including the presence of pathogenic microorganisms which lead to the low availability of phosphate in the soil. This condition become a limiting factor for plant growth and production. Endophytic fungi can be used as antagonistic agents in inhibiting pathogenic fungi and to increase the efficiency of phosphate solubilization known as phospholytic fungi. This study aimed to find antagonistic agents from endophytic fungi that can suppress the growth of pathogenic fungi and test the ability of endophytic fungi to dissolve phosphate. Fungi isolates used were BB-Biogen collection isolates, consisting of 42 endophytic fungi isolates, and 3 pathogenic fungi isolates (*Fusarium* sp., *Colletotrichum acutatum*, *Phytophthora capsici*) on chili plants (*C. annuum*). The antagonism test was carried out using the dual culture method in the Potato Dextrose Agar (PDA) medium for 5 days incubation at temperature (± 28 ° C). The parameters measured were based on the formation of inhibition zones and the calculation of the percentage of growth inhibition of fungi isolates. The test results obtained 7 representative fungi isolates (RIVA4, RIVA5, MIVD2, *Aspergillus niger*, *Cladosporium sp.*, *Cladosporium oxysporum*, and *Chaetomium globosum*). Based on the calculation of the percentage of growth inhibition, fungi isolates with RIVA5 code have a higher potential in inhibiting the growth of all three pathogenic fungi. Calculation of the percentage of endophytic fungi inhibition of RIVA5 were 70.3% (*Fusarium* sp.), 63.3% (*C. acutatum*), and 60% (*P. capsici*). Phosphate test was carried out by the cork borer method in pikovskaya medium for 4 days incubation at 27-28 ° C. The parameters measured were based on the formation of clear zones around the colony. There were 4 endophytic fungi isolates (MIVA4, MIVF7, *Aspergillus sydowii*, and *A. niger*) formed a clear zone around the colony, which indicates the presence of phospholytic activity. Based on the calculation of the phospholytic index, *A. niger* isolates have a high phosphate solubility index value of 5.

Keywords: *Capsicum anuum*, endophytic fungi, pathogenic fungi, phospholytic fungi

INTRODUCTION

Chili plants (*Capsicum annum*) is one of the most popular vegetable commodities in Indonesian and has a high economic value (**Mariyono *et al*., 2015**). Chili is one of the main commodities for Indonesian farmers because it can be planted in various fields and known to have high adaptability (**Ali, 2006**). Chili productivity in Indonesia is still very low, due to many factors such as pathogenic microorganisms which can reduce the quality and quantity of production (**Than *et al.,* 2008; Kim *et al*., 1999**).

Indonesian farmers generally still uses a lot of synthetic pesticides because of the ease of obtaining and effectiveness. Even though many research results show that the excessive use of synthetic pesticides results in environment problems and endanger to human health (**Nantawanit *et al.,* 2010**). A fairly safe and environmentally friendly control is needed as an alternative control, one of which is by using endophytic microbes. Endophytic microbes are microbes found in plant tissue systems such as leaves, flowers, twigs, or plant roots. Endophytic microbes grow and get nutrient from their host plants. Endophytic microbes can be in the form of bacteria or fungi, but in the last decade, fungi gained more attention to be explored (**Putra *et al*., 2015**). Endophytic fungi can produce bioactive compounds such as antibiotic, enzyme, plant growth promoting substances **(Sun *et al*., 2008; Bezeerra *et al*., 2015, and Hwang *et al*., 2011)**. As antibiotic producer, endophytic fungi have a potency to inhibit the growth of pathogenic fungi and are expected to be able to effectively become biocontrol agents for pathogenic microorganisms (**Gao *et al*., 2010**). Pathogenic microorganisms can be a limiting factor that can reduce the quality and quantity of production in plants, in addition to these factors, the form of P availability in the soil is also a limiting factor for growth and production in plants. The increase in phosphate causes the phosphate fertilizer given is inefficient, which as the result, phosphate needs to be given in high quantities (**Brindaban *et al.,* 2020***)*.

An alternative that can be used to improve the efficiency of phosphate fertilization is to utilize microorganisms that can dissolve phosphate. Phosphate solvent microorganisms are microorganisms that can extract phosphate from an insoluble form into a form available to plants through the secretion of organic acids produced to release P from the sorption complex (**Khan, *et al*., 2009**). This study aims to find the antagonistic agent of endophytic fungi against pathogenic fungi as well as their ability to dissolve phosphate.

MATERIAL AND METHODS

Sample Collection

Endophytic fungi isolates and pathogenic fungi were obtained from Biogen Culture Collection. About 18 isolates of endophytic fungi were obtained from *Rhodomyrthus tomentosa*, and 15 isolates from *Melastoma malabathricum*, 9 isolates from the *Alpinia malaccensis*. while as many as 3 isolates of pathogenic fungi (*Fusarium* sp., *Colletotrichum acutatum*, *Phytophthora capsici*) were taken from *Capsicum annuum*.

Morphology Observation of Fungal Endophyte

The fungi were firstly grouped on the bases of their colony appearance on PDA such as colony shape, color, elevation, texture, mycelia type, edges, density, and diameter. Fungal colonies with similar characteristics were grouped into the same morphotypes.

Antagonism test of fungi

Antagonism test of fungi was carried out by the dual culture method based on **Naik *et al*., (2009**). Pathogenic and endophytic fungi were inoculated on PDA medium in a Petri dish (diameter 9 cm) with a distance of 3 cm. Incubation was carried out at room temperature (± 28 °C) for 5 days. Percentage inhibition of endophytic fungi against pathogen was calculated using the formula:

$$H = \frac{J1 - J2}{J1} \times 100\%$$

H= Percentage of inhibition
J1 = the radius of the pathogenic fungi colony towards the edge of the Petri dish
J2= the radius of the pathogenic fungi colony that is headed toward the fungus

Phosphat sollubilization activity test

Endophytic fungal isolates were inoculated using sterile straws and transferred into Pikovskaya medium in a petri dish. The media was incubated at room temperature (± 28 °C) for 7 days. Indications of phosphate dissolution by fungi can be characterized by the formation of clear zones around colonies. The activity of fungi in phosphate degradation is expressed by the value of the Phospholytic Index measured using the following formula:

$$\text{Phospholytic Index} = \frac{\text{diameter of clear zone (cm)}}{\text{diameter of fungi colony (cm)}}$$

RESULTS AND DISCUSSION

A total of 41 endophytic fungi were characterized based on their morphology on PDA medium. Each endophytic colony shows a unique and varied appearance of colonies. Most of endophytic fungi have circullar shape, flat elevation, hypha cottony texture, aerial myselium, and average colony size after 11 weeks by 6.8 cm (Fig. 1; Table 1).

