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SHORT COMMUNICATION

PRODUCTION OF ALPHA AMYLASE AND CELLULASE FROM SOLID STATE CULTURE OF *ASPERGILLUS OCHRACEUS*: A FEASIBILITY ANALYSIS

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ABSTRACT

The growing demand and application of industrially important enzyme necessitate the need to explore new sources with diverse enzymes ranging in their specificity and activities. Enzymes are safe alternatives to chemical synthesis due to minimum side effect and ease of manufacturing. Solid state fermentation (SSF) is a cost-effective alternative to submerged fermentation with agro-residues or waste, often being used as substrate for growing diverse organisms for production of metabolites. Current study is one of the scarce report on exploring alpha amylase and cellulase production ability *Aspergillus ochraceus* (MTCC 1877) using wheat bran as substrate at relative humidity of 90% and at 30 ºC, for 7 days. Result showed the potential of *Aspergillus ochraceus* (MTCC 1877), as potential source of the two enzymes. Results revealed comparatively higher alpha amylase activity in the SSF extract of *Aspergillus ochraceus* (MTCC 1877) in comparison to *Trichoderma longibrachiatum* (ITCC 7839). On the contrary, comparatively higher cellulase activity was observed in the SSF extract of *Trichoderma longibrachiatum* (ITCC 7839). The results showed the potential of *Aspergillus ochraceus* (MTCC 1877) as a source of the two enzymes. Variation in enzymes activity may be attributed to the experimental culture conditions and may be further optimized to enhance the enzymes yield.

Keywords: Enzymes, Solid state, *Aspergillus ochraceus*, *Trichoderma longibrachiatum*, cellulase, alpha amylase

INTRODUCTION

Solid state fermentation (SSF), is a well explored technology, used for production of value-added products, by growing bacterial and fungal strains on solid substrate (or support) with insignificant or no free water. The technique had been used for centuries, for developing fermented food **(Hyseni *et al.*, 2018; Pablo *et al.*, 2020)**. The 'GRAS' strains mediated development of fermented food, feed and pharmaceutical products had been well explored using SSF **(Barrios-González *et al.*, 1988; Ramachandran *et al.*, 2004; Sitanggang *et al.*, 2020)**. Enzymes are one of the most well explored and utilized biomolecules, in various industrially important sectors, and are economically produced using SSF **(Pandey *et al.*, 2008; Liu and Kokare, 2017)**. These biocatalysts are primarily proteins in nature and are produces from diverse range of organisms **(Liu and Kokare, 2017).** It is this diversity of biomolecules from different sources and their associated properties that obtrude them as a suitable safe alternative to chemical catalysts **(Vanacek *et al.*, 2018).** It is this diversity in the catalytic properties of enzymes that had been a boon for their application in various commercial sectors ranging from food, feed and pharmaceutics to paper and pulp, jute and textile sectors, environmental remediation, and others **(Koyani and Rajput, 2015; Raveendran *et al.*, 2018).**

Diversity in the habitats of different microorganisms is associated with the diversity in the reaction conditions variability of the enzyme **(Müller *et al.*, 2015; Garcia-Garcera and Rocha, 2020).** It is these diversities and wide acceptability of enzymes in different sectors that provide an impetus to explore new or novel organisms as source of enzymes.

The enzyme production from fungal strains on agro-residues as substrate, through SSF had been reported to be higher in yield and cheaper in cost, thereby making the overall process economical **(Pandey *et al.*, 2008; Agrawal *et al.*, 2013; Pablo López-Gómez *et al.*, 2020).**

Previous studies have shown the vast varieties of enzymes, that can be successfully produced using *Aspergillus sp.* **(Hu *et al.*, 2011; Lopes *et al.*, 2011; Shinkawa and Mitsuzawa, 2020).** The fungi, *Aspergillus ochraceus* is, well known for its mycotoxic effects on plant and chicks owing to the production of secondary metabolite, Ochratoxin A, extensively reported for its hazardous effects **(Doupnik, 1970; Hocking, 2006; Martínez-Rodríguez and Santiago, 2011; de Almeida *et al.*, 2019).** However, recent interest in *A. ochraceus* is primarily due to the therapeutic potential of other metabolites from the fungi. Studies by **Hu *et al.*, (2021)** had shown the anti-Parkinson's effects of alkaloids and other metabolites produced by *A. ochraceus*. In another study by **Aracri *et al.*, (2019),** the potential of *A. ochraceus*, as a potential source of enzyme Tannase had been reported.

Current study is the first feasibility analysis of the fungal strain, *Aspergillus ochraceus* MTCC 1877, as a potential source of industrially important enzymes (*viz.* alpha amylase and cellulase) on agro-residues, using solid state fermentation.

MATERIALS AND METHODS

Fungal strain and culture media

The current studies were performed using *Aspergillus ochraceus* (MTCC 1877), purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The fungi *Trichoderma longibrachiatum* (ITCC 7839), was used to compare the output of SSF among the two fungi. All further studies were performed using the two fungi, under similar experimental conditions. The growth and maintenance of the strain was done using Potato dextrose agar (HiMedia Laboratories Pvt. Ltd., India). The solid-state fermentation studies were performed using wheat bran as substrate. All other chemicals used in the studies were of analytical grade, unless specified.

Growth and maintenance of fungal strain

The fungi were revived from the cryostocks of fungi, preserved at – 20 ºC. Both the fungi, *Aspergillus ochraceus* (MTCC 1877) and *Trichoderma longibrachiatum* (ITCC 7839), were revived from the cryostock and propagated on potato dextrose agar (PDA) plate at 30 °C for 7 days in incubator (Kühner, Switzerland). After revival of the strains, the culture plates were stored at 4 °C.

Solid state fermentation studies

The humidity is an important component of solid-state fermentation and largely depends on nature of substrate. In current study, the change in relative humidity (RH) with increasing volume of water was measured using Winner Thermo – hygrometer TH-402 (Fig. 1). The wheat bran was autoclaved twice at 121 ºC for 30 minutes to reduce the microbial load. The plastic petri plate with 20 g wheat bran and RH of 90% was used for solid state fermentation studies. The plates were inoculated with two cuboidal pieces (1cm × 1 cm; length × width, height depends on thickness of agar plate) each, and the plates were incubated at 30 ºC for 7 days. After the 7 days of culture, the fermented solid materials were harvested in a muslin cloth that was used for enzyme extraction.

Extraction of Enzymes

The feasibility of SSF harvest as a source of enzymes, alpha amylase and cellulase, were analyzed by suspending 40 g harvest and tied in muslin cloth, in

200 ml distilled water at 270 rpm, 4 ° C for 2 hours. The liquid extract was used further for enzyme activity.

Enzyme assay for Alpha Amylase

The activity of α-amylase in the extract was analyzed using the alpha amylase assay method reported by **Luo *et al.*, (2019),** with modification. The assay method involves estimation of reducing sugars, produced on hydrolysis of starch using Dinitrosalicylic acid (DNS) reagent. The reaction mix for prepared by mixing 1 ml of 1`% (w/V) starch with 1 ml of extract. The reaction mixture was incubated for 10 min. Further 1 ml DNS reagent and 5 ml distilled water was added, and the solution was boiled. The solution was cooled and the absorbance at 540 nm was monitored. The enzyme activity was defined to be 1 U/ml when 1 mg of reducing sugar was liberated per unit time, per unit volume of enzyme under optimum conditions of temperature and pH. The quantity of reducing sugar was estimated using the standard plot, prepared using glucose as standard.

Enzyme Activity of Cellulase

The activity of Cellulase in the extract was estimated using the assay method suggested by **Lone *et al.*,(2012),** with some modifications. Briefly, 1ml of cellulose (1% microcrystalline in water) was mixed with 1 ml extracted and incubated at room temperature for 10 minutes. The mixture was further supplemented with 1 ml DNS Reagent and 5 ml water. The tubes were placed in boiling water bath at around 80 ºC – 100 ºC. The solution was cooled and the absorbance at 540 nm was monitored. The enzyme activity was defined to be 1 U/ml when 1 mg of reducing sugar was liberated per unit time, per unit volume of enzyme under optimum conditions of temperature and pH. The quantity of reducing sugar was estimated using the standard plot, prepared using glucose as standard.

RESULTS AND DISCUSSION

Cellulase and alpha amylase are widely used in food, feed and pharmaceutical sectors. The vast application of these industrial enzymes, obtrude them as highly demanded enzymes. Current study explored the potential of *Aspergillus ochraceus* MTCC 1877, as a suitable alternative source of the enzymes. The choice of using *Trichoderma longibrachiatum* ITCC 7839, in the study, for comparisons of results, was based on previous reports highlighting *Trichoderma longibrachiatum* to be the source of cellulase **(Tegl *et al.*, 2016)** and amylase **(Kovacs *et al.*, 2004)** enzymes. Results showed that the *Aspergillus ochraceus* MTCC 1877, had the potential of being a source of two industrially important enzymes, *viz.*, cellulase and alpha amylase. The comparative analysis of the results of the two fungi (Figure 1), showed comparatively higher activity of alpha amylase in the extract from *Aspergillus ochraceus* MTCC 1877. The results suggest the importance of culture conditions in the variation in yield of enzyme among the two fungal strains. Further, the analysis of cellulase activities among the two organisms (Figure 2), revealed the opposite response of two fungi. The cellulase activity after the SSF was observed to be higher in the extract of *Trichoderma longibrachiatum* ITCC 7839 than *Aspergillus ochraceus* MTCC 1877.

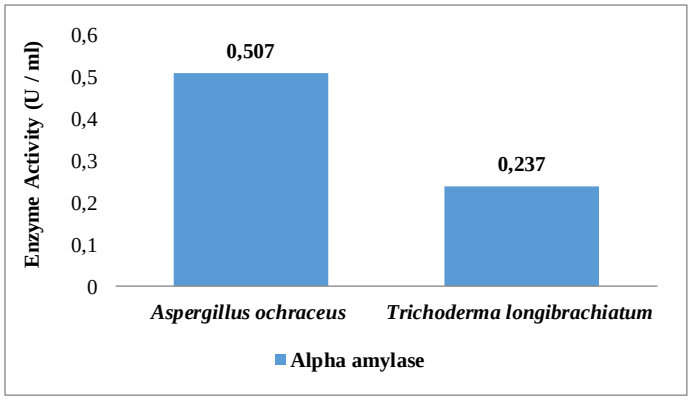

Figure 1 Comparison of the alpha amylase activity (U / ml) of *Aspergillus ochraceus* and *Trichoderma longibrachiatum* in solid state fermentation studies

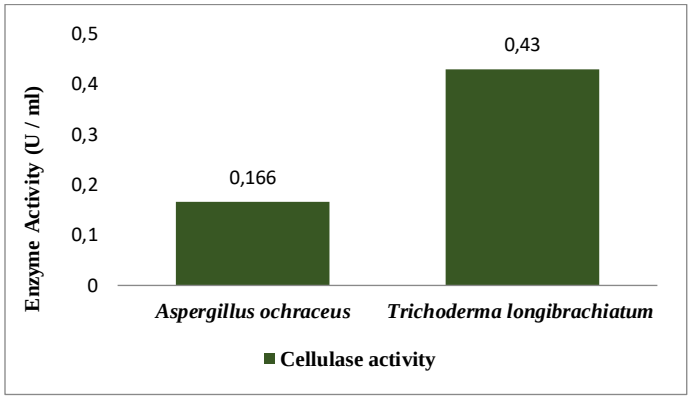

Figure 2 Comparison of the cellulase activity (U / ml) of *Aspergillus ochraceus* and *Trichoderma longibrachiatum* in solid state fermentation studies

The role of bioprocess parameters in the growth, metabolism and production of various primary and secondary metabolites from different industrially important organisms have been well explored **(Bailey and Ollis, 1986).** The organisms can produce both the enzymes. However, difference in enzyme activity of the two strains may be attributed to variation in the microenvironment around the growing fungal strains and their response to abiotic parameters (90% RH in wheat bran at 30 ºC after 7-day culture). The feasibility analysis suggests *Aspergillus ochraceus* MTCC 1877 to be a potential source of alpha amylase and cellulase.

CONCLUSION

Aspergillus ochraceus strain had been well explored for its mycotoxic effect and associated mycotoxins. The fungal metabolites had also shown some significant effect against neurological diseases. However, scarce reports are available on their potentials of being a source of enzymes. Current study explored the significance of co-production of alpha amylase and cellulase on the agro-residue, 'wheat bran', under controlled experimental conditions. The study will pave the way for exploration of other commercially important enzymes from the organisms and strategic approach to optimize culture conditions for improving the yield of enzymes.

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REGULAR ARTICLE

INTERPLAY BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI AND POULTRY MANURE ON GROWTH AND YIELD OF SOYBEAN (GLYCINE MAX (L) MERILL) UNDER SCREENHOUSE CONDITIONS

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ABSTRACT

A pot experiment was carried out at the University of Ibadan, Oyo State Nigeria using the Completely randomize design to study the growth of soybean (*Glycine max* L.) as affected by mycorrhizal species and poultry manure interplay under screenhouse conditions. The treatments include five levels of mycorrhizal inoculum, and two levels of poultry manure. Data collected include plant height, number of leaves, number of branches, leaf area and biomass yield. Analysis of variance was carried out on all data and means were separated using the Duncan's multiple range test at $p < 0.05$. *Glomus gigaspora* with 12.5 g poultry manure, *Glomus gigaspora* with sole 6.25 g poultry manure significantly enhanced the growth of soybean. Fresh and dry biomass of soybean were also significantly enhanced by *Glomus deserticola* with 12.5 g poultry manure. Number of seeds / pot and grain yield/pot were significantly enhanced when compared with the control. Mycorrhizal spore count in the soil at harvest were significantly different with highest spore observed under *Glomus clarum* and the least under control. This study conclude that mycorrhizal species-poultry manure combination varies in their effect on the growth and yield of soybean.

Keywords: Arbuscular mycorrhiza fungi, plant growth promotion, principal component analysis, Soybean, sustainable agriculture, yield

INTRODUCTION

Soybean (*Glycine max* L.) is a leguminous crop. It belongs to the family *Fabaceae*, subfamily *Papilionoideae*, genus *Glycine* and subgenus soya. It is an herbaceous annual plant which is one of the world's most valuable crop used as a source of dietary protein **(Park *et al.*, 2020)**. Tropical Soybean varieties can be categorized into three groups based on the maturity period. Early maturing which matures in less than 105 days after planting, medium maturing which matures between 105-120 days after planting and late maturing which mature after 120 days after planting. The present production of this crop and many other crops are not adequate to meet the growing world population. Climate change and urbanization are the two major factors affecting food production **(Borrell *et al.*, 2020)**. Conventional farming cannot achieve the production of enough food if the population continues to increase at this pace hence, the need to find better and suitable means of food production so that enough food can be made available. Consequently, the use of organic agriculture and plant growth promoting microorganisms (PGPM) has been adopted.

Organic agriculture is important in achieving food security **(Muller *et al.*, 2017)** and it is regarding as being environmentally friendly than conventional agriculture **(Meemken and Qaim, 2018)**. Application of organic residues to soils increase soil organic matter, buffer soil, improve aggregate stability and enhance water retention capacity of soils. Organic agriculture is environmentally friendly and excludes use o of inorganic fertilizer. Manure has been used effectively as organic fertilizer for centuries and their nutrient content varies depending on source and nutrient content **(Reganold and Wachter 2016; Zhang *et al.*, 2020)** organic manures are efficient in increasing soil nutrient contents, ensuring positive residual effects and enhancing soil's physical and chemical characteristics **(Mahmood *et al.*, 2017)**. The utilization of the inorganic manure is an integral part of agriculture that has been exploited overtime and across ages because of its ability to restore soil fertility, supply major plant nutrients, such as N, P, K, Ca, Mg and stabilizes soil pH.

The PGPM are majorly of bacteria and fungi microbes. They have been successfully implemented in the promotion of growth in various plants **(Igiehon *et al.*, 2020; Olanrewaju and Babalola, 2019)**. Different mechanisms such as growth hormone production, siderophore production, nitrogen fixation, phosphorus solubilization, ACC deaminase production, etc. are employed by PGPMs in improving plant growth and controlling pathogens **(Olanrewaju *et al.*, 2017)**.

One major fungus that has been implemented in plant growth promotion is Arbuscular mycorrhizal fungi (AMF). Many agricultural and horticultural crop species form a mutualistic symbiotic relationship with AMF in their roots **(Igiehon and Babalola, 2017)**. AMF are efficient in acquisition of nutrients resulting in improved plant growth **(Kim *et al.*, 2017; Liang *et al.*, 2019)**. Furthermore, root colonization with AMF enhances plant tolerance to abiotic stress **(Al-Arjani *et al.*, 2020; Begum, Ahanger, *et al.*, 2019; Begum, Qin, *et al.*, 2019; Mathur *et al.*, 2018; Yasmeen *et al.*, 2019)**, thus improving crop growth and productivity. AMF are of immense importance in maintaining soil fertility which are influenced by the effect of climatic and seasonal changes on the physical and chemical properties of soil.

The objective of the study therefore is to know the effect of selected AMF and poultry manure on growth, yield, and nutrients uptake of soybean under screenhouse conditions.

MATERIALS AND METHODS

Soil collection and analysis

The experimental soil was collected on the agricultural land site of the University of Ibadan, Nigeria (7°24'N; 3°48'E) and pot experiment conducted in the screenhouse of the Department of Agronomy, University of Ibadan, Ibadan, Nigeria. Soil sample was collected at a depth of 0-15 cm, air-dried and sieved with 5 mm mesh size to remove debris before weighing into 5 kg pots. The bulk soil was sub-sampled, air dried at room temperature and grounded to pass through 2 mm and 0.5mm diameter sieve for analysis. The particle size analysis was done by hydrometer method **(Gee and Or, 2002)**. The pH was determined in a 1:2 soil/water suspension using digital pH meter. Organic carbon was determined by Walkley-Black dichromate wet oxidation method **(Nelson and Sommers, 1996)**. Total nitrogen was by micro-Kjeldahl distillation technique as described by **Bremner and Mulvaney (1982)**. Available phosphorus was determined by Bray No 1 method as described by **IITA (1982)**. Exchangeable K, Ca and Mg were extracted using ammonium acetate, K was determined using flame photometer, Ca and Mg determined using atomic absorption spectrophotometer.

Poultry manure preparation

Poultry manure was air – dried and characterized by nitric and sulfuric acids before further analysis to determine the micronutrients following standard protocols **(Cater; Kalra and Maynard, 1991)**.

Mycorrhiza source

Sample of mycorrhiza propagules was collected from Soil Microbiology Laboratory of the Department of Agronomy, University of Ibadan, Nigeria. They are *Glomus clarum* (G.c), *Glomus gigaspora* (G.g), *Glomus deserticola* (G.d), and *Glomus mosseae* (G.m). Arbuscular mycorrhizal inocula (20 g each) were applied at third-quarter central top of the soil 24 hours before soybean seed were sown into the soil **(Carling *et al.*, 1978).**

Experimental design and treatments

The experimental was a factorial with mycorrhiza having five levels, poultry manure with three levels of poultry manure viz 6.25 g (1 pm), 12.5 g (2 pm) and a control. The experiment was arranged in a completely randomize design and replicated three times to give a total of 45 pots. The variety of soybean planted was (TGX 1448 -2E). Two soybean seed were sown in 5 kg pot filled with topsoil. The mycorrhiza inoculum was applied alone, and the two levels of poultry manure were also applied alone after which each inoculum was applied in consortia with each level of poultry manure. There was a control in which nothing was applied. Standard agronomic practices were followed.

Statistical analyses

All data were analyzed using analysis of variance (ANOVA) with the statistical analysis software (SAS) version 9.4 (SAS Institute, Inc., Cary, NC, USA), and Duncan's Multiple Range Test (DMRT) at *P<0.05* was used to separate the means. Multivariate analysis was done using the R statistical package **(R Core Team, 2019)**. Principal component analysis (PCA) was done using the FactoMineR package **(Lê *et al.*, 2008)** and Pearson correlation was performed using the core function of the stats package in R. A hierarchical cluster analysis was performed using the ward. D2 method in a cluster R package **(Maechler, 2019)**.

RESULTS

Soil and poultry manure nutrient analysis

Table 1 Nutrient content of poultry manure, soil chemical characteristics and particles size distribution

Poultry manure		**Soil**	
Nutritional components		**Chemical properties**	
Total nitrogen (%)	3.5	Nitrogen (g/kg)	3.00
Total phosphorus (%)	3.45	Bray P (mg/kg)	16
Ca (%)	0.32	Exc Ca (cmol/kg)	7.56
Mg (%)	0.52	Exc Mg (cmol/kg)	0.22
K (%)	2.24	Exc Na (cmol/kg)	0.56
Mn (%)	0.02	CEC (cmol/kg)	10.03
Fe (%)	0.47	Exc k (cmol/kg)	0.56
Cu (%)	0.03	Mn (mg/kg)	76
Na (%)	0.23	Fe (mg/kg)	145
Zn (%)	0.03	Zn (mg/ kg)	17
pH in H_2O	8.30	Cu (mg/kg)	2
pH in KCl	8.10	pH in H_2O	6.85
Organic carbon (%)	18.1	pH in KCl	6.13
Organic matter (%)	31.2	Organic carbon (g/kg)	12.4
		Organic matter	2.1
		Sand (g/kg)	789
		Silt (g/kg)	91
		Clay (g/kg)	120
		Soil textural class	Sandy loam

Plant growth characteristics

For At 2 weeks, the mean values of the plant height of the control and the inoculated plants are relatively close (Table 2). Starting from 3 weeks, we start seeing clear disparities in the mean values up to the 5th week. At 2 weeks, combination of *Glomus mosseae* and 6.25 g poultry manure followed by sole application of poultry manure. At the 5th week, combination of *Glomus mosseae* and 6.25 g manure still have the highest value, the same as the combination of *Glomus deserticola* and 12.5 g manure which is 66.33 cm followed by single application of 6.25 g manure which is 61.67 cm. In relation to the control, at 2, 3, 4, and 5 weeks, the control mean value is higher than 4, 6, 1, and 0 numbers of treatments respectively.

Table 2 Effect of mycorrhizal and poultry manure application on plant height (cm) at 2,3,4 and 5 WAP

Treatments	**2**	**3**	**4**	**5**
Control	21.67 cd	28.50 bc	34.33 e	35.67e
G.d	21.00 cd	23.83 cd	38.67 de	55.00 cd
G.m	21.83 cd	26.50 bc	42.00 cd	56.67 cd
G.c	20.73 d	24.67 cd	34.16 e	48.33 d
G.g	23.00bc	27.17 bc	41.67 cd	57.67 bc
1pm	26.67 a	32.67 a	50.33 a	61.67 ab
2pm	23.67 ab	28.83 bc	39.33 a	58.67bc
G.d + 1pm	21.00 cd	24.17 cd	36.67 a	47.00 d
G.m + 1pm	27.33 a	33.50 a	48.83 ab	66.33 a
G.c + 1pm	21.33 cd	25.83 cd	38.00 a	51.67 d
G.g + 1pm	24.17 ab	28.50 bc	45.50 bc	57.33bc
G.d + 2pm	24.00 ab	31.50 ab	50.00 a	66.33 a
G.m + 2pm	26.50 a	31.83 ab	45.00 bc	57.67bc
G.c + 2pm	22.00 bc	30.00 ab	41.83 cd	57.67bc
G.g + 2pm	26.83 a	30.67 ab	39.38 de	55.00 cd
F Statistics	0.48^{ns}	0.51^{ns}	0.52^{ns}	0.69^{ns}
C.V.	25.32	26.75	27.89	21.87

Legend: Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola,* G.m = *Glomus mosseae*, G.c = *Glomus Clarum*, G.g = *Glomus* gigaspora, 1 pm= 6.25 g poultry manure, 2pm = 12.5 g poultry manure, WAP = Week After Planting

The number of branches increases at the different weeks (Table 3). At 2 weeks, treatments *Glomus mosseae* and combination of *Glomus clarum* with 12.5 g manure are statistically different while the control is not like any of the other treatment at 3 and 5 weeks, the values recorded are not statistically different for the number of leaves (Table 4) at 2 weeks, all treatments are significantly higher than the control, same for the period of the experiment. However, the combination treatments have significant values than the single treatments and the control. Statistically, the responses of the leaf area of the plants to the various inoculations at the different weeks are not different at each week (Table 5). However, the combined treatments gave higher leaf area than the other treatments.

Table 3 Effect of mycorrhizal and poultry manure application on number of branches at 2,3,4, and 5 WAP

Treatments	**2**	**3**	**4**	**5**
Control	1.67 b	3.00 a	3.33 c	5.66 a
G.d	2.00 ab	2.66 a	4.00 bc	6.33 a
G.m	2.33 a	3.33 a	4.33 abc	5.66 a
G.c	2.00 ab	2.66 a	4.00 bc	5.66 a
G.g	2.00 ab	2.66 a	4.33 abc	6.67 a
1pm	2.00 ab	3.67 a	5.00 ab	6.67 a
2pm	2.00 ab	3.00 a	4.67 abc	5.67 a
G.d + 1pm	2.00 ab	3.33 a	4.00 bc	6.33 a
G.m + 1pm	2.00 ab	3.33 a	5.00 ab	6.33 a
G.c + 1pm	1.67 ab	2.67 a	4.00 bc	6.33 a
G.g + 1pm	2.00 ab	3.67 a	4.67 abc	6.33 a
G.d + 2pm	2.00 ab	3.33 a	4.00 bc	7.00 a
G.m + 2pm	2.00 ab	3.33 a	5.00 ab	6.33 a
G.c + 2pm	2.33 a	2.67 a	4.66 abc	6.00 a
G.g + 2pm	2.00 ab	3.67 a	5.67 a	6.00 a
F statistics	1.07^{ns}	1.15^{ns}	1.67^{ns}	0.64^{ns}
C.V	14.91	20.33	17.49	14.43

Legend: Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola,* G.m = *Glomus mosseae,* G.c = *Glomus Clarum*, G.g = *Glomus gigaspora*, 1 pm= 6.25 g poultry manure, 2 pm = 12.5 g poultry manure, WAP = Week After Planting

Table 4 Effect of mycorrhizal and poultry manure application on number of leaves at 2, 3, 4, and 5 WAP

Treatments	**2**	**3**	**4**	**5**
Control	6.67 b	10.00 bc	15.33 bc	16.67 b
G.d	8.33 ab	10.33 bc	14.33 c	19.67 ab
G.m	9.00 ab	10.67 bc	15.33 bc	18.00 ab
G.c	8.33 ab	10.67 bc	14.00 c	21.33 ab
G.g	8.00 ab	11.00 ab	16.00 ab	26.00 a
1pm	9.00 ab	13.00 a	16.67 ab	23.33 ab
2pm	8.00 ab	11.00 ab	13.33c	18.33 ab
G.d + 1pm	8.67 ab	11.66 ab	13.00 c	18.33 ab