Table 1 Culture characteristics of the Endophytic Fungi on PDA

Isolate code	Shape	Colour		Elevation	Texture	Mycelium	Edge	Size (cm) of colony after 11 days
		Above	Reverse					
RIVA1	circullar	brown	cream to yellow	raised	Cottony	aerial	entire	7.2
RIVA2	circullar	dark green	cream to yellow	raised	Cottony	aerial	entire	7.2
RIVA3	irregular	white	Cream	raised	fluffy	aerial	dentate	8
RIVA4	circullar	white	Cream	Flat	cottony	aerial	filamentous	8.5
RIVA5	circullar	white	Cream	Flat	velvety	aerial	filamentous	9.5
RIVD1	irregular	white,black	cream to yellow	convex	rocky	immersed	dentate	9
RIVD2	circullar	white	Cream	raised	rocky	aerial	entire	3
RIVD3	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	7.4
RIVD5	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	2.5
RIVD6	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	2.5
RIVD7	irregular	black,white	Cream	convex	rocky	immersed	dentate	2.8
RIVD8	circullar	white	Cream	raised	cottony	aerial	entire	2.7
RIVD9	irregular	black,white	cream to yellow	raised	rocky	immersed	dentate	9
RIVD10	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	2.6
RIVD11	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	3.2
RIVD14	circullar	white	Cream	raised	cottony	aerial	entire	1.5
RIVD15	circullar	white	Cream	raised	cottony	aerial	entire	8.4
RIVD16	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	9
MIVF3	irregular	grey	Grey	Flat	fluffy	aerial	undulate	2
MIVF4	circullar	white	Cream	raised	cottony	aerial	undulate	8.3
MIVF5	circullar	white	Cream	convex	fluffy	aerial	filamentous	8.3
MIVF6	circullar	white	Cream	convex	cottony	aerial	dentate	8.2
MIVF7	circullar	white	Cream	raised	cottony	aerial	dentate	8.5
MIVA1	circullar	white	Cream	raised	orcky	immersed	entire	2.5
MIVA2	irregular	white	Cream	Flat	cottony	aerial	dentate	9
MIVA3	circullar	white	Cream	raised	cottony	aerial	entire	9
MIVA4	circullar	white,cream	cream to yellow	raised	cottony	aerial	entire	9
MIVB1	irregular	white	Cream	raised	cottony	aerial	dentate	6.5
MIVB2	circullar	grey	Grey	Flat	fluffy	aerial	filamentous	7
MIVD1	irregular	white	Cream	Flat	fluffy	aerial	entire	7
MIVD2	circullar	white	Cream	Flat	cottony	aerial	entire	9
MIVF1	circullar	white	Cream	Flat	cottony	aerial	filamentous	9
MIVF2	irregular	white	Cream	Flat	cottony	aerial	entire	9
Colletotrichum boninense	circullar	black, White	cream to yellow	Flat	fluffy	aerial	entire	7.7
Aspergillus sydowii	circullar	black	Yellow	Flat	cottony	aerial	entire	7.5
Cladosporium oxysporum	circullar	black	Yellow	Flat	cottony	aerial	entire	8.5
Aspergillus niger	circullar	black	Yellow	Flat	cottony	aerial	entire	8.8
Cladosporium sp.	circullar	black	Yellow	Flat	cottony	aerial	entire	8.3
Guignardia mangiferae	circullar	black	Yellow	Flat	cottony	aerial	entire	8.7
Chaetomium globosum	circullar	grey, cream	cream to yellow	raised	cottony	aerial	entire	8.7
Diaporthe anacardii	circullar	white	cream to yellow	raised	cottony	aerial	entire	7.6

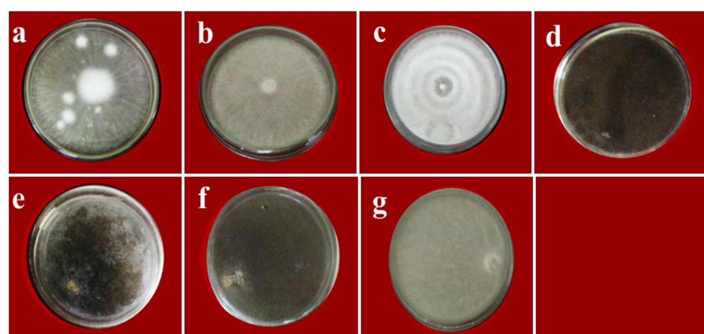

Figure 1 Colony of endophytic fungi isolates a) RIVA4, b) RIVA5, c) MIVD2, d) *Aspergillus niger*, e) *Cladosporium* sp., f) *CladosporiumOxysporum,* g) *Chaetomium globosum* on PDA medium for 7 days.

Antagonistic testing was carried out by the dual culture method, in which endophytic fungi isolates and pathogenic fungi isolates were grown together in a petri dish (**Naik *et al*., 2009**). It aims to create a mechanism of competition that occurs between the two. When pathogenic fungi isolated are inoculated in a medium that already contains endophytic fungi, the growth, and development of endophytic fungi are inhibited due to reduced space and nutrients.
The results confirmed that 7 isolates have a higher antagonistic ability than others in inhibiting the growth of pathogenic fungi. The parameters observed in antagonistic testing are the presence of inhibitory zones between pathogenic and endophytic fungi, and the reduction of pathogenic fungi mycelium. Based on the data obtained, it is known that the most potential endophytic fungi isolates in inhibiting the 3 pathogenic mold isolates are RIVA5 isolates with inhibition values of 70.3% (*Fusarium*), 63.3% (*Colletotrichum acutatum*), 60% (*Phytophthora capsici*) (Table 2).

Table2 Value of growth inhibition of pathogenic fungi by potential endophytic fungi

No	Isolate code/ name	Growth inhibition (%) of pathogenic fungi isolates		
		Fusarium sp.	*Colletotrichum acutatum*	*Phytophthora capsici*
1	RIVA4	16	61	-6
2.	RIVA5	70,3	63,3	60
3.	MIVD2	7,4	45,4	37
4.	*Aspergillus niger*	40	20	0
5.	*Cladosporium* sp.	40	50	0
6.	*CladosporiumOxysporum*	66	31,52	0
7.	*Chaetomium globosum*	52	56	0

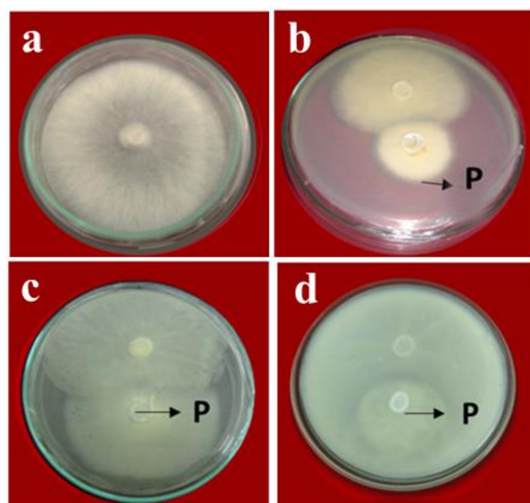

Figure 2 Antagonistic test results a) control of RIVA5 endophyticfungi isolates, antagonist test of RIVA5 endophytic fungi isolates against b) *Fusarium*, c) *Colletotrichum acutatum*, d) *Phyopthora capsici* on PDA medium aged 5 days at room temperature ± 28°C. *P= Pathogen).

Control fungi isolates without antagonistic treatment and fungi isolates with dual culture antagonist treatment had differences in colony growth (Fig. 2). Treatment fungi isolates showed reduced mycelium, non-sporulating, and had smaller diameters while control isolates without antagonistic treatment showed normal my growth of mycelium, which was not reduced, and sporulated. That is because there is an antagonist interaction between endophytic fungi isolates and pathogenic fungi isolates in antagonistic testing.
Endophytic fungi isolates in dual culture treatment have antagonistic ability to inhibit the growth of pathogenic fungi colonies. This is in line with **Talapatra *et al*.**, (**2017**) which stated that the inhibition of the growth of mycelium colonies of pathogenic fungi is due to the antagonistic nature of endophytic fungi. Antagonistic interaction is a form of defense that includes self-defense, territory, and nutrition.
The reduction of hyphal or mycelium width that occurs in pathogenic fungi is suspected due to the antagonistic nature of endophytic fungi. Pathogenic fungi lack nutrients to grow when they are grown with endophytic fungi in the same medium so that the mycelium that is formed becomes less and there is no sporulation. The inhibition zone formed is due to the antagonism characteristic of endophytic fungi isolates. The inhibition zone is a clear zone that indicates inhibition of fungi growth due to the secretion of metabolite compounds by endophytic fungi isolates. The presence of antagonistic mechanisms in endophytic fungi against pathogenic fungi is a form of antibiosis. Secondary metabolites are metabolite compounds that function as inhibitors of growth of pathogenic fungi. These compounds are not essential for growth and are produced at certain times. Secondary metabolites are a form of self-defense from adverse environmental conditions. Secondary metabolites are in the form of pelysis enzymes, volatile compounds, sidospores, or other toxic compounds. compared to bacteria and plants, fungi are among the most productive producers of secondary metabolites (**Keller *et al*., 2005**).
Differences in the ability of antagonism between fungus can be caused by many things, including the speed of spore formation, the number of antibiotic compounds produced and the differences in specific enzymes produced. Some antagonistic mechanisms are space and nutritional competition, production of antifungal compounds, and lytic enzymes (glucanase, chitinase, and protease) (**Chet and Chernin, 2002**). The mechanism of space and nutrient competition occurs when endophytic fungi try to obtain limited space and nutrients when grown together with pathogens so that the growth activity of pathogenic fungi colonies is disrupted due to lack of nutrients and space to grow (**Janisiewicz & Korsen, 2002; Sharma *et al*., 2009**). Lytic enzymes cause degradation of protein components making up the fungi cell walls, resulting in inhibition of cell wall growth in mold mycelium (**Chet and Chernin, 2002**; **Nunes 2012**).
Regarding to the phosphate test, it was found that endophytic fungi isolates which can dissolve phosphate, including 4 fungi isolates, namely MIVA4, MIVF7, *Aspergillus sydowii*, and *A. niger. A. niger* has the highest phospholytic ability among other fungi isolates, with a phospholytic index value of 5.0 (Table 3).
The media used for phosphate testing is psychovaya media, which turbid into white because it contains insoluble P such as calcium phosphate. After 48-72 hours of incubation, the potential for microorganisms to grow on tricalcium phosphate agar will indicate the presence of a clear zone (Fig. 3), while other microorganisms do not exhibit this characteristic.
The presence of clear zones seen around the colony is an indicator that the fungi can dissolve phosphate in the media. Hydrolysis activity qualitatively illustrates the ability of mold isolates to remodel phosphate by comparing the size of the clear zone around the colony with the size of the colony's diameter.
The mechanism of biological phosphate dissolution occurs because these microorganisms produce enzymes such as the enzyme phosphatase and phytase enzyme. The activity of the enzyme phosphatase produced by these fungi is known through the phospholytic index.