Legend: Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola,* G.m = *Glomus mosseae,* G.c = *Glomus Clarum*, G.g = *Glomus gigaspora*, 1 pm= 6.25 g poultry manure, 2 pm = 12.5 g poultry manure, WAP = Week After Planting

Table 4 *Cont.*

Treatments	2	3	4	5
G.m +1pm	8.00 ab	13.33 a	16.33 ab	18.00 ab
G.c +1pm	7.33 ab	11.33ab	14.67 c	22.00 ab
G.g +1pm	10.33 a	13.33 a	17.33 a	23.33 ab
G.d +2pm	9.00 ab	14.00 a	18.67 a	23.33 ab
G.m +2pm	8.33 ab	13.33 a	18.00 a	21.00 ab
G.c + 2pm	8.67 ab	11.67 ab	16.67 ab	20.33 ab
G.g + 2pm	8.33 ab	12.66 ab	16.67 ab	20.00 ab
F Statistics	0.77^{ns}	0.87^{ns}	1.06^{ns}	1.16^{ns}
C.V.	19.44	20.33	18.75	20.24

Legend: Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola*, G.m = *Glomus mosseae*, G.c = *Glomus Clarum*, G.g = *Glomus gigaspora*, 1 pm= 6.25 g poultry manure, 2 pm = 12.5 g poultry manure, WAP = Week After Planting

Table 5 Effect of mycorrhizal and poultry manure application on leaf area at 2, 3, 4, 5 WAP

Treatments	2	3	4	5
Control	11.32 a	20.57 a	21.27 c	25.53 b
G.d	12.63 a	13.77 a	22.30 bc	26.76 ab
G.m	12.00 a	19.63 a	23.80 abc	29.13 ab
G.c	11.43 a	17.60 a	21.23 c	26.83 ab
G.g	14.67 a	17.47 a	27.36 abc	33.56 ab
1pm	14.80 a	25.90 a	38.33 a	42.63 a
2pm	11.86 a	27.33 a	27.77 abc	33.63 ab
G.d + 1pm	11.86 a	27.33 a	23.47 abc	29.13 ab
G.m +1pm	17.26 a	20.87 a	28.67 abc	33.63 ab
G.c +1pm	12.03 a	15.07 a	27.77 abc	31.67 ab

Legend: Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola*, G.m = *Glomus mosseae*, G.c = *Glomus clarum*, G.g = *Glomus gigaspora*, 1 pm= 6.25 g poultry manure, 2 pm = 12.5 g poultry manure, WAP = Week After Planting.

Table 5 *Cont.*

Treatments	2	3	4	5
G.g + 1pm	12.18 a	16.63 a	27.30 abc	33.10 ab
G.d + 2pm	15.07 a	22.67 a	35.00 abc	39.96 ab
G.m +2pm	19.97 a	21.97 a	37.57 ab	43.40 a
G.c + 2pm	13.60 a	22.97 a	30.63 ^{abc}	35.77 ab
G.g + 2pm	16.67 a	24.60 a	30.93 abc	37.77 ab
F Statistics	0.83^{ns}	0.92^{ns}	1.40^{ns}	1.30^{ns}
C.V.	33.95	34.35	28.62	25.48

Legend: Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola*, G.m = *Glomus mosseae*, G.c = *Glomus clarum*, G.g = *Glomus gigaspora*, 1 pm= 6.25 g poultry manure, 2 pm = 12.5 g poultry manure, WAP = Week After Planting.

Single treatments of *Glomus deserticola*, *Glomus gigaspora*, *Glomus mosseae*, and combination treatments of *Glomus mosseae*+6.25 g manure, *Glomus gigaspora*+6.25 g manure, Glomus deserticola+12.5 g manure, *Glomus mosseae*+12.5 g manure, *Glomus clarum*+12.5 g manure, and *Glomus gigaspora*+12.5 g manure are not statistically different from one another (Figure 1). However, single treatments of 6.25 g manure gave the highest fresh and dry biomass yields. Furthermore, treatments with *Glomus clarum* inoculum, gave the highest mycorrhizal count. The control and the *Glomus deserticola* treated plants are not statistically different in their mycorrhizal count. It was observed that combination treatment of *Glomus clarum*+6.25 g manure gave the highest root colonization percentage and all treatments with 12.5 g of manure were seen to show higher root colonization than those with 6.25 g or those without manure. This suggests that high manure content influences the root colonization ability of the mycorrhiza species. Furthermore, treatment *Glomus gigaspora*+6.25 g manure produced the highest number of seeds followed by *Glomus clarum*+12.5 g manure and *Glomus mosseae*+6.25 g manure. The grain yield is similar in all treatments except for the control which is much lower than the other treatments. Like the grain yield, days to 50% flowering is similar for all treatments in their effects.

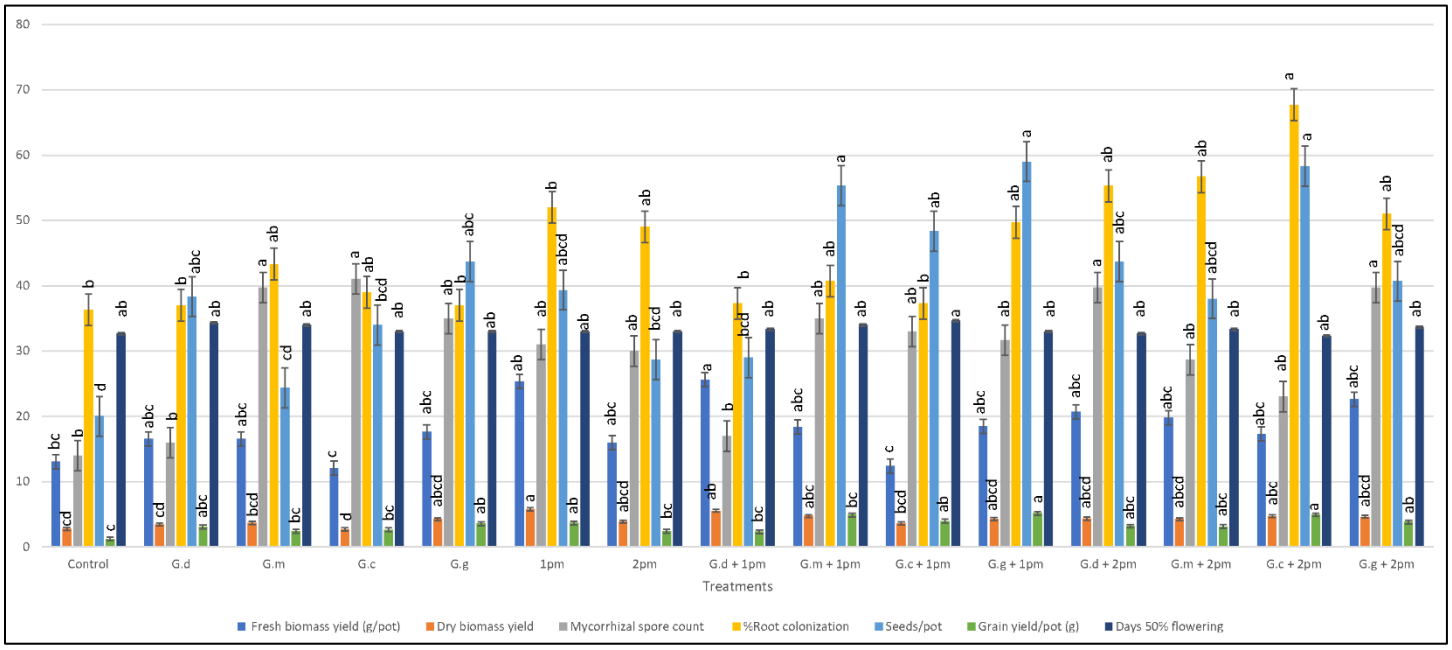

Figure 1 Effect of mycorrhizal and poultry manure application on biomass yield, grain yield, mycorrhiza spore count, root colonization, and days to 50% flowering. Means in the same column with the same letter are not significantly different at 5% level of probability using DMRT. G.d = *Glomus deserticola*, G.m = *Glomus mosseae*, G.c = *Glomus Clarum*, G.g = *Glomus gigaspora*, 1 pm= 6.25 g poultry manure, 2 pm = 12.5 g poultry manure.

Principal component analysis

The principal component of the variances taken by component responses of the plants on the traits over the treatments used in the study are represented in a biplot (Figure 2). PC1 and PC2 account for 67.9% of the total variances observed. Among the variables, the plant height, number of branches, leaf area, and dry biomass yield are the major contributing components of PC1 while number of seeds, fresh biomass yield, and grain yield contribute highest to PC2 (Table 6). Treatments that improve fresh biomass yield and number of seeds also increases the grain yield.

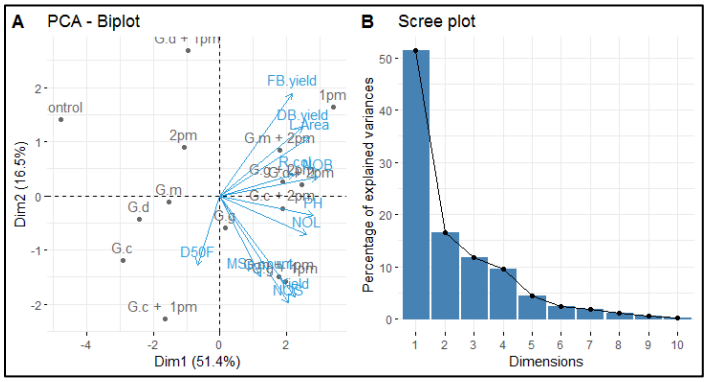

Figure 2 Biplot representation of the PCA analysis. (A.) PC 1 and PC 2 biplot using quantitative trait scores (B.) Scree plot of the loadings explaining the variances at each PC

Table 6 Trait contributions, eigenvalues, and cumulative percentage of the first five components

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
PH	13.707967	0.6997785	0.2128521	3.6608263	5.4231327
NOB	15.099067	0.6241507	5.0838851	1.3247173	0.0085977
NOL	11.749025	2.7852839	2.7084376	1.3353652	23.230957
L.Area	12.434389	6.3353539	0.4489379	3.9602473	12.312132
FB.yield	8.1813151	19.462155	9.2958483	0.360694	7.7038025
DB.yield	10.618197	8.6955659	8.4509858	5.7459467	1.9552202
MSp.count	2.5356375	11.942743	0.0019746	52.073125	3.2838357
R.col	8.7506308	0.8676604	21.793444	0.3613102	28.22655
NOS	7.3013965	21.120961	0.4069789	17.496557	0.4241143
D50F	0.7951592	8.803347	51.394001	0.4938005	17.420647
Yield	8.8272156	18.663	0.2026554	13.187411	0.0110105
eigenvalue	5.6580151	1.8121661	1.2975681	1.0577207	0.4843258
Percentage variance	51.436501	16.474238	11.796074	9.6156427	4.4029622
Cumulative percentage variance	51.436501	67.910738	79.706812	89.322455	93.725417

Cluster analysis

The treatments were clustered into 4 based on their effects on the plant responses (Figure 3). Each group of clusters is distinct from the others by their color. Higher number of the combination treatments are represented close to each other while the same is observed for single treatments.

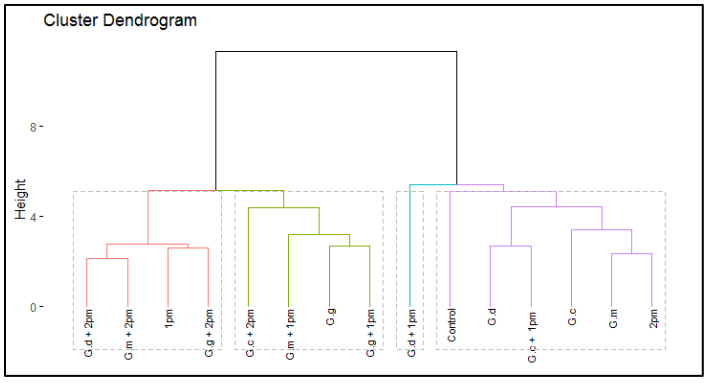

Figure 3 Hierarchical clustering dendrogram analysis; Euclidean distance was used and the associations between groups were done by the Ward method for the treatments with respect to the plant responses

Correlation analysis

There are lots of significant correlations among the traits scored (Figure 4).

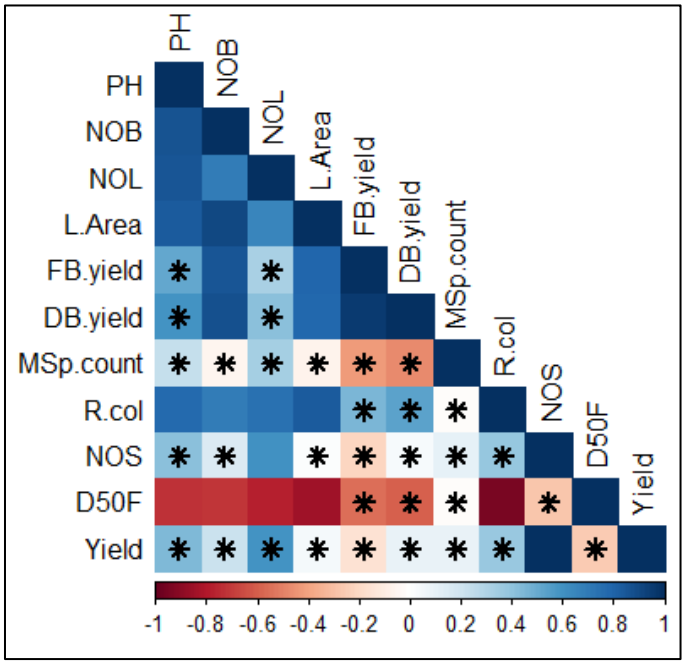

Figure 4 Correlations among the traits scored; Pearson's rank correlation matrix and performance analytic chart of the variables showing the relationship among the variables scored on the plants. (Blue color indicates a highly positive correlation and red color indicates a highly negative correlation among the different traits. PH: plant height(cm), NOB: number of branches, NOL: number of leaves, L.Area: leaf area, FB.yield: fresh biomass yield (g/plant), DB.yield: dry biomass yield (g/plant), MSp.count: mycorrhiza count, R.col: percentage root colonization, NOS: number of seeds, D50F: days to 50% flowering, Yield (g/plant)), *= significant, *p<0.05*.

The level of significance of the correlations at *p<0.05* is shown by the asterisks. Either positive or negative correlations, the asterisks indicate if it is statistically significant or not. The red colors show negative correlations while the blue colors show positive correlations. The deeper the colors, the stronger the correlations. Plant height has a positive correlation with all other traits except days to 50% flowering but only correlations with fresh and dry biomass yield, mycorrhiza spore count, number of seeds, and yield that are significant statistically. Number of branches on the other hand has a positive correlation with number of leaves, leaf area, fresh and dry biomass yield, root colonization, number of seeds, and yield with the last two being only the positive significant correlations. Number of leaves is positively and significantly correlated with fresh and dry biomass yield, mycorrhiza spore count, and yield while leaf area is significantly positively correlated with yield. Fresh biomass yield is significantly positively correlated with percentage root colonization but significantly negatively correlated with mycorrhiza spore count, number of seeds, days to 50% flowering, and yield. Dry biomass yield correlates positively and significantly with percentage root colonization and yield but negatively and significantly with mycorrhiza spore count and days to 50% flowering while mycorrhiza spore count and root colonization both correlate positively and significantly with number of seeds and yield but negatively with days to 50% flowering. Furthermore, number of seeds correlates positively with yield while days to 50% flowering has negative correlations with all the traits.

DISCUSSION

Crop yield is affected by nutrient availability, pests and diseases, water availability, and poor soil condition among others **(Donatelli *et al.*, 2017; Röös *et al.*, 2018; J. Zhang *et al.*, 2020)**. AMF enhances plant ability to absorb minerals and improve growth through mineral solubilization, production of growth hormones, etc. Manure from various sources have been identified in plant growth promotion **(Abbas *et al.*, 2020; Ekinci *et al.*, 2019)** with the sources playing a crucial role **(Bibiano *et al.*, 2019)**.

Principal component analysis

Biplot represents the association among different traits, and the length of the lines shows the contribution of each trait in the variations observed (Figure 2). The eigenvalues and the corresponding factors are sorted by descending order of how much of the initial variability they represent. The Eigenvalue significance

criterion, as described by **Kaiser (1960)** was used to select statistically significant principal components. The biplot analysis can be used to select the best treatment in breeding for a trait.

Clustering and Correlation analysis

The clustering grouped majority of the combined treatments together in the same clusters (Figure 3), hence we can say that they produce similar responses different from the sole treatments. The correlation matrix shows traits that have impact on each other (Figure 3). It aids the selection of traits without over-picking traits for improved breeding. From the traits, tall plants produce high yield hence, farmers will prefer treatments that favor tall plants (Figure 2) so they can have high yield.

CONCLUSION

The study concludes that the treatments have effects on the growth and yield parameters of soybean. It was also observed that combination treatments of AMF with higher quantity of manure produced the best result on growth and yield.

This study was conducted in a screenhouse however due to diverse environmental factors, diversity in soil microbiome of the field, it is suggested that further the study should be replicated on the field to know if these treatments will still yield the same result.

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REGULAR ARTICLE

TITLE OF MANUSCRIPT: ENHANCEMENT OF LIPOLYTIC CHARACTERISTICS OF A PALM-OIL MILL EFFLUENT-SOURCED *KODAMAEA OHMERI* PL2

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ABSTRACT

The hydrolysis of long-chain triglycerides and fats is catalyzed by lipases, important enzymes which have essential biotechnological, medical, and industrial roles. Fungi were isolated from palm-oil mill wastes and screened for their lipid-hydrolyzing potentials. Lipase production and activity characteristics were determined using different substrates. Sixteen, out of the 75 fungi isolated, exhibited lipolytic characteristics. Yeast isolate PL2 gave the highest zone of hydrolysis (44.5 mm) and was identified as *Kodamaea ohmeri*. This yeast was cultured under diverse media conditions for lipase production and the enzyme produced under each physicochemical condition was determined spectrophotometrically. The optimum parameters for lipase production (1% Tween-80, Ca2+, peptone, 37°C and pH 6) were used to produce lipase which was further purified through (NH4)2SO4 precipitation, membrane dialysis and gel chromatography. The activity of the partially purified lipase increased progressively with each purification stage. The lipase had optimum activity at 50oC and was alkalophilic with peak pH for enzymatic hydrolysis at 8.0. The lipase was supported by 0.5% concentrations of SDS and Triton X-100 (129.9 and 129.4%, respectively), and a 100-fold activity increase with 0.5% Tween-80. Lipase activity was augmented by Manganese and calcium ions (10 mM) but inhibited by NH4+, Fe3+ and Na+. The use of Tween-80 resulted in increased enzyme yield as well as better enzyme characteristics compared to other substrates. The knowledge obtained in this study may be harnessed for commercialization of *Kodamaea ohmeri* lipase.

Keywords: Palm-oil mill wastes, Fungal lipases, *Kodamaea ohmeri*, Enzyme characterization

INTRODUCTION

Lipases are hydrolytic enzymes which catalyze esterification reactions in lipids (water insoluble energy sources important to all life) to generate simpler products of lipid hydrolysis (**Das *et al.*, 2016**). Over the years, the study of lipases has gained attention as it finds multipurpose applications in various sectors. Lipases possess outstanding economic properties such as the ability to function in micro-aqueous environment, catalyze aminolysis, acidolysis, esterification and transesterification reactions (**Joseph *et al*. 2008; Asih *et al.* 2014; Alami *et al.* 2017**). Unlike some other enzymes, lipases do not require cofactors to catalyze hydrolytic reactions and can remain active in the presence of organic solvents (**Liu and Kokare, 2017; Chandra *et al.* 2020**). Lipases hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acid (**Gilham and Lehner, 2005; Alami *et al.* 2017**) and hence, have attracted continued extensive research on their detection methods, production and optimization, assay, purification strategies and industrial applications (**Hasan *et al.* 2006, Hasan *et al*. 2009; Alabdalall *et al.* 2020**). Lipases have been numerously applied in the biodiesel industry, wastewater treatment, de-oiling lipid-clogged drains, the hydrolysis of milk and fat in the dairy industry, leather industry to remove lipids from hides and skin, as a component of detergent for the removal of oil and fat stains, in the textile industry, in pharmacy and medicine as diagnostic tools, (**Kumar *et al.*, 2005; Alabdalall *et al.* 2020**). Lipases are widely distributed and present in animals, plants, and microorganisms (**Lascano-Demera *et al.* 2019**). Yeasts are vegetative budding unicellular eukaryotic organisms which are safe desirable producers of enzymes because of their lack of production of toxic secondary metabolites (**Pham *et al.* 2019**). For thousands of years, they have been employed in many traditional fermentative, industrial and biotechnological processes (**Turker, 2015; Lascano-Demera *et al.* 2019**), such as in the production of enzymes, pharmaceutical proteins, bioremediation, biocatalysts, to mention a few (**Turker, 2015; Alami *et al.* 2017; Lascano-Demera *et al.* 2019; Alabdalall *et al*. 2020)**. Many fungi have been reported to produce lipase and the *Pichia, Candida, Saccharomyces, Yarrowia* and *Kodamaea* species isolated from a waste sample collected from a grease trap were documented to exhibit lipolytic activities (**Sakpuntoon, 2020**). Microorganisms abound in many organic wastes and the isolation of new microorganisms with unique characteristics from these wastes is highly probable. Nigeria has many cottages palm-oil processing farms which generate vast volumes of organic wastes from palm fruit processing. Palm-oil mill effluent (POME) is the voluminous liquid/wastewater obtained from palm-oil processing. The production ratio of this 'waste' to palm-oil is 5:2. When discharged, the organic POME, rich in organic matter, is a brown sludge and contains suspended particles with a pH range between 4 and 6 (**Asih *et al.* 2014; Setiadi *et al*. 2015**). The effluent contributes to environmental pollution in neighboring soil and water bodies resulting in increased demand for the total oxygen required to breakdown organic contaminants (**Setiadi *et al*. 2015**). The organic nature of the POME also makes it a rich source of microorganisms which would utilize the nutrients in the waste for growth and metabolism, hence a candidate for the isolation of lipolytic organisms.

The production of enzymes has been reported to be influenced by diverse cultural conditions and their activities seen to be in response to the physicochemical changes they are exposed to during growth. The attributes of lipases necessitate continuous search for microbial sources which can contribute to meet the market demand (**Geoffry and Achur, 2018**), hence, much work is still required to understand the potentials of microorganisms in the production of valuable resources. Also, there is an ever-increasing demand for lipases in many industrial applications which encourages further search for new lipase-producing microorganisms from diverse sources. In this work, the characteristics of a lipolytic *Kodamaea ohmeri* PL2 strain, isolated from palm oil mill effluent, and the contributions of physicochemical amendments to cultivation media for enzyme production and lipase characteristics were investigated.

MATERIALS AND METHODS

Sample collection

Samples were collected from a cottage palm oil processing mill site in Igbo Oloyin, Ibadan, Nigeria. From the palm oil mill, different types of waste samples generated during palm-oil processing were obtained from different locations including the freshly released palm-oil processing effluent: A (7°32'35.724"N, 3°58'15.648"E), old/abandoned effluent site: Eo (7°32'39.564"N, 3°58'7.494"E), mud-like sludge generated from settling of the effluent over time: L (7°32'41.442"N, 3°58'11.58"E; 7°32'37.846"N, 3°58'15.972."E) and soil: S (7°32'33.648"N, 3°58'12.966"E) over which waste water had been discharged for a long period of time. These were collected in sterile Ziploc bags and analyzed microbiologically in the laboratory.

Isolation of microorganisms and screening for lipase producers

Using the pour plate method, 10 g samples were appropriately diluted in cooled sterile water to generate 10-fold dilutions. Aliquots (1 mL) of the dilutions were introduced into sterile, 9 cm disposable Petri dishes after which cooled, sterilized agar (Yeast Extract Agar and Potato Dextrose Agar) amended with 0.1g/L streptomycin were poured into the plates, swirled, and allowed to set. The Petri dishes were incubated at 27oC and examined for growth over 96 hours. Morphologically unique yeast and mold isolates were subjected to lipase plate screening assays by stab-culturing in 1% sterilized (a) olive oil medium and (b) Tween-80 medium both with basal medium constituents of 10 g/L Peptone; 5 g/L NaCl; 0.1 g/L CaCl2; 0.5 g/L Congo red; 20 g/L Agar-agar and 1 L distilled

water, while the Tween-80 medium additionally had 3 g/L Yeast extract. The inoculated plates were incubated at 27oC for 48 hours.

Culture selection and identification of lipolytic microbe

A detection of fungal lipolytic potential was confirmed by the formation of clear/pale halos around the point of fungal inoculation in the colored agar mat. The isolate with the highest diameter of clearance was selected for further work and identified based on morphological, fermentative, and molecular characteristics. The morphological characteristics of the colony of the selected isolate was observed to determine the size, color, elevation, opacity, texture, edge, pigmentation, and shape (**Yarrow, 1998, Alami *et al.*2017**). This was followed by wet mount microscopic morphological examination using lactophenol cotton blue stain under x40 and x100 magnification. Biochemically, the isolate was examined for its sugar fermentation patterns by inoculating 24 hours old culture in filter-sterilized sugars and steam-sterilized gelatinized starch in screw-capped tubes of sterile yeast extract-methyl red medium (g/L: yeast extract 23; methyl red 0.5, carbohydrate sugars 10) with inverted Durham tubes. The carbohydrates included glucose, lactose, maltose, arabinose, mannitol, sucrose, fructose galactose, glycerol, and starch. A medium in which no organism was inoculated served as control for the experiment and the tube was observed daily for color change and gas formation. For molecular characterization, the yeast genomic DNA was extracted, and the ITS universal primer set (ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'; ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3') was used (**Looke *et al.* 2011**). These were sequenced using a Genetic Analyzer 3130XL sequencer (Applied Biosystems) with BigDye Terminator V3.1 cycle sequencing kit following the manufacturers' manual. A Bio-Edit software and MEGA 6 was used for all genetic analysis.