Table 3 Value of Phospholytic Index

No.	Isolate code/ name	Diameter of clear zone (cm)	Diameter of fungi colony (cm)	Phospholytic Index
1	MIVA4	3.2	2.9	1.1
3.	MIVF7	1.6	0.9	1.7
4.	*Aspergillussydowii*	2.13	0.87	2.4
5.	*Aspergillusniger*	2.15	0.43	5.0

The observations showed a high Phosphate dissolving index and had a fast-growing ability obtained from *Aspergillus niger* isolates. *Aspergillus* are known to be everywhere and grow on almost all substrates. *Aspergillus* is a dominant group of phosphate solvent fungi found in acid soils in Indonesia. This *Aspergillus* genus has high potential in dissolving Phosphate bound to become Phosphate available in the soil.

The difference in the value of the phospholytic index in each isolate shows the difference in the activity of the enzyme phosphatase in hydrolyzing phosphate. Isolates with high index values indicate high extracellular phosphatase activity, and vice versa in isolates with low index values, phosphatase activity is also low extracellular.

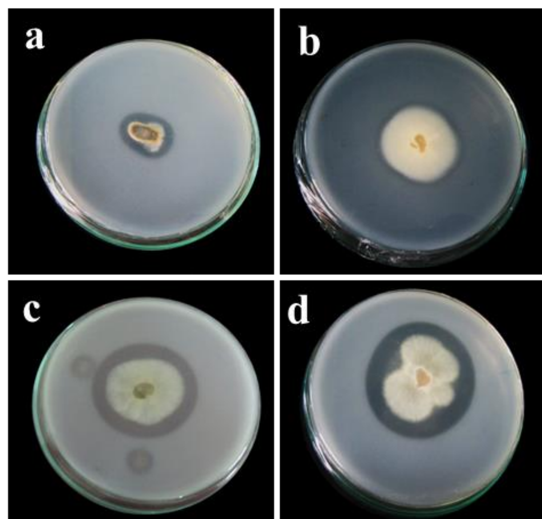

Figure 3 Phospholytic fungi isolates at 4 days after inoculation a) MIVA4, b) MIVF7, c) *A. sydowii*, d) *A. niger*

The amount of phospholytic enzyme activity produced by endophytic fungi is shown by the increasing width of the clear zone. The result of the phosphate breakdown is only indicated by the presence of a clear zone which indicates that phosphate has been overhauled into peptide compounds and amino acids which are dissolved in the medium.

The wide clear zone around the colony explains the ability of fungi to qualitatively dissolve Phosphate varies depending on the genetic nature of each microbe in producing organic acids that play a role in determining the ability of Phosphate dissolution (**Mittal *et al*., 2008**). The superior phosphate solvent microbes will produce the largest diameter of the clear zone and are faster than other colonies.

CONCLUSION

Based on the results RIVA5 has the highest potential in inhibiting the growth of all three pathogenic fungi. Calculation of the percentage of hyphal or mycelium width inhibition of RIVA5 were 70.3% (*Fusarium* sp.), 63.3% (*C. acutatum*), and 60% (*P. capsici*). In the phosphate test, it is known that *A. niger* endophytic fungi isolates have the highest ability among other fungi isolates in hydrolyzing phosphate, with phosphate solubility index value of 5.

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FUNGAL TERRITORY
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REGULAR ARTICLE

MICROBIOLOGICAL SURVEY AND DISSEMINATION OF FILAMENTOUS FUNGI OF PUBLIC HEALTH SIGNIFICANCE FROM WATER RESERVOIR (STORAGE TANK) IN CROWN ESTATE, IGBINEDION UNIVERSITY, OKADA, NIGERIA

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ABSTRACT

Results of this investigation revealed that several species of filamentous fungi are present in the reservoir waters. Samples were collected from the surface and walls of the inner storage tank. The isolation process was done by a range of techniques and media, monitored by trained taxonomist to achieve broad arrays of water-borne fungi classified to species level. The direct plate enrichment counts and filtration technique recorded the highest number of counts (59 % and 32 %) respectively. The sabauraud dextrose Agar (SDA) was observed as the medium that recorded the highest colonies (60 cfu/100 ml) for incubation period of 5 d at 30 ºC. Six different fungal taxa were recovered from the three examined sites (ST1, ST2 and ST3) and the ST3 was reported to have the highest number of isolated species. However, certain fungi were observed to be more distributed than others, specifically in species of *Aspergillus* and *Penicillium* which tended to be the most common. Although, species of other genera such as *Acremonium* sp., *Rhizopus stolonifer, Mucor racemosus* and *Trichophyton* sp. were also present but in low counts. The significance of fungi in water systems is lowly expressed as many of the species isolated from water sources are confirmed to possess the potentiality of secreting toxic secondary metabolites like patulin, produced by *P. espansum* causing immune-suppression in hosts, *A.flavus* secrets aflatoxins that can be carcinogenic, *A. versicolour* releases the musty odours in homes, while some moulds are concerned in food deterioration.

Keywords: Filamentous fungi, isolation techniques, mycotoxins, reservoir and heath relevance

INTRODUCTION

The filamentous fungi consists of long structure known as filamentous hyphae which are extended to derive nutrients from substrates and produced for vegetative non-sexual reproduction with spores or cornidia and a vast are human pathogens. Fungi are constantly present in different water sources and their impacts lead to many biological processes such as biodegradation, biodeterioration, biofilm and its aesthetic health issues that are of enormous challenges to water-related regulatory agencies.
Fungi secrete digestive enzymes to breakdown biomolecules into simpler elements, prior to utilization as nutrients and in production of mycotoxins such as aflatoxins, fuminisins and zearalenons that are inimical to humans' health. The adverse effects ranged from being used as biological and chemical weapons introduced into water distribution systems, production of cadaverine and putrescine with pungent irritating odour. Though, they are useful in synthesis of antibiotics and other β-lactams and as biological pesticides to control weeds and pests (**Espinale & Montero, 2004**).
An alarming increase in the number of pathogenic diseases due to filamentous fungi is attributed to the increase in patients undergoing immunosuppressive therapies, organ transplants and acquired immune deficiency syndrome (AIDS) patients. The most common organisms responsible for this trend are the *Aspergillus* and *Penicillium*. The systematic investigation of fungi in drinking water system is on the increase, because it is usually performed when cases of contamination are reported. A limited number of tests revealed that, fungi are present in a significant amount in stored water samples, but the range of species is extremely variable (**Ferrti & Demoraes, 2001**). The objective of this study was to detect whether the storage tank in Crown Estate of Igbinedion University, Okada, Nigeria is a source of filamentous fungi of public health importance.

MATERIALS AND METHODS

Sample Collection

Water samples were collected from three water reservoirs (storage tanks) with each above 100,000 litres in the month of March, 2019 on weekly basis in the Crown Estate, Igbinedion University, Okada, Nigeria. Sterile bottles were sufficiently quantified with sodium thiosulphate to neutralize the chlorine. Five hundred millilitre (500 ml) water samples were obtained and stored at 4 ºC before the isolation was performed within 24 hr. A direct collection approach was used whereby the bottles were inserted onto the storage tank from the top and allowed to fill gently.