Effect of physicochemical conditions on lipase production

Effect of lipid substrates as carbon sources on lipase production and optimization of production conditions: For enhanced production of the enzyme, a study of various parameters such as temperature, pH, carbon sources, nitrogen sources, metallic ions and incubation time on the production of lipase was done. The initial production medium composed of Peptone: 10 g/L, NaCl: 5 g/L, $CaCl_2$: 0.10 g/L, Olive oil: 10 mL, Distilled water: 1000 mL at a pH of 7. Each of these parameters were modified as shown below (**Musa and Adebayo-Tayo, 2012; Bakir and Metin, 2015**). A loopful of the chosen isolate was taken from a 24-hour old culture, into 0.85% NaCl and the suspension turbidity was then adjusted to an equivalent of No. 2 McFarland standard solution which then served as the seed inoculum for the fermentation studies.Various oils (castor oil, coconut oil, olive oil, melted shea butter and Tween-80) were enlisted as substitutes to olive oil in lipase production. These served as the sole carbon source in the fermentation medium used in the cultivation of the selected yeast and were compared to the effect which glucose exerted. Fifty milliliters each of the respective media were inoculated with 1% overnight culture, incubated for 24-120 hours, and after each day of incubation, samples were withdrawn from the production broth and assayed spectrophotometrically to determine lipase production (**Bakir and Metin, 2015**). The effects of increasing the substrate concentration of the best carbon source (1-5% v/v) was also determined to know what concentration would be continued with in subsequent lipase production studies.

Effect of metallic ions on lipase production: The contribution of metallic ions to lipase production was determined using chloride salts of Manganese, Zinc and Iron. These were used as substitute to the Ca^{2+} in the control production medium described above and for each of the reaction, 0.01% of the metal to be varied was supplied in the reaction medium. These were sterilized and each conical flask was seeded with 1% of the inoculum and incubated for 120 hours at 27°C. The lipase assay was carried out at 24 hours intervals.

Effect of different nitrogen sources: The effect of selected organic and inorganic nitrogen sources (Peptone, Yeast extract, Urea and Tryptone; $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$,) on the enzyme production was determined over five days. The reaction medium contained 1% w/v of the nitrogen compound which was inoculated and incubated as described above.

Effect of temperature: The cultivation temperature was varied in order to obtain the optimum incubation temperature that favored the production of the lipase enzyme. The fermentation medium was inoculated with the yeast and incubated at 27°C, 37°C, 40°C and 50°C for five days.

Effect of media pH on lipase production: The optimum pH for the enzyme production was determined by incubating the selected yeast in culture medium whose pH was adjusted between 3 and 10. Three different buffers were used to attain the ranges of pH enlisted: pH 3-5 (Sodium-citrate), pH 6-8 (Potassium Phosphate) and pH 9-10 (Carbonate-Bicarbonate) (**Bakir and Metin, 2015**). The fermentation medium was prepared with the buffers at the different pH ranges and sterilized. On cooling, the medium was inoculated and incubated at 27°C for 120 hours. Samples were assayed to determine lipase production at 24-hour intervals.

Assay for lipase activity: Using *p*-nitrophenyl palmitate (*p*NPP) as substrate and the method of **Sumarsih *et al.* (2019)**, assay of the lipase product in broth was carried out by determining the activity of the lipase using to produce *p*-nitrophenol. Following each incubation time, the enzyme solution was obtained by centrifugation at 4000 rpm for 30 minutes. A reaction mixture was formulated which consisted of 0.1 mL of the enzyme solution, 0.8 mL 50 mM Phosphate buffer (pH 8.0) and 0.1 mL 10 mM *p*NPP dissolved in ethanol. The reaction mixture was incubated at 30°C for 30 minutes. After incubation, 0.25 mL of 100 mM Na_2CO_3 was added to stop the reaction. The mixture was centrifuged at 4,000 rpm for 15 minutes and read spectrophotometrically at 410 nm. One unit of lipase activity (U/mL) was defined as the amount of lipase that caused the release of 1µmol of *p*-nitrophenol from *p*-nitrophenyl palmitate (*p*NPP) in one minute.

Production and characterization of lipase

Lipase was produced by submerged fermentation using the optimum culture parameters from the various culture conditions above. The medium was prepared, sterilized, cooled, inoculated, and incubated at 37◦C for 48 hours. After production, the reaction medium was centrifuged at 4,000 rpm for 30 minutes to obtain the cell-free supernatant which was used as enzyme solution for characterization studies.

Effect of temperature on the lipase activity: The lipase was maintained at different temperatures (27, 35, 40 and 50°C) for different time intervals (15, 30, 45 and 60 minutes) and then used in reaction mixtures containing 0.1 M phosphate buffer (pH 8.0) and the enzyme solution and afterwards, an assay was carried out using the standard assay procedure earlier described.

Effect of pH on lipase activity: Lipase activity was determined at various pH ranging from 3.0 - 10.0. Each of the adjusted pH buffers was introduced, using equal volumes, into test tubes containing the enzyme and incubated for 30 minutes at 27°C. Afterwards, standard assay procedure was used to quantify the activity of the lipase.

Effect of surfactants on lipase activity: Surfactants (Tween-80, SDS and Trixton-X 100) were used at 0.05%, 0.1%, 0.5% concentrations by mixing equal volumes of the enzyme and surfactants, then allowing the mixture to incubate for 30 minutes, and then assayed.

Effect of cations on the lipase activity: Varying concentrations (10 mM, 25 mM, 50 mM) of Mn^{2+}, Ca^{2+}, Na^{+}, Fe^{3+}, and NH_4^{+} salts were introduced into test tubes containing an equal corresponding volume of enzyme. These were incubated for 30 minutes and quantified using the standard lipase assay.

Effect of increasing concentration of reaction substrate on the lipase activity: Increasing concentrations of pNPP (0.1, 0.25, 0.5% w/v) dissolved in 0.1 M potassium phosphate buffer (pH 8.0) were added to a corresponding equal volume of enzyme solution and left to incubate for 30 minutes. The released P-nitrophenol was monitored spectrophotometrically.

Lipase purification and protein determination: The lipase present in the crude cell-free extract (100 mL) was partially purified by ammonium sulphate precipitation, allowed to stay overnight at 4°C (**Dixon and Webb, 1971**). The precipitate recovered by centrifuging this initial mixture at 10,000 rpm for 10 minutes using a cold centrifuge (Eppendorf centrifuge 5427R) was used for dialysis and column chromatography using a pre-activated dialysis bag submerged in 0.1M phosphate buffer solution of pH 8.0 overnight. A 10 mL solution of the partially purified enzyme was eluted in a column packed with solution of Sephadex G-100 (in PBS solution). The samples were eluted with the same buffer at a flow rate of 75 mL/hour and the eluted fractions were used for determination of the enzyme activity and protein concentration using bovine serum albumin as the protein standard. The protein concentration readings were taken in duplicates in tubes containing 0.1- 1.0 mL of 0.2 mg of protein stock solution (2 mg/mL bovine serum albumin) and brought up to 1mL with distilled water. For the test mixture, 0.5 mL of Phosphate buffer saline pH 7.0 was added to 0.5 mL of the crude enzyme. To the reaction and test mixtures, 5 mL of bovine serum albumin solution was then added, and the mixture was allowed to stand for 10 minutes. Dilute Folin ciocalteu reagent (0.5 mL) was added, followed by the thorough mixing of the solution which was then allowed to stand for 25 minutes at room temperature. The absorbance was read at 660 nm and the protein concentration was determined afterwards (**Lowry *et al.*, 1951**).

Data were subjected to descriptive statistics.

RESULTS

Isolation of, screening for and identification of lipolytic fungi from palm-oil mill waste samples

The palm-oil containing samples recorded a total fungal count between 1.4×10^3 and 3.0×10^5 per gram. A total of 75 fungi were isolated from the palm-oil mill

wastes. Lipase screening, using olive oil and Tween-80 as substrates showed that 16 isolates (13 yeasts and 3 mold) had zones of hydrolysis (Table 1). The production of zones served as a confirmation of the lipase producing potentials of the isolates obtained. Isolates with high clear zones (in reducing order), indicated by a color change of the chromogenic oil-containing medium were isolates PL2, YA3, YA1, with 44.5 mm, 42 mm and 26.5 mm zones of hydrolysis, respectively, at 72 hours. Also, six of the lipolytic organisms (37.5%) originated from the sludge sample (PL1, PL2, PL3, PSSL1, YL1 and YSSL3) while YA1 and YA3 originated from freshly discharged watery mill effluent component.

Table 1 Zone of lipid hydrolysis by lipolytic fungi on Tween-80 and olive oil screening medium

Fungal Isolate	Zone of hydrolysis (mm)	
	Tween-80	Olive oil
YA1	4	26.5
PL1	3	15
PE2	5	24
YA3	9	42
PL2	9	44.5
PA2	1.5	21
YSSL3	6	10
PEO2	3	14.5
PEO3	3	15
PSSL1	12.5	8.5
YL1	6	6
PA4	18.5	18.5
YA2	0	8.5
PE1	4	21.5
PS3	3.5	20.5
PL3	1.5	8.5

Among these active isolates, PL2 was selected for further lipase production studies. The selected yeast strain PL2 was morphologically cream colored, raised, entire, circular, glistening. Microscopically, the spores were spherically shaped and budding. The yeast was able to ferment glycerol, fructose, sucrose, glucose, and maltose and produced gas with glucose and was presumptively identified as *Pichia pastoris* PL2. Molecular analysis confirmed the presumptive *Pichia* yeast strain as *Kodamaea ohmeri* PL2 with the assigned accession number MW192826 and a phylogenetic tree to show the relatedness of *Kodamaea ohmeri* PL2 to some other selected organisms (Fig 1).

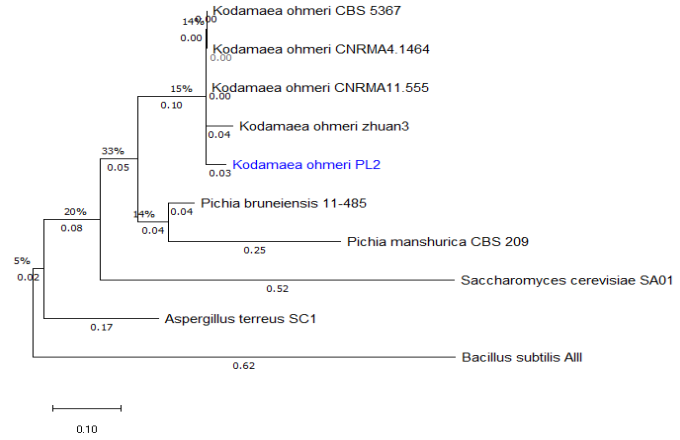

Figure 1 Phylogenetic tree of *Kodamaea ohmeri* PL2 in relation to other microbial strains

Lipase production by *Kodamaea ohmeri* PL2 in modified media

The highest quantity of lipase (262 U/mL) was produced by *K. ohmeri* PL2 at the fourth day of incubation in a medium containing Tween 80 (Figure 2a). Other production media containing castor oil and olive oil also recorded high enzyme activity at this time. At 24 hours (Day 1), the best lipase production by *Kodamaea ohmeri* PL2 also occurred in the Tween-80 medium (166.6 U/mL), closely followed by that in the Olive oil containing medium (160 U/mL) while the least was produced in the glucose medium. Hence, Tween-80 was selected as the substrate for lipase production in subsequent studies. Varying the concentrations of Tween-80 in the production medium, lipase production was not significantly different between 1-5% (P value 0.0977), hence, 1% Tween-80 concentration was continued with (Figure 2b).

From Figure 2c, Calcium ion stimulated the highest lipase production. The other cations also supported the production of lipase especially at 96 hours. On the effect of different sources of organic and inorganic nitrogen, it was observed that peptone had the highest stimulatory effect on lipase production (342 U/mL) compared to the other sources (Figure 2d). In addition, the ammonium-containing inorganic compounds (especially ammonium sulphate and ammonium chloride) also supported enzyme production, but with lower yields. The medium in which urea was utilized gave the least enzyme yield. From Figure 2e, 37°C favored the optimum yield of the lipase throughout the incubation period with the highest yield observed at 24 hours (148.5 U/mL). The lipase yield increased gradually with increase in the pH of the production medium until it peaked at pH 6 with the highest lipase yield (117.53 U/mL) realized at 96 hours (Figure 2f). The values obtained at pH 7 and 8 were close, but lower, presenting the same pattern over the incubation days. The least enzyme quantity was obtained at pH 10.

Characterization of *Kodamaea ohmeri* PL2 lipase

The effect of various physicochemical conditions on the activity of the *Kodamaea ohmeri* PL2 lipase is shown in figures 3a-d. Figure 3a shows the effect of enzyme incubation at increasing temperatures and incubation period on the activity of the PL2 lipase. The highest activity was observed at 50°C with increasing activities between 15 minutes to a peak at 30 minutes (160.62%) and a slight reduction thereafter at 45 and 60 minutes, respectively, (160.26 and 158.08%). At 40 ℃ a slight increase in the enzyme activity was recorded at 15 and 30 minutes. The lipase therefore was temperature stable over one hour. The lipolytic activity was neutral to slightly alkaline and peaked at pH 8 (Figure 3b). The lipase activity increased steadily up till pH 8, where the maximum activity was observed with a relative lipolytic activity of 163.05%. The values obtained at pH 7 and 9 were slightly less (with 35 and 21% increases, respectively), making the lipase an alkaliphilic enzyme. Figure 3c shows the effect of surfactants on the activity of the *Kodamaea ohmeri* PL2 enzyme. The lipase activity increased consistently with increasing Tween-80 concentration. Tween-80 stimulated lipase activity giving an activity of 130, 147 and 210% at 0.05, 0.10 and at 0.50 mM Tween-80 concentration, respectively, relative to the untreated enzyme. TritonX-100 and SDS augmented lipase activity at their highest concentration (0.5mM).