Isolation

This involved the concentration of fungi propagules and filtration processes.

Concentration

This is the trapping of propagules found in the sample by centrifugation at 2000 resolution per minute (rpm). The fungi contained in the sample were observed directly or allowed to grow and seen as colonies.

Filtration

One hundred milliliter of reservoir water samples was filtered through a pre-sterilized 0.45 μm Millipore filter paper and aseptically transferred onto an agar medium in 10 cm petri dishes for 5 d at 30 ºC. Four media that are cheap, simple and with good results were used in the test and these include half corn meal agar (CMA/2), neo-pentose-glucose-rose Bengal agar (NGRBA), sabouraud dextrose agar (SDA) and oomycetes selective medium (OSM). The observation of the filtered sample was carried out by microscopy.

Baiting

This test was mainly to determine the zoospore fungi (Oomycetes) and conducted as described (**Virginin *et al*., 2011**) with slight modifications. Many pieces of 1.0 cm sterilized snake skin and hallucinogenic seeds of tobacco plants were added to 100 ml fraction of samples in shake-flask incubated for 5 d at 30 ºC, during which period, any baits accompanied with presumed evidence of fungi colonies were aseptically accepted and transferred onto oomycetes selective medium (OSM) and samples plated out in duplicates.

Swapping

The swab was collected by using a scalpel-fixed with cotton wool at the tip to scrap the wall of the inner surface of the water reservoir. The obtained swab was then rinsed in saline solution (Nacl, 1.0 % w/v). A sample (0.1 ml) spread evenly over the medium surface, then dispensed into petri-dishes and incubated for 5 d at 30 ºC. The media employed were CMA/2; NGRBA and SDA.

Enrichment

This was done by inoculating 10 ml of diluted sample suspension onto 90 ml of SDA in shake-flask. Sub-samples (1.0 ml) were then inoculated onto the medium in plates, incubated for 48 hr at 25 ºC and observed for the growth of fungal colonies. This test was conducted to cater for samples with low fungi colonies.

Enumeration and Identification

All colonies on agar plates were counted and recorded as colony forming unit (cfu/100 ml) of water. The fungi were identified on the platform of macroscopic and microscopic characteristics. However, most *Aspergillus* was identified to species level according to procedures outlined (**Kimsey *et al*., 2010**).

RESULTS

Table 1 Number and percentage of different fungal colonies recorded by various methods and media

Media	Filtration	baiting	Plating	swabbing
CMA/2	18	-	20	1
NGRBA	10	2	34	3
OSM	2	3	14	3
SDA	25	1	32	2
All media	55 (32.0 %)	6 (3.5 %)	100 (59.0 %)	9 (5.3 %)

Key: NGRBA – Neo-pentose- glucose Bengal agar; OSM – Oomycetes selective medium; SDA – Sabouraud dextrose agar; DP – direct plating NGRBA – Neo-pentose- glucose Bengal agar; OSM – Oomycetes selective medium; SDA – Sabouraud dextrose agar; DP – direct plating

The baited and swabbed methods employed in this study recorded low number of fungal isolates. There was an estimated reduction of counts compared to the overall fungal counts (Table 1). The surfaces of the baits tended to be completely covered with bacteria and yeast cells which reduced the number of counts and the dryness of the inner wall of the water reservoir also contributed to the low counts recorded. Nevertheless, 6 fungal (CFU/ml) was obtained from baiting and 9 fungal (CFU/ml) was recorded from swabbing methods respectively. The direct plating and filter techniques recorded highest values of fungal colonies (100 and 55 CFU/ml). The NGRBA provided the highest recovering of filamentous fungi (34 out of 100) were detected using enrichment plate method (Table 1).
The fungal propagules microscopically examined showed that, the cornidia were more produced in all the media used with average score (5.3) and the sclerotia recorded the least mean (0.3) (Table 2)

Table 2 Mean of propagules on 1.0 cm pieces of filter employed from stored water sample sites

Isolation	hyphae	Spores (cornidia)	sclerotia
ST1	-	4	1
ST2	3	10	-
ST3	2	2	-
Mean	1.7	5.3	0.3

The table below showed the dissemination of organisms based on the isolation methods and media used; the species obtained were of 5 genera, the most abundant were *Aspergillus* and *Penicillium* which accounted for 63 %. The other organisms were *Acremonium simplex*, *Rhizopus stolonifer, Mucor racemosus* and *Trychophyton* sp. estimated at 37 %. The *Aspergillus* identified to species level included; *A.flavus, A fumigatus* and *A. versicolour*, while the *Penicillium* species were *P. brevicompacum* and *P. espansum*. Overall, all fungi were accordingly isolated from the three surveyed sites (ST1, ST2 and ST3) though the highest number of fungi was recorded in ST3, *A. fumigatus* and *P. brevicopacum* were present in all the sites investigated.

Table 3 Methods and media used in isolation for various fungal group

Organisms	Sites of isolation	Methods	Media
Acremonium simplex	2, 3	Filtration, direct Plating(DP), swab	CMA/2, SDA
Aspergillus flavus	1, 3	Baiting filtration	NGRBA, CMA/2, OSM
Aspergillus fumigatus	1, 2, 3	Baiting, DP, Filtration	CMA/2, SDA, NGRBA
Aspergillus versicolor	1, 2		
Penicillium brevicompacium	1, 2, 3		NGRBA, OSM
Penicillium espasum	1, 3	DP, filtration, baiting	NGRBA, OSM, SDA
Mucor racemosus	1, 3	DP, filtration, swab	CMA/2, SDA
Rhizopus stolonifer			
Trychonphyton sp.	3	DP, filtration, swab	All media

Key: NGRBA – Neo-pentose- glucose Bengal agar; OSM – Oomycetes selective medium; SDA – Sabouraud dextrose agar; DP – direct plating

DISCUSSION

The identification of vast number fungi is primarily based on morphological and microscopic convectional techniques of spore-bearing structures. Six genera were recovered from the three sites analyzed to include *Aspergillus, Penicillium, Acremonium, Rhizopus, Mucor* and *Trychophyton*. However, they contain variable number of species and therefore needed much attention to identify them to species level. The investigative methods and resources available as enunciated by **Mirian *et al.* (2007)** have enabled broad spectrum of isolation and identification realistic and a lot of fungi observed in the reservoir which were not water-related microorganisms.
The method employed that gave the highest count was the serial dilution enrichment plate method involving different media at incubation time of 5 d for 30 ºC. This study disclosed that, reservoir of Crown Estate, Igbinedion University, Okada is a source of filamentous fungi (ff) ultimately due to prevailing environmental conditions, isolation and growth media used. The growth of ff could be inhibited by the presence of bacteria and yeast cells competing for nutrients and toxins often released by ff can clear the proliferation of other microbes in the system. In this analysis, few organisms were recorded by the swabbing approach, thereby indicating that biofilm development had not occurred on the inner wall of reservoir.
The media devoid of antibiotics with raised ff level may indicate that chemotherapeutic substances have been produced by the fungi. The *Aspergillus* and *Penicillium* recorded highest number of occurrence (Table 3). **Briza *et al.* (1989)**; **Jose (1967)** declared that *P. espansum*, secretes chemicals that damage post-harvest diseases of apples and patulin (immune-suppressive toxin), *P. bravicompactum* produces mycophenolic acid that causes animal and human diseases such as oesophageal cancer, hormonal disorder and benign endemic nephropathy. *Aspergillus flavus* produces gliotoxin that suppresses the function of immune system and aflatoxin responsible for chronic granulomatous sinusitis, keratitis and wound infection. A. versicolour causes musty odours in homes and produces hepatotoxic and carcinogenic mycotoxins, while *A. fumigatus* produces gliotoxin that is immuno suppressive, and fumagillin that is cyclohexane derivative, known as inhibitor of endothelial cell proliferation. The *Acremonium* releases octenol, linked to odours, *Trychophyton* sp. is concerned with skin, toe, hair and nail infections and the *Rhizopus* and *Mucor* are involved in decomposition and spoilage of food substances.
In the study of **Virginin et al. (2011)** *Alternaria alternata, Aspergillus niger, P. espansum, P. bravicompactum*, few *molds* and *Trychophyton mentagrophyte* were detected in water systems in low counts. Differences in genera observed is adduced to conditions such as raw water sources, system hydraulics, water temperature patterns, maintenance and infrastructural systems from which the samples were collected. Despite the fact that all the fungi were obtained from the suspension, deriving reliable data for this analysis was difficult. This is thought to be due to the mycelial type of filamentous fungi which is capable of disintegrating into irregular structures to initiate their own growth and also as a result of the difference in fungi cornidia hydrophobicity which are uniformly distributed or aggregated, as some agglutinate due to thick gelatinous coatings (**Cast, 2013**). Realizing this fact, it is not possible to suggest a specific medium for growth, though numbers were recovered in all media. Although, all the fungi were recovered from the suspensions, obtaining ideal data for these organisms proved tedious.