The effect of various cations on the activity of the enzyme is shown in figure 3d. From the observations, Calcium ion and Manganese ion had a 24% stimulatory effect on the lipase activity at 10 mM concentration. However, Fe^{3+}, NH_4^+ and Na^+ suppressed lipase activity. In most of the cations, the enzyme activity reduced with increasing cation concentration.

When a fixed volume of lipase was reacted with increasing *p*NPP concentrations, the highest activity of the *Kodamaea ohmeri* PL2 lipase was recorded at the 10 mM *p*NPP concentration (96.43 U/mL). The lipase activity thereafter reduced, and the least activity (49.5 U/mL) was observed using 50 mM concentration (results not shown).

From the purification of the *Kodamaea ohmeri* lipase, the maximum specific activity recovered from chromatographic fraction was 3.0 U/mg with a purification fold of 2.01 and lipase activity of 83.9 U/mL (Table 2).

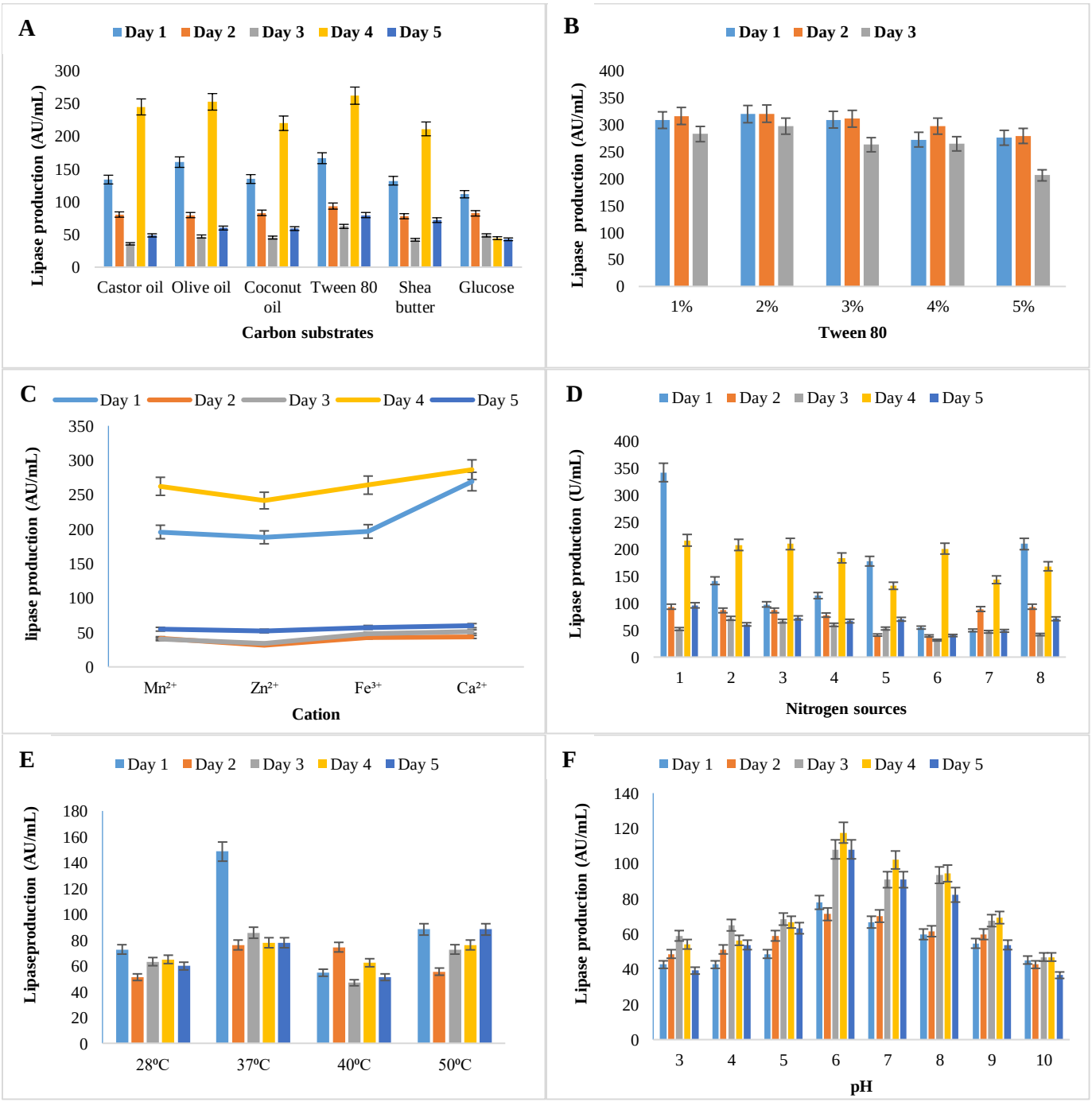

Figure 2 The effect of (a) various oil-based carbon sources, (b) increasing Tween 80 concentrations, (c) diverse metal ions, (d) organic and inorganic nitrogen sources [1: Peptone; 2: (NH4)2SO4; 3: NH4Cl; 4:NaNO3; 5: Yeast extract; 6: Urea; 7: Tryptone; 8: NH4NO3], (e) culture cultivation temperatures and (f) effect of modifications of cultivation medium pH on lipase production by *Kodamaea ohmeri* PL2 with increasing incubation days

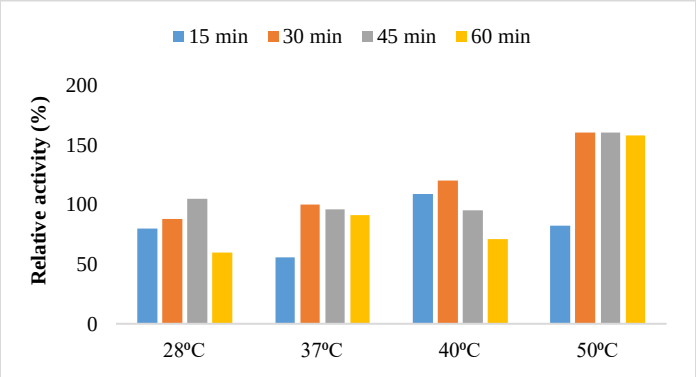

Figure 3a Effects of increasing temperature with corresponding increasing enzyme incubation time on the lipolytic activities *Kodamaea ohmeri* PL2

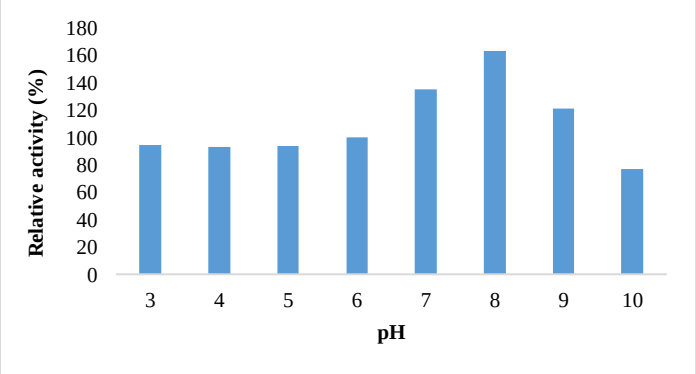

Figure 3b Effects of increasing pH regimes on the characteristics of the *Kodamaea ohmeri* PL2 lipase

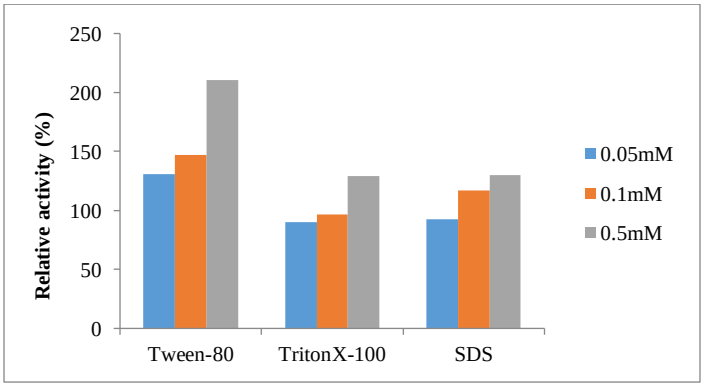

Figure 3c Effects of increasing concentrations of different surfactants on the lipolytic activities *Kodamaea ohmeri* PL2

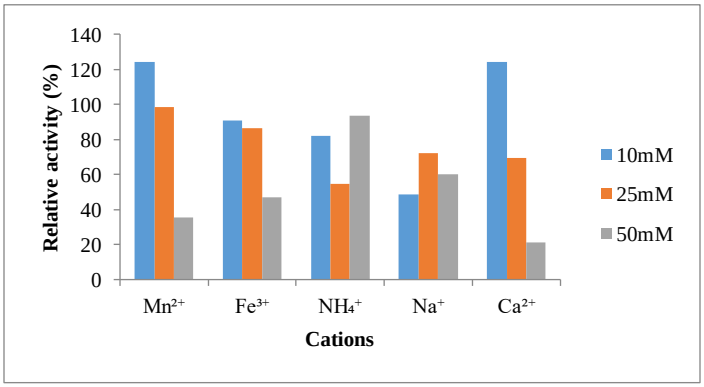

Figure 3d Effect of increasing cation concentrations on the activity of lipase produced by *Kodamaea ohmeri* PL2

Table 2 Protein Content and the effect of purification on Lipase produced by *Kodamaea ohmeri* PL2

Purification Step	Total lipase activity (U/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Lipase	202.5	132.7	1.53	1	100
$(NH_4)_2SO_4$ Precipitation	148.6	85.7	1.73	1.14	73.4
Dialysis	94.7	34.1	2.78	1.82	46.8
Gel Chromatography	83.9	27.9	3.00	2.01	41.4

DISCUSSION

An abundance of fungi was obtained from the different palm-oil mill wastes sampled and 21.3% of lipid-degrading organisms were present in the lipid-rich samples. When screening for lipolytic organisms and in lipase production, samples rich in oil and fatty carbon are essential **(Alabdalall *et al.*, 2020**). The highest number of lipolytic microorganisms was recorded in the sludge-like effluent of palm-oil processing area. This observation was in line with the work of **Salihu *et al.* (2011)** and **Asih *et al.* (2014)**, who respectively isolated lipolytic *Candida cylindracea* from oil effluent and produced lipase from a palm-oil mill waste. The fungal isolates in this work exhibited zones of hydrolysis on the olive-oil and the Tween-80 screening medium (shown as clear halos around the producing isolates and a color change of the medium from red to yellow). The use of olive oil and Tween-80 as screening substrates was reported in the work of **Zouaoui *et al.* (2012).** Even though the biochemical tests suggested that best lipolytic isolate, PL2, was probably *Pichia pastoris*, it was molecularly confirmed to be *Kodamaea ohmeri* PL2. Some members of the genus *Pichia* were reclassified as belonging to the genus *Kodamaea* (class: *Ascomycetae*; family: *Saccharomycetaeae*) based on the mating types and formation of spores having both spherical and hat shapes. **Corbu *et al.* (2019)** also reported the occurrence of an isolate CMGB-ST19 which was conventionally identified as *Pichia ohmeri* but reported using ITS-PCR as *Kodamaea ohmeri* CMGB-ST19. *Kodamaea ohmeri* has been reported in association with traditional fermentation of dairy products **(El-Sharoud *et al.*, 2009)**. Strains of *Kodamaea ohmeri* have been documented to have possible biotechnological applications and produce D-arabitol (**Zhu *et al.*, 2010**) A xylose-producing antimicrobial *Kodamaea ohmeri* was described by **Corbu *et al.* (2019)** and two strains of *Kodamaea ohmeri* were reported to successfully control rice seedling rot disease (**Limtong *et al.*, 2020**).

The production of fungal lipases and their lipolytic characteristics have been documented by many authors and the sources of carbon are reported to considerably impact enzyme production. In this work, *Kodamaea ohmeri* PL2 successfully utilized all the oils supplied as carbon sources and produced lipases which yielded titers higher than what was obtained when glucose was used as controlled substrate. Out of the six sources of carbon tested, Tween-80 supported the best lipase production, followed closely by olive oil. This is in line with the work of **Geoffry and Achur, (2018)** who reported that the highest lipase activity was obtained in the presence of Tween-80 as a carbon source in their research. Cell permeability is increased by the addition of surfactants such as Tween-80 to the medium, hence, enhancing the release of various molecules across the cell membrane. Although Tween-80 is a surfactant, it can serve as the sole carbon source in a medium for lipase production because it contains fatty acids. When surfactants are added to an oil-containing production medium, they increase the lipase activity (**Ramani *et al.*, 2010; Geoffry and Achur, 2018**). As opposed to olive oil being the second-best carbon source for lipase production after Tween-80 as observed in this study, **Zouaoui *et al.* (2012**), reported that olive oil supported the maximum lipase production for all the isolates used, although with values close to those in the Tween-80 medium. **Das *et al.* (2016)** reported the stimulation of lipase using vegetable oils. Also, the medium with glucose had the least lipase production, since it did not contain fatty acids which would induce lipase production, a finding supported by **Asih *et al.* (2014)**. The increasing concentrations (1-5% v/v) of Tween-80 did not translate to increased lipase production and 1% Tween-80 concentration was continued with. **Kutyla *et al.* (2021)** used Tween 80 as sole carbon source in optimized production for lipase by a *Chrysosporium pannorum*A-1 and reported highest lipolytic activity on day 4 of the culture using 0.4–0.9% Tween-80. The work by **Das *et al.* (2016)** reported no appreciable variation in the activity of lipase produced in a coconut oil-based fermentation setup at 0.1-1.5% v/v.