CONCLUSION

Fungal proliferation in drinking water may be influenced by raw water source, system hydraulics, nutrient concentration and biotic interactions causing severe myco-infections in immunosuppressed hosts resulting from drinking, contact and inhalation of aerosol spores. If further scientific studies suggest an increase in disease risk, pilot epidemiological analysis and surveillance could be performed to checkmate the deleterious menace of fungi dissemination in drinking water systems.

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REGULAR ARTICLE

EFFECTS OF COMBINED INOCULATION OF BAMBARA GROUNDNUT (*Vigna subterranean L. Verdc.*) WITH *GLOMUS MOSSEA* AND *BRADYHRHIZOBIUM JAPONICUM* ON NITROGEN AND PHOSPHOROUS UPTAKE IN SHOOT, PLANT BIOMASS, LEAF CHLOROPHYLL AND MYCORRHIZAL INOCULATION EFFICIENCY (MIE).

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ABSTRACT

The response of Bambara groundnut to co-inoculation with Abuscular mychorrhizal fungi (*G. mossea*) and *Bradhyrhizobium japonicum* (strain USDA110) with regard to leaf chlorophyll, percentage shoot nitrogen and phosphorus, nodule weight and plant biomass was studied. Bambara plants were grown under screen house conditions in pots. Plants were inoculated with 1ml of *B. japonicum* USDA 110 strain (10^9 cfu /ml), Mychorrizal was applied to the plants 10g, and 20g (90 spores/g) and water was applied at 10ml, 20 ml and 50ml every other day. The obtained results showed that dual inoculation activity was able to improve both nitrogen and phosphorus in plant shoot, MIE, but did not improve biomass and leaf chlorophyll when compared with plants subjected to single inoculation with only *G. mossea* and only *B. japonicum.* More Nitrogen and Phosphorus was retained in the shoot of plants co-inoculated with *B. japonicum* and 20g *G. mossea* when given 50ml of water and also had higher biomass. Leaf chlorophyll reduced in plants as flowering approached. *B. japonicum* was able to positively influence and establish symbiosis with *G.mossea* and synergistically effectively act as "mycorrhiza helper bacteria" (MHB) when both were co-inoculated in Bambara plant.

Keywords: *G. mossea*, *B. japonicum, Water stress,* Bambara groundnut

INTRODUCTION

Bambara nut (*Vigna subterranean* L. Verdc.) is a leguminous seed crop of African origin (Nwanna *et al*., 2005), that is highly underutilized and been found to have a high nutritive value and drought tolerance (Anchirinah *et al*., 2001 and Ocran *et al*., 1998). It is considered to be a famine culture crop probably because it associates with mycorrhiza. Synergy between mycorrhizal fungi and rhizobia micro symbionts (nitrogen fixers) in legumes has been studies by (Jesus *et al.,* 2005; Kaschuk *et al.,* 2010) and their association described as a tripartite (Vega *et al.*, 2010), where the Mycorrhizal help to increase the absorption and solubilisation of phosphorus to rhizobia in plant nodules (Scotti; 1997), while Rhizobia fix nitrogen provide it as ammonia to the plant, which provides carbohydrate to micro-symbionts (Silveira *et al*., 2001; Gross *et al*., 2004). The benefit of these microorganisms to the host plant depends on the compatibility between the rhizobial strain and mycorrhizal fungi inoculated. Positive symbiosis formed with mycorrhizal fungi (Frey-Klett *et al.,* 2007), when found to be synergistically effective they are called "mycorrhiza helper bacteria (MHB) (Garbaye; 1994). Fungal-rhizobial inoculant has been able to increase N2 fixation in soybean by 30 % as compared to conventional use of rhizobia (inoculant) (Jayasinghearachchi and Seneviratne, 2004). For legumes cultivation, the relationship between mycorrhiza and rhizobia and is highly importance because it affects the rate of infection and mineral nutrition as well as the chemical and physical conditions of the soil by adding organic waste and increasing the growth of these plants (Andrade *et al*., 2000). When there is deficiency of phosphorus, there is low nodulation and nitrogen fixation in legumes have except if their roots are colonized by mycorrhizas or an alternative source of phosphorus in soil is made available. Moreover, the mycorrhizal condition influences the efficient competition among strains of rhizobia to occupy the nodules in the roots of the host (Miranda and Miranda, 2002; Garg and Manchanda, 2008). Kaschuk *et al.,* (2010) studied the AMF-rhizobia symbiosis in 12 legume species, and they reported an increase in the photosynthetic rate and grain yield of legumes. However, according to Scotti (1997), the benefit of these microorganisms to the host plant depends on the compatibility between the strain of rhizobia and mycorrhizal fungi inoculated. Experiments are needed to study the compactibility of symbiotic microorganisms (bacteria and mycorrhizal fungi while considering water availability) for Bambara groundnut which will enhance the understanding of symbiosis (Gueye; 1992) in the plant and provide knowledge about their role in enhancing the plant resistance to drought this is because increasing the use of BNF is a major way to increase or maintain the yield of legumes (Ngakou *et al*., 2012), reduce the footprint of agriculture on the environment and may be used to address the current challenge of meeting the fast-growing demand for agricultural products worldwide.

MATERIALS AND METHODS

Prepation of broth for inoculation

Pure cultures of the *rhizobia* isolates was obtained and inoculated into 100ml Erlenmeyer flasks containing 50 ml of yeast-mannitol. The inoculated broth was incubated at 28°C on a Rotary shaker for 7 days after which the bacterial count when satisfactorily determined to be about 10^9 cfu/ ml, was then used to inoculate plants at 1 week of growth (Woomer *et al*., 2012).

Pot Experiment

Sea sand was washed repeatedly with water to remove debris and to reduce pH to between 6.6 - 6.8 which is most suitable for *rhizobia* growth. The crushed gravel and medium sized gravel were also washed till the water was clean. The sea sand, crushed gravel and peat were mixed in a ratio 6:6:1 and mixed until it was evenly distributed. The mixture was then sterilized at 121°C and 1.05 kg cm^{-2} for 15 minutes. The medium sized gravel was also sterilized (Woomer *et al*., 2012). Sterilized 500 ml pots were filled with the sterile sand and sterilized seeds were planted in in them and allowed to germinate. One week after planting (WAP), the BG plants were thinned to one viable plant per pot.1 ml of the inoculums which were already prepared as described above, was introduced into the cowpea plants. Bambara groundnut seeds were sterilized and planted in sterilized soil in pot under screen house conditions and allowed to germinate. Dual inoculation of *B. japonicum* (USDA110 strain) (1ml) (Somasegaran, and Hoben, 2012)) using sterile pipette and 10 g and 20g of mychorrhiza (*G. mossea)* (Carine *et al*., 2017 and Gomoung *et al*., 2017) was applied to plants and limited amount of water (10ml, 20ml, 50ml) was also applied. A completely randomized block experimental design was used treatments (see Table 1) (including three controls, KNO_3 treatment to which nitrogen was applied, Rhizobial application alone and the un-inoculated control) were replicated in each of the 4 blocks.

Application of nutrient and water to Plants

Cowpea plants were allowed to grow for 8 week during which they were given 20 ml of nutrient solution consisting of both micro and macro nutrient. To prepare nutrient solution given to plant, the stock solutions were mixed using 100 ml of

macro- stock solution and 10 ml micro- stock solution made up to 10 liters using distilled water. The nutrient solution was sterilized at 121°C and1.05 kgcm^{-2} for 15 minutes and was aseptically given to the plants weekly.
The solution for the N+ treatment (control containing nitrogen) was prepared using 5% of N in KNO_3 this was sterilized at 121°c and 1.05 kg cm^{-2} for 15 mins after which 50 ml of the solution was added to the plant weekly. Water application was done by giving 10ml, 20ml and 50ml of water (according to the treatment) every other day to the plants (Table 1).

Harvesting

Plants were allowed to grow for 10 weeks, after which they were harvested by cutting at the base with a secateur. The shoots and roots were placed in labelled paper bags in an oven were they were dried at 68°C for 72 hours until constant weight was obtained. The shoot dry weights were recorded.

Determination of chlorophyll

Chlorophyll readings were taken from leaves using a Spad meter at the 2ND 4TH and 6TH weeks after planting.

Determination of %N and %P in plant shoots

The total % Nitrogen in the shoots of the plants were determined using the micro Kjeldahl method (Kjeldahl; 1883) while phosphorus was determined by the molybdenum blue method (Murphy and Rilley, 1962). The three control treatments plants were used as a reference plant.

Statistical Analysis

The data collected was analysed for correlation (Pearson's) using SPSS version 20 and Fisher's least significance was used to compare means at $p \leq 0.05$.

RESULTS

3.1 %Nitrogen

the %N in the shoot increased with increase in the amount of water in both 10g and 20g G. mosses applications. 10g/10ml treatment had a lower %N than the treatment that had only 10g with no rhizobia inoculation, but the 10g/20ml and 10g/50ml treatments had higher %N than the 10g only treatment. All 10g *G. mosseae* had lower %N than the USDA110 only treatment but were higher than the un-inoculated control and the KNO_3 control while the 20g/10ml, 20g/20ml, 50g/ml all had higher %N than the KNO3 and un-inoculated control but only 20g/10ml had a %N that was lower than that obtained in the USDA110 only treatment.

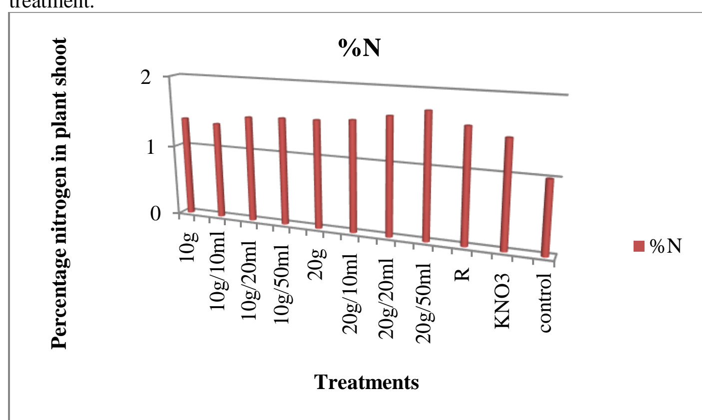

Figure 1 Uptake of Nitrogen in shoot of plants

3.2 %Phosphoru

%P increased with increase in the amount of water at both 10g and 20g *G. mosseae* applications. The %P of USDA110 only was lower than that of all other treatments except that of the un-inoculated control. While the 10g/50ml and 20g/50ml treatments had higher %P than their corresponding treatments that had only *G. mosseae* with no USDA110 applied in them.

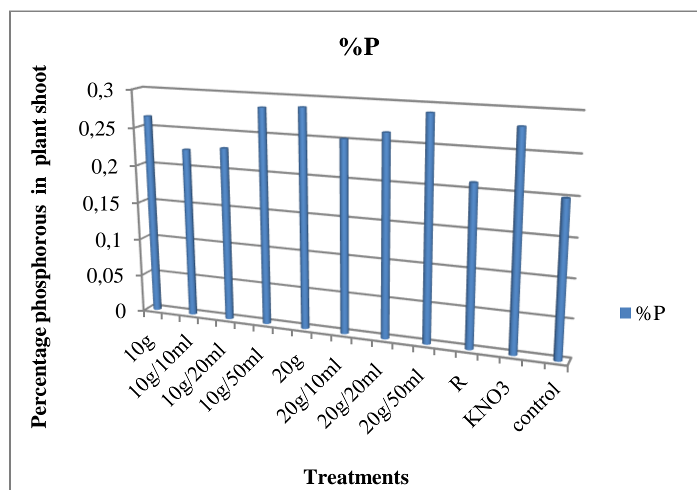

Figure 2 Uptake of Phosphorous in shoot of plants

Leaf chlorophyll

Leaf chlorophyll reduced steadily from the 2ND week to the 6TH week in all treatments and also decreased with increased application of water.

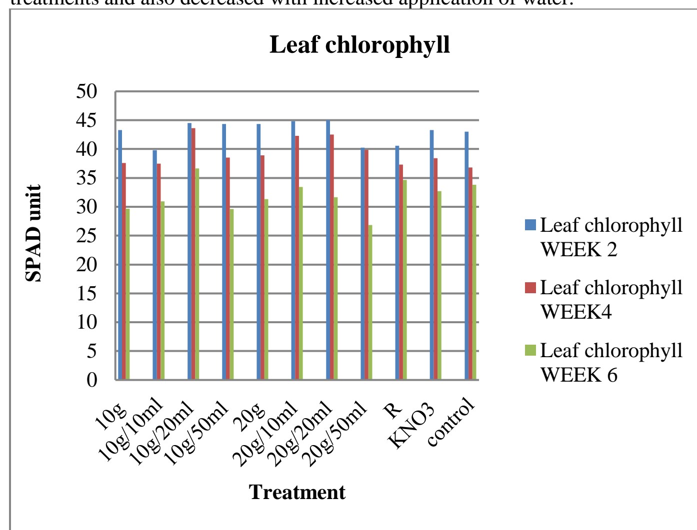

Figure 3 Leaf chlorophyll of experimental plants

Shoot weight

Shoot weight increased with increased application of water in both 10g and 20g *G. mosseae* treatments but plants with USDA110 inoculation had higher weights than their counterparts with no USDA110 inoculation.

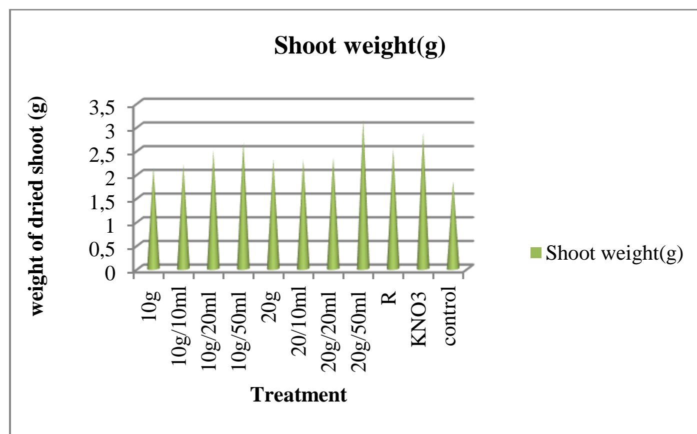

Figure 4 Dried weight of plant shoot

Mycorrhizal Inoculation Efficiency (MIE

MIE increased significantly with increase in the amount of water application when both 10g and 20g of *G. mosseae.* When 20g *G. mosseae* was applied there was no significant difference in the MIE on application of 10ml, 20ml and 50ml of water while there was significant difference for the 10g application.

Correlation

There was a positive correlation between leaf chlorophyll and shoot dry weight, %P and negative correlation between %N and MIE which were not significant. Significant negative correlation was found between the % N and shoot dried weight, and no significant correlation between the MIE phosphorous but n and other parameters although it had a negative correlation with the leaf chlorophyll at the 2nd and 6th week after planting.