The presence of trace quantities of mineral salts in the production medium either stimulates or inhibits enzyme synthesis depending on the nature of the enzyme under study. Calcium ions supported the highest lipase production, an observation like the findings of **Ramani *et al.* (2010)**. According to **Celligoi *et***

***al.* (2016)**, calcium ions enhance the specificity of ion binding process on an enzyme's active site.

From the diversity of nitrogen sources used, both the organic and inorganic nitrogen sources stimulated lipase production with the highest lipase titre in a medium containing peptone. These findings were like the reports of **Kumar *et al.* (2011)** and **Sharma *et al.* (2016)** when studying the effect of nitrogen sources on lipase production.

The incubation temperature affects lipase production probably by modulating the growth of the cell and thus its metabolic activities (Gupta *et al.,* 2004). *Kodamaea ohmeri* PL2 had its highest lipase production at 37ºC. **Sooch and Kauldhar, (2013)** and **Rasmey *et al.* (2017)**, reported best lipase production by some fungi at the mesophilic range (30-40ºC). The considering the source of isolate (POME sludge), it is likely that the environmental adaptation of *Kodamaea ohmeri* PL2 to a mesophilic condition also contributed to this finding.

The pH of a production medium determines microbial growth and the enzyme yield since a change in the extracellular pH will impact the intracellular pH equilibrium, pH can thus affect the mechanism of enzyme synthesis. From this study, the optimum pH for lipase production was reached at pH 6. **Gupta *et al.* (2004), Sooch and Kauldhar (2013), Celligoi *et al.* (2016), Liu and Kokare, (2017)** and **Rasmey *et al.* (2017)** all reported highest lipase production between pH 6 and 9.

Investigating the effect of incubation time on the production of enzyme revealed that the lipase production was highest at 24 and 96 while lower values were obtained at days 2 and 3. The longer an organism is incubated with its substrate, the greater the amount of product, however, the rate of product formation is not usually a simple linear function of time of incubation as the enzyme could suffer denaturation and loose a significant amount of their activity during the period of incubation. In a study by **Massadeh and Sabra (2011)** it was observed that the maximum lipase activity was obtained after 24 hours.

The lipase of *Kodamaea ohmeri* PL2 had its highest activity at 50ºC and was thermostable over 60 minutes with activities higher than what obtained at 15 minutes. **Islam *et al.* (2008)** reported a lipase active between 30-60ºC and **Tripathi and Thakur (2014)** also supported the temperature activity findings of this research signifying that lipase could be moderately thermo-active. The activity of this lipase peaked at pH 8 but values recorded at pH 7 and 9 were higher than those recorded at other pH regimes.

Tween-80 successfully augmented the catalytic activity of the *Kodamaea ohmeri* PL2 with a 110% increase at its 0.5% concentration. This is because of the reaction occurring at the aqueous/organic interface. **Liu and Kokare, (2017)** also reported a Tween-80 augmented lipolytic activity. Unlike the suggestion of **Sharma and Goswami (2020)** that a reduction in catalysis likely occurs as surfactant concentration increases, the three concentrations enlisted in this research (all below 1.0%) resulted in increasing lipase activity, mostly profound in Tween-80. At times, divergent/mixed responses may be recorded by a particular lipase upon different non-ionic surfactants. Hence, the lower activity seen in this work using Triton X-100 (29% increase) compared to Tween-80. It is therefore possible to apply this lipase in the catalysis of various industrial processes and product formation. **Borkar *et al.* (2009)** reported a lipase stable in the non-ionic Tween-80 along with a reduced activity using Trixton-X-100.

In the presence of Ca^{2+}, the maximum hydrolytic activity of the *Kodamaea ohmeri* PL2 was enhanced. **Islam *et al.* (2008)** reported the best activity using 10 mM Ca^{2+} concentration. **Amanda *et al.* (2001)** reported that Ca^{2+} may help in the structure formation of active enzymes leading to a higher enzyme activity. Lipases have been documented to possess a Ca^{2+} binding site **(Salameh and Wiegel, 2007; Liu and Kokare, (2017)** and this could have been responsible for the high lipase activity recorded in this work. The Ca^{2+} probably featured as ligands thereby facilitating the linkage to adjacent residues and in the formation of improved stability in lipase structure. Manganese also enhanced lipase activities and could also contribute to enzyme thermostability. Other cations (Fe^{3+}, NH_4^+ and Na^+) had inhibitory effects on the *Kodamaea ohmeri* PL2 lipase. Also, **Salameh and Wiegel, 2007** reported that cations were found necessary for lipase activities and supported the thermostability of lipases.

The highest specific lipase activity was recorded in the lipase subjected to gel chromatographic analysis, an indication that the specific activity improved with increasing purification steps. **Lukong *et al.* (2007)** suggested that the increase in the activity of enzyme after purification might be due to the removal of more impurities at each step of purification. Hence, the level of purification achieved can be measured by the increase in enzyme activity.

There was a reduction in lipolytic activity of the *Kodamaea ohmeri* PL2 lipase with pNPP concentrations above 10mM. This reduction may be because of the high starting concentrations employed in this study (10 mM) since generally, the rate of a reaction increases when higher concentrations of the substrate is made available to the reaction mixture until a certain peak after which the rate reduces. **Sumarsih *et al.* (2019)** who used 0.5-1.75 mM concentrations of p-nitrophenyl palmitate recorded highest lipase activity at a 1.5 mM pNPP concentration.

CONCLUSION

The importance of lipase cannot be overemphasized as it is one of the top three most commercialized enzymes, relevant in several industries in which it finds various applications. It is therefore important that cost effective methods should be harnessed as well as microorganisms with effective lipolytic abilities, this will allow for the increased production and application of this essential enzyme. In this research, the lipolytic potential of fungi isolated from waste sites of a local palm fruit processing factory was reported. The selected lipolytic yeast, molecularly confirmed to be *Kodamaea ohmeri* PL2, was able to utilize a variety of lipid substrates for lipase production. The study also outlined optimum parameters for lipase yield which included cationic additions, especially calcium ion, 1.0% Tween-80 concentration, peptone and ammonium nitrate, pH 6 and a culture incubation temperature of 37ºC. The moderately thermoactive *Kodamaea ohmeri* PL2 lipase was enhanced by a pH range of 7-9, stable at 50ºC for 60 minutes and had improved specific activity with increasing lipase purification steps. These optimum conditions can thus be explored for large-scale/industrial applications and commercialization of *Kodamaea ohmeri* PL2 lipase.

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REVIEW

MYCOTOXINS-CAUSES, PREVENTION AND CONTROL: MATHEMATICAL MODELING STRATEGIES

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ABSTRACT

For many decades ago, planting, harvesting, storing, transporting, distributing, and processing agricultural produce into useful products such as foods and feeds have been plagued by various contaminations and spoilages. Most often, these contaminants are fungi and molds-based microbes producing toxic contaminants that result in severe deterioration of some of the quality characteristics of these agro products. These toxic metabolites are called mycotoxin. Many fungi toxic in food and feed are known to be hazardous to human and animal's health. To prevent the contamination of mycotoxins in foods and feeds, primary, secondary, and tertiary methods are required. Similarly, certain treatments are equally necessary to control the continuous growth of these toxins in the products. This study deals with the review of these various preventive and corrective methods with the view of providing useful insight to the current practices of mitigating the production and contamination of mycotoxins in food and feed products. The study discusses the tendency of an integrated Taguchi model for predicting or studying mycotoxin through the combination of various preventive activities to emerge the optimum preventive procedure.

Keywords: mycotoxins cause, prevention, control, treatment, physical, chemical, quantification, integrated Taguchi-data envelopment analysis, robust parameter procedures

INTRODUCTION

According to **Bennett and Keller (1997)**, mycotoxins are the results of the metabolites formed by fungi and mold. These are those belonging to the species *Aspergillums*, *Penicillium* and *Fusarium*. Mycotoxins are produced in cereal crops, animal feed and forage products either before or during harvesting. They are known to show different chemical types and about 400 mycotoxin fungal metabolites are known to be toxic in nature **(Moss, 1997)**. The fungi contamination of food and feed majorly results in decline in produce yield, value, and mostly importantly economic losses (**Atanda *et al.*, 2010)**. Several hundred diverse mycotoxins have been recognized, but the most usually observed mycotoxins that are constitute a threat to human health and that of livestock are aflatoxins. Human and animal exposure to mycotoxins can happen either directly by eating infected food or indirectly from animals that are fed with infected feed, for instance from milk, meat, and egg **(Abdel-Wahhab *et al.*, 2004)**.

The potential risks of mycotoxin's presence in plants could be controlled or prevented by different methods. This is simply through checking for infected parts of the plant and removing it from the plant, by practicing enhanced cultivation, proper harvesting, and good storage conditions **(Abdel-Wahhabet *et al.*, 2008)**. To prevent crop contamination of fungal from the feed and food, pre- and post-harvest strategies which include annual crop rotation, proper use of pesticides, aeration of the crops effectively after harvesting, storing at a secure humidity level, and providing good protective storage **(Whitaker *et al.*, 2005)**. To understand the menace of mycotoxins, European Union published a list of mycotoxins that are of interest; with those causing major concern for the safety of animal feed and foods **(EU SCAN, 2003)**. The list mentioned Aflatoxin B1 (AFB_1) and Ergot sclerotia (subject to the Commission Regulation (EC) No 1881/20062); Zearalenone (ZEA), Deoxynivalenol (DON), Ochratoxin A (OTA) and Fumonisins (especially Fumonisin B_1, FB_1). The recommended tolerance levels of these mycotoxins have been published in the **Commission Recommendation 2006/576/EC3**. Furthermore, mycophenolic acid, cyclopiazonic acid and moniliform in mycotoxins have been identified as those with high possibility for emerging threats. Unfortunately, their occurrence and toxicological data are still scares, limited, and highly required to combat the menace.

The secondary metabolic products of molds (especially of *Aspergillus*, *Penicillium* and *Fusarium* genera) are called mycotoxins. There are over 300 of such secondary metabolites; however, just around 30 are toxic. Most of these molds producing mycotoxins derive their toxicogenic tendencies at all climatic conditions when attaching to their hosts which could be any solid or liquid in the presence of adequate nutrients and moisture. Mycotoxins are usually referred to as poisonous compounds in foodstuffs created by some fungal type thus posing direct and indirect health threats to human and animal **(Moss, 1997; Scudamore, 2005)**. By direct, mycotoxin contamination occurred through cereal crops and plants. Furthermore, indirect could be by animal feeding contaminated feeds containing mycotoxin residues to animal and human contract it through consumption of contaminated plant and animals' products such as milk, meat, eggs **(Galvano *et al.*, 2005; Scudamore, 2005)**. The fungal species responsible for mycotoxin production mainly belongs to five (5) species namely *Aspergillums*, *Fusarium*, *Claviceps*, *Stachybotrys* and *Penicillium* **(Sweeney and Dobson, 1998; Santin, 2005)**. The environment where pre-harvest and postharvest are conducted is also an essential factor to mycotoxin contamination of grain and oilseed crops **(Anonymous, 2003a)**.

Environmental stress during the production of cereal grain and other products consequently reduced the strength requires to characterize and predispose plants to infestation and colonization by toxigenic fungal species. Some of fungal strains are known to produce more than one mycotoxin and a single mycotoxin produces more than one fungal spp. In certain instances, single species could produce multiple mycotoxins **(Devegowda and Murty, 2005; Santin, 2005)**. Environment where that are prone to frequently irregular increase of the temperature and humidity can also affected the colonization of mycotoxin **(Russell *et al.*, 1991)**. During storage of food and feed products, the occurrence of toxigenic fungal contamination is facilitated through some factors related to the prevalent environmental conditions such as moisture, temperature, substrate aeration, oxygen and carbon dioxide concentration, microbial interactions, mechanical injure, great quantity of fungal and pest invasion **(Ominski *et al.*, 1994; Anonymous, 2003a; Santin, 2005)**. High temperature and humidity are the most important factors of mycotoxins fungal colonization and production.

It is usual to categorize toxigenic fungi into "field" (or plant-pathogens) and "storage" (saprophytic/spoilage) organisms **(D'Mello, 2001; Santin, 2005)**. Field fungi are group of fungal species that inhabit seeds while the crop is still in the field and require high moisture conditions (20-21 %). These include species of *Claviceps*, *Neoitphodium*, *Fusarium*, *and Alternaria*. Storage fungi (also called storage molds) are group of fungi that infiltrate grains or seeds during of storage. The group of fungi that invade seeds during storage relatively needs less moisture than field fungi (13-18 %) and in most cases they do not present any serious threat before harvest, and they were those that could grow at moisture contents in equilibrium with relative humidity of 70 to 90% where no free water is present. Storage fungi include species *Aspergillus* and *Penicillium* **(Anonymous, 2003a; D'Mello, 2001; Santin, 2005).** Fungi grow at temperatures between 20 °C and 30 °C. It should be noted that if the grain is at high temperature during time of harvest, high temperature can be maintained for many days or week after harvest unless the storage has regulated room temperature for cooling **(Santin, 2005)**.

Therefore, prevention of mold growth and its mycotoxin production relies on elimination of environmental factors that can favor the growth of mycotoxin. Preventing mycotoxin accumulation in stored grains and oilseeds depends primarily on humidity or moisture control. If the product of feed and foods is too dry to allow fungal growth and it is kept dry, no further deterioration can occur. However, if there is pest activity, moisture migration, condensation, or water

leaks, fungal growth that would be able to bring mycotoxin contamination can happen. Most of the contamination in storage comes from infections that began in the field **(Anonymous, 2003a)**. For the period of collecting and processing food and feed products, adequate care must be ensured to prevent excess damage to protective part and kernels from breaking or bruises to the protective shie of the crop as this may lead to contamination during storage. Moreover, the highest rates of invasion of fungal species are known to be linked with broken and insect-damaged kernels **(Munkvold and Desjardins 1997; Malone *et al.*, 1998b; Anonymous 2003a)**. Urgent attention is advocated to removing contaminated kernels collected in the field, with minimum loss of sound kernels highly recommended **(Sauer *et al.*, 1992; Widstrom 1996; Munkvold and Desjardins 1997; Anonymous 2003a)**.

Pests are known to have contributed immensely to fungal growth due to physical damage of grain barriers, which renders it liable to mold invasion of the exposed endosperm. The biological activity of pests can bring about a raise in both moisture and temperature of the invaded grain. Pests can also carry spores of mold and their fecal materials that can serve as a substrate for mold growth **(Santin, 2005)**. Appropriate grains moisture reduction and well-regulated storage is able to minimize fungal development and mycotoxin infestation after harvesting **(Anonymous 2003a; Santin, 2005)**.

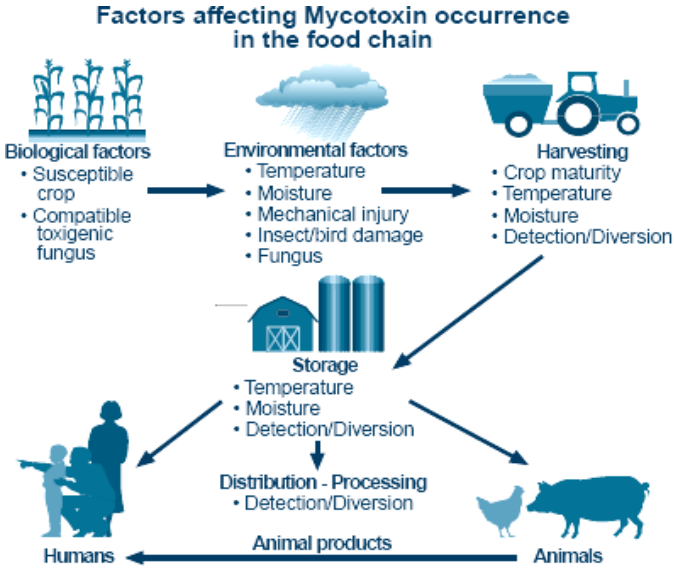

Figure 1 Factors affecting mycotoxin occurrence in the food and feed chain **(Anonymous 2003a**; adapted from **Pestka and Casale 1989)**

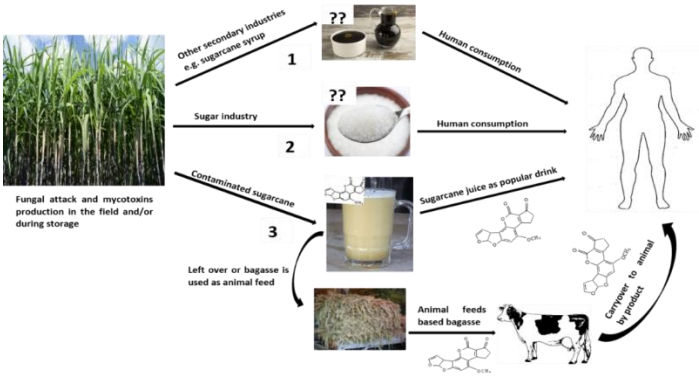

Figure 2 Mycotoxins contamination in sugar cane direct and indirect expose to human being **(Abdel-Wahhab et al., 2008).**

According to **Adegoe (2004)**, more than 300 mycotoxins that have been separated and characterized. These are not limited to the followings: aflatoxins, ochratoxins, deoxynivalenol, zearalenone, fumonisin, patulin**,** trichothecenes and alternariol.

Toxicological syndromes of mycotoxins ingestion are grouped as acute and chronic type toxicity. The acute one generally has short time of onset and clear toxic response, but the chronic toxicity seen by time-consuming and identified diseases which are cancers **(James, 2005)**. In humans particularly the liver and kidneys can be affected **(Bankole and Adebanjo, 2003)**. Aflatoxins have been shown to be involved with and to exaggerate hepatitis B infection, while the fungi fumonisins spices contaminated food have shown to account for esophageal disease in South Africa **(Makaula *et al.*, 1996)**. Similarly, F. *sporitrichoides* and *F. poae* have been identified with alimentary toxic aleukia. Some of the symptoms include esophagealpain, laryngitis, asphyxiation, and vertigo **(Lewis *et al.*, 2005)**. Mycotoxin contamination generates a broad range of harmful effects in animals causing different health problems such as lesions in the mouth, unnecessary enlargement of liver and kidney, pale aspect of liver, immune disorder, dysfunction of nervous system, weakness of bones, reduction in pigmentation, diminish of egg production and egg weight, poorer growth rate to mention a few. These signs depend on the animal species and mycotoxins contamination level **(Abdel-Wahhab *et al.*, 2008)**.

Good Agricultural Practices (GAP) in farming system reduces the contamination of mycotoxin in feed and food products **(Abdel-Wahhab *et al.*, 2013)**. To prevent or reduce of the contamination of animal feed, good practice and activity at farmer level action must be taken before and after harvesting and storage condition must be carefully **(Negashe *et al.*, 2018)**. According to **Abdel *et al.* (2008)**, the first primary prevention is before of the fungal mycotoxin contamination and infestation of the feed and food at the beginning. Primary prevention is the most important for retarding mycotoxin fungal growth in feeds and foods **(Atanda *et al.*, 2013)**. Several procedures can be undertaken. These are planting of anti-fungal plants, timely sowing, harvesting, and weed control, appropriate storage, and transportation, control of insect, moisture, and temperature. Humidity during harvesting is also a main factor for the formation of mycotoxin in feed and food **(Negedu *et al.*, 2011)**. The procedures include (a) planting fungal resistant plants for feed total field control of mycotoxin causing fungi, (b) arranging the proper time of harvest, (c) ensuring proper moisture contents of the feed and food during harvesting, post harvesting and storage condition, administering of fungicides to retard the growth of fungi, (d) managing pest and insects' infestation in storage by using standard insecticides, pesticide and (e) modifying the atmosphere.

According to **Kabak *et al.*, (2006)**, harvesting strategies are most significant in the prevention of mycotoxin contamination of foods and feeds. If the attack of some fungi begins in product of feed and food in beginning phase, this secondary prevention level of will be then necessary. The presented toxigenic fungi should be eliminated, or its development be stopped to put off further deterioration and mycotoxin production **(Abdel *et al.*, 2008)**. Actions such as drying the product could be necessary. These secondary prevention methods include (a) discontinuation of the development of contaminated fungi by re-aeration the products of feed and food and (b) elimination of infected seeds from the feed and food. If the feeds and foods are greatly infested by contaminated fungi-mycotoxins, the first and second preventive actions need to be initiated **(Atanda *et al.*, 2013)**. Commonly, agricultural products' contamination of mycotoxin can be banned by means of good pre-harvest and post-harvest management practice **(Kabab *et al.*, 2006)**.

The physical, chemical, and biological control means are usually employed to treat infected plants and crops. However, the action has its own restrictions, since the treated products should be safe from the chemical's contaminants and the essential nutritive value should not be compromised **(Abdel *et al.*, 2008)**. The products of feeds and foods are separated by mechanical means, color categorization of the feed and food, elimination of the small or screenings from the bulk shipments of grains and nuts considerably reduce the mycotoxin content of grains. Gamma irradiation has successfully been used to control ochratoxin levels in animal feeds **(Refai *et al.*, 1996)**. After the crops or plants are harvested, aerated, dried, stored, then and suitable transportation of the products are major importance physical treatment needed thereafter to re-recontamination. The technique should be sure that the removing of toxic substance system can change the contaminants to a non-hazardous derivative without harmful change in the food and feed products. A broad variety of chemicals have been shown to decrease, demolish or inactivate mycotoxins in feed and food **(Samarajeewa *et al.*, 1990)**. The ammonization process uses ammonium hydroxide or gaseous ammonia, both of which are uniformly successful in detoxifying aflatoxins in peanut, cotton, and maize meals **(Piva *et al.*, 1990)**. Major in-roads have been the use of structure difference by natural or bio-control strategies. For instance, growth of non-toxicogenic bio-control fungi, toxigenic strains within field can help in the decreasing of mycotoxins in the crops (Cleveland *et al.*, 2003). The use of microbe-free mycotoxins has been reported by (Murphy *et al.*, 2006) to be shows possible improvement to the treatment of mycotoxins. Fusarium pathogens can be reduced or dropping by using the choice of the production of food and feed cereal plant **(D'Mello and Macdonald, 1998; Baker, 1987)**. Biocontrol is one of the best naturally secure and in some cases is the only choice offered to protect plants in opposition to pathogens **(Heydari, 2010)**.

Existing risk management measures to avoid the direct adverse effects (both acute and chronic) on livestock as well as the indirect effects due to the presence of these toxins in animal products and foods and have been well developed and documented. These includes regulations and recommendations of the tolerable limit of the mycotoxin in foods and feeds, monitoring and enforcing compliance to good production practices by feed and food producers, development of standardized analytical methods to determine the mycotoxin content of a feed lot, developing and encouraging farmers on all the preventive measures and decontamination of mycotoxins contaminated foods and feeds. Many analytical techniques have been designed and applied for determining and predicting mycotoxins in feed and foods. However, most of these known methods have not been thoroughly validated and structured by the European Union of CEN (http://www.cenorm.be). Certain procedures have been standardized for analyzing mycotoxins in human food by the CEN Technical Committee 275 (CEN/TC 275); one protocol **(EN ISO 17375:2006)** has been provided by CEN Committee, the CEN/TC 327 standardized techniques for analyzing mycotoxins in feeds. Standardized procedure such aspr-EN 15791:2009 and ISO 14718:1998with the use of High-Performance Liquid Chromatography (HPLC) has also been sanctioned. Similar HPLC techniques include immune-affinity column clean-up, fluorescence detection and immune-affinity column clean-up, RP-HPLC with fluorescence detection during pre- or post-column determinations (pr EN 16006), OTA estimation by immune-affinity column clean-up and HPLC fluorescence detection (pr EN 16007) just to mention a few.

The best way to control mycotoxin to know the amount of the toxin ingested or that could be ingested by human and animal. This has been done through experimentation and mathematical modeling. The most common experimentation is the in vitro analysis. This procedure involves the analysis of mycotoxin adsorption in the screening of potential mycotoxin detoxifying agents. The idea behind these techniques is that if the detoxifying aid could not adsorb mycotoxin *in vitro*, then such aid has slime or no tendency to detoxify mycotoxin *in vivo*. It is a method used for assessing, identifying, and ranking effective mycotoxin-detoxifying aids as well and the detoxifying conditions and viable mechanism (Diaz and Smith, 2005). Many researchers have adapted and published these experimental applications at different forms such as single-concentration, classical isotherm (those involving binder concentration fixed, toxin concentration increasing) gastro-intestinal tract models, variable loading binding experiments and beyond. These applications are described as follows:

- **Single-concentration methods**
  Under single-concentration method, a known level of mycotoxin is reacted with a known amount of the sample in an aqueous solution. The adsorption of purified toxin solutions in these aqueous media is measured as percent adsorbed (%ads) which the fraction of the toxin bound to the adsorbing aid. This value is determined based on the loading of the adsorbing aid used.
- **Adsorption isotherms**
  According to **Grant and Phillips (1998)** and **Ramos and Hernandez (1996)**, adsorption isotherms have been applied to evaluate mycotoxin-detoxifying aids. This involves plotting mycotoxin adsorbed per unit weight versus constant temperature at stables conditions. It took into consideration the reversibility in the chemical equilibrium of the detoxification process. Freundlich, Langmuir and Hill are frequently used models for interpreting the results.
- **Food matrix method: Modified adsorption isotherms**
  Isotherms could be modified with the view of comparing adsorbed mycotoxins in the presence and absence of a food matrix. This would yield results of whether a matrix-mycotoxin related could affect adsorption efficiency. Here, Freundlich, Langmuir and Hill fitted curves are obtained for mycotoxin adsorbed. A thorough precaution for the efficiency of this method is to ensure that a mycotoxin-free food matrix is used.
- **Gastro-intestinal models: Static and dynamic experiments**
  The purpose of the in-vitro is to assess the efficiency of mycotoxin-detoxifying aids in attaching to the mycotoxins for analysis through simulated gastrointestinal model. This will identify the physiological conditions that are germane such binding. In these static and dynamic in-vitro techniques, gastro-intestinal models are used to examine the efficacy of the detoxifying aids. In the work of **Vekiru *et al.* (2007)**, it was revealed that the efficacy of mycotoxin adsorbing aids is strictly dependent real set of conditions at which it passes through the gastro-intestinal tract.

Retrospectively, mycotoxin production is difficult to be prevented; however, certain prevention activities can be adopted to limit its contamination on food and feeds, bio availability or toxic effects. **Abrunhosa *et al.* (2009)** has revealed vast number of microorganisms that could destroy or reduce several mycotoxins. Biological methods were concluded to be the foremost among all preventive methods. The technique is known for rendering mycotoxins ineffective by bio-transforming the toxin to produce non-toxic metabolites that less harmful when ingested.

Recent revelations confirm the need prioritize modeling mycotoxin in all ramifications **(Battilani, 2016)**. These efforts could be justified due to (i) mycotoxin contamination is vastly becoming a global phenomenon (ii