Table 1 Mycorrhizal Inoculation Efficiency (MIE) in plants

Treatments	Water (ml)	Inoculum amt (ml)	*G. mossea* (g)	MIE(%)
10g	100	-	10	15.14
10g/10ml	10	1	10	18.9
10g/20ml	20	1	10	34.1
10g/50ml	50	1	10	43.78
20g	100	-	20	24.89
20g/10ml	10	1	20	24.89
20g/20ml	20	1	20	27.57
20g/50ml	50	1	20	69.73
R	100	1	0	0
KNO3	100	-	0	0
control	100	-	0	0

Table 2 Correlation between plant parameters

	leaf chlorophyll Week 2	leaf chlorophyll Week4	leaf chlorophyll Week 6	%Nitrogen	%Phosphorus	Shoot dried weight(g)	Mychorrhizal Inoculation Efficiency (%)
leaf chlorophyll Week 2	1	.110	.116	-.269	.393	-.112	-.146
leaf chlorophyll Week4	.110	1	.299	.411	.147	.177	.426
leaf chlorophyll Week 6	.116	.299	1	-.371	-.645*	-.382	-.593
%Nitrogen	-.269	.411	-.371	1	.582	.699*	.586
%Phosphorus	.393	.147	-.645*	.582	1	.643*	.569
Shoot dried weight(g)	-.112	.177	-.382	.699*	.643*	1	.600
Mychorrhizal Inoculation Efficiency (%)	-.146	.426	-.593	.586	.569	.600	1

*Correlation is significant at the 0.05 level

DISCUSSION

Availability of water had effect on the amount of Nitrogen and phosphorus uptake and shoot weight which all increased as the availability of water increased similar to the findings of Carine *et al*., 2017. Higher amounts of *G. mosseae* also had effects on the N, P, shoot weight and MIE (Fig 1, 2, 3 and Table 1) this is similar to the findings of Moila; 2018. The Mycorrhizal Inoculation Efficiency increased with the amount of water and amount of mycorrhizal when dual inoculation was used in plants and is similar to the report given by Esale *et al*., 2015 and Tsoata *et al*., 2015.

There was no significant increase in leaf chlorophyll when dual inoculation was applied differing from the result obtained by Kaschuk *et al*., 2010 were there was an increase in the photosynthetic rate. Although Bambara did not necessarily depend on mycorrhiza for satisfactory growth and nodulation with rhizobia as observed by Jesus *et al*., 2005, its presence enhanced the development, growth, %N, %P, and MIE especially under conditions of limited water.

The treatment with 20g/50ml of water had the highest value for all the parameters taken. The Mycorrhizal Inoculation Efficiency increased with the amount of water and amount of mycorrhizal when dual inoculation was used in plants and is similar to the report given by Esale *et al*., 2015, Tsoata *et al*., 2015.

CONCLUSION

Although Bambara groundnut did not necessarily depend on mycorrhiza for satisfactory growth and nodulation with rhizobia, its presence enhanced the development, growth, %N, %P, and MIE especially under conditions of limited water. In addition, *B. japonicum* was able to positively influence and establish symbiosis with G.mossea and synergistically effectively act as "mycorrhiza helper bacteria" (MHB) when both were co-inoculated in Bambara plant.

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REGULAR ARTICLE

YEAST DYNAMICS AND PHYSIOCHEMICAL EVALUATION OF CARROT WINE PRODUCED WITH *Saccharomyces cerevisiae*

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ABSTRACT

Yeast dynamics and physiochemical evaluation of carrot wine produced with *Saccharomyces cerevisiae* were assessed. Fresh ripe and health carrot (6kg) were sequentially processed (washed, preheated, blended and sieved) into juice and fermented for 60 days with *Saccharomyces cerevisiae*. Airtight glass jars composed of juice (2000g), distilled water (2000mL) and sugar (200g) at controlled temperature (20 -25 ^{0}C) was used for fermentation. Wines were clarified (siphoning), aged (45 days) and pasteurized (50^{0}C – 60^{0}C) to stop fermentation. Proximate analysis, yeast dynamics, physiochemical and wine qualities were assessed. Result showed that juice extraction process reconstitute nutritional composition of carrot, such that moisture, ash and total carbohydrates increased, while others (fat, crude fiber and crude protein) decreased. A trendy progressive yeast dynamic model of ***Yeast load*** *= -0.195* ***(Day)*** ^{2} *+ 1.822* ***(Day)*** *+ 4.566* with coefficient (R^2 = 0.907) was observed. Fermentation significantly decreased pH and increased total acidity. Observed wine qualities include alcoholic content (7.88 - 9.19% v/v), attenuation (121% - 142%) and calories (0%). Clarification and ageing have diminishing effect on alcohol content. Carrot wine was judged as physically appealing moderate alcoholic beverage, with smooth consistent taste (authors' opinion), and could be modeled with yeast dynamics. Thus this wine is recommended to calories sensitive people.

Keywords: Carrot, wine, Saccharomyces *cerevisiae*, yeast dynamic, physiochemical

INTRODUCTION

Wines other than grape wine are classified as fruit and vegetable wines. Alcoholic beverage of fermented carrots is among the vegetable and fruit wines. Across African, Asian and Latin American countries, there are many locally fermented alcoholic wines brewed from popular fruit and vegetables such as banana, dates pineapples and grape (**Swami *et al*. 2014**), and unpopular among them is carrot. Carrot (*Daucuscarota*) is one of the edible root vegetables classified as tubers; with prehistory from central Asia region. Popular bright orange varieties (used in this study) are among others (purple, red, white, and dark) colour varieties (**Siemonsma**, **1994**) composted of high level of ß-carotene (pre-vitamin A) and carbohydrates (sugars), vitamin B complex and minerals (**Bystrická *et al.* 2015**). Preheating of carrot to tenderness improve the natural sweetness and nutritional value (Snodgrass. 2004). Cooked or raw carrot vegetable have varying medicinal purposes (Carlos and Dias 2014) and as well reduces the risk of oxidative diseases due to high antioxidants (**Shukla *et al*. 2014; Kumari *et al.*, 2014**).
Most winery processes are sequentially in three operational stages such as before fermentation, during fermentation and after fermentation. Typically, fermentation stage of wine production, yeasts convert sugar to carbondioxide gas and alcohol (**Swami et al. 2014**) within varying time duration in control or uncontrolled chamber. Optimization of wine processes is mostly targeted at controlling the fermentation environments of physiochemical (pH, temperature, salt, chambers etc), redox (O_2, H_2 etc) yeast growth dynamics, nutrition, time and organoleptic parameters (**Keller, 2010**). Proliferation of some species of yeast (*Zygosaccharomyces* and *Brettanomyces*) in wines, sometimes leads to failed fermentation (**Loureiro, *et al.* 2003**), due to synthesis of array of volatile phenolic metabolites. These metabolites poison the yeast and add odd flavours to the wine. *Saccharomyces cerevisiae* is most popular yeast used in bakery and winery industries; it is single celled eukaryotic budding yeast (**White *et al.,* 2010**) initially isolated from the surface of grapes. *Saccharomyces cerevisiae* are used as primary fermenter due to their ability to produce CO_2 and alcohol in aerobic or microaerobic conditions by metabolizing sugar. For predictability of fermentation, sufficient primary fermenters are initially added to repress the wild yeast (**Vaughan-Martini *et al.,* 1995**; **Gonzalez *et al*., 2001**). Varying fermentative and physiological properties of different strains of S. *cerevisiae*, necessitate the selection of appropriate strain to achieved required impact on finished wine (**Swami *et al*., 2014; Dunn *et al*., 2005**).

MATERIAL AND METHODS

Sample Collection/preparation

A total of 6kg of fresh ripe and healthy carrot vegetables were obtained from community markets within Lapai, LGA Niger state, Nigeria. Samples (bright yellow carrots) were transported to the laboratory in clean plastic bags (< 4^{0}C), then sorted, washed, weighed and stored in refrigerator prior fermentation processing.

Preparation of Inoculum Starter Culture

S. cerevisiae pure colonies were obtained from repository, Microbiology laboratory, Ibrahim Badamasi Babangida University, and verified using potato dexterous broth (PDB) in 250ml Erlenmeyer conical flask at room temperature incubation for 24hrs with relevant biochemical assay. Verified Isolates were primed for 3 to 6hrs in PDB media and yeast cell concentration (mean of triplicate counts) was determined by direct counting method using hemacytometer (Levy, USA). The stock solutions were reconstituted to cell count of ~10^6cfu/mL and stored (4 ^{0}C) as described by **Balogu et al., (2016).**

Fermentation Protocol

A total 6kg of three sets(2000g) of fresh carrots root vegetable were separately chopped (~2cm^3), steamed for 10 mins, minced with industrial blender for 20mins to achieve fine slurry. The slurries were sieved in muslin bag to obtain the carrot juice. Each juice (500 mL), distilled water (2000 mL) and sugar (200g) were discharged in a clean glass jar (steam sterilized at 15 psi and 121^{0}C for 15 minutes) and allowed to cool to room temperature. The glass jars were sealed with stoppers (fitted with gas-valve), agitated and allowed to stand for 24 hr. The 24 h old mixture was degassed (mild sulfur gas) before 200mL of starter culture (~10^6cfu/mL) was added. Culture fermented for 60 days at 25^{0}C - 30 ^{0}C, with agitation and degassing every 48 h. Wine was clarified by siphoning the supernatant of wine sediments into a clean steam sterilized long neck round bottom glass bottle sealed with gas-valved stopper. Citric acid (to limit spoilage by bacteria) added to the clarified wine and allowed to continued fermentation for 7 weeks (46days) and pasteurized (50^{0}C – 60^{0}C) to stop fermentation (**Balogu and Towobola 2017**).

Specific Gravity

50ml of each sample were discharged into volumetric flasks at 20⁰C and hydrometer was used to determine specific gravity (appropriate correction factor was factored in with temperature variations). The alcohol content (%), apparent attenuation and calories were determined (**Balogu and Towobola 2017**).

Proximate composition and Physiochemical analysis parameter

Nutritional composition of fresh carrot root vegetable was analysed and selected physiochemical parameters such as pH, temperature and titratable acidity were assessed. Temperature and pH were determined using standard methods. (**AOAC, 1999, Balogu and Towobola 2017**).

Microbial Analysis

Yeast assays (isolation, characterization and enumeration) was conducted on samples collected at 15 days intervals of fermentation using PDA (yeast) and relevant biochemical tests in accordance with the methods of **Cheesbrough (2010).**

Statistical Analysis

Data generated were subjected to ANOVA, Duncan's Multiple Range Test and Chi – square using SPSS software version 20 of 2014.

RESULTS AND DISCUSSION

Proximate composition of Carrot (juice and whole) showed that moisture were 89.25% and 78.06%, ash(1.42% and 1.26%), fat (0.91% and 1.83%) crude fiber (1.24 % and 13.36%), crude protein (1.12 % and 2.44%) and total carbohydrates were 6.06% and 3.05% respectively (Table 1).

Table 1 Proximate analysis of carrot (*Daucus carota*) juice

Component	Carrot (%)	
	Whole	Juice (extract)
Moisture	78.06	89.25
Ash	1.26	1.42
Crude fiber	13.36	1.24
Crude protein	2.44	1.12
Fat	1.83	0.91
Total carbohydrate	3.05	6.06

Composition of whole carrot were significantly ($P<0.05$) different from the carrot juice. The discarded carrot chaff after juice extraction accounts for these discrepancies. This was obvious in the values of crude fiber (major constituent of the chaff) between the whole carrot and the juices. High moisture component decreases shelf life of beverages due to enhanced yeast proliferation. Invariably, whole carrot drinks are likely to have longer shelf life than carrot juice drinks. **Olalude et al. (2015)**, observed similar composition of carrot juices and expressed related opinion on the correlation of moisture and microbial growth. Also higher carbohydrate composition in juice than whole carrot would further enhance the rate of microbial spoilage.
Yeast profile increased steadily from 6log10 cfu/mL to 8log10 cfu/mL within 60 days fermentation. Within the five interval sampling, the yeast load were 7.95log10 cfu/mL, 7.86$\log_{10}$ cfu/mL, 8.75$\log_{10}$ cfu/mL, 8.84$\log_{10}$ cfu/mL observed at day 15, 30, 45 and 60 respectively. Dynamic yeast model [***Yeast load** = -0.195 **(Day)**^{2} + 1.822 **(Day)** + 4.566*] with $R^2 = 0.907$ was observed (Fig 1). Application of models in winery processes would optimize the process and minimize production cost. The coefficient (R^2 = 0.907) indicated more than 90% optimization of yeast profile within 60 days fermentation. This means that the conditionings of intrinsic and extrinsic factors are responsible for the optimization.

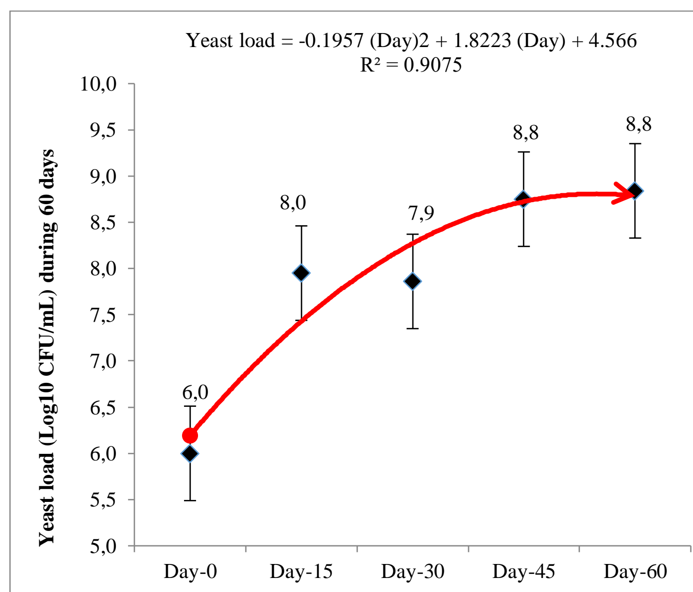

Figure 1 Yeast load dynamic of carrot wine fermented for 60 days. NB: ◆ = mean plot of triplicate values ⌶ = standard error of mean plot

Among the physiochemical properties, pH significantly decrease from 7.47 to 5.21 and TTA increased significantly from 1.20 to 2.12, while temperature was not significantly ($P<0.05$) altered after fermentation. (Table 2). Since temperature predetermines yeast activities, the relative stability in temperature (24.7 ± 0.02) attributes to the trendy typical yeast climax observed in this study.

Table 2 Physicochemical properties of carrot juice and wine after 60 fermentation

Parameter	Fresh carrot juice (0 day)	Fermented carrot wine (60days)
Temperature (⁰C)	24.6 ± 0.17	24.7 ± 0.02
pH	7.47 ± 0.16	5.21± 0.02
TTA (%v/v)	1.20 ± 0.016	2.12 ± 0.0721

Matunda (2015) and Idise, (2012) collaborated that alcoholic beverage of pineapples are optimized at temperature range of 20 - 30⁰C. The observed change in pH depicts that carrot wines are acidic alcoholic beverages though, less acidic than pineapple produced under similar parameters. **Goswami and Ray (2011)** optimized alcoholic yield of grape wine at pH 5.0 - 5.5, validating this study's average pH (5.21). It is not surprising that fermentation increased TTA by more than 56%, confirming that fermentation unlocked most of the acidic antioxidants (organic acids) abundant in carrots. This strengthens the finding of **Grooper et al., (2012),** that most vegetable fruits rich in antioxidants are acidic (at pH 1-5).
Alcoholic strengths of carrot wine were evaluated using the specific gravity (SG) of the samples. Within 15 and 60 days interval, SG were 0.9800 and 0.9900, Alcoholic content (9.19 and 7.88), attenuation (142% and 121%) respectively. However, carrot wine has no calories after 60 days fermentation (Table 3). Making carrot wine a good option for those that are calorie and weight sensitive. Alcoholic content decreased by 14% within 46 days of ageing, due to utilization of ethanol after depletion of chapitilized sugar. Reduction in percentage attenuation (rate of sugar conversion to ethanol) of aging wine, further strengths this argument.

Table 3 Alcoholic content of Carrot Wine

Duration	Specific gravity	Alcoholic (%v/v)	Calories (/355ml)	Attenuation (%)
15 days	0.9800	9.19	0.0	142
60 days	0.9900	7.88	0.0	121

Initial SG = 1.050

CONCLUSION

Carrot wine processed with *Saccharomyces cerevisiae* would yield a moderate alcohol and low calories wine. Carrot wine is physically appealing (Fig. 2),

consistent smooth feeling (authors' opinion only) and could be modeled with yeast dynamics.

Figure 2 Bottled carrot wine after 60 days fermentation Adjudged as physically appealing and smooth taste feel.

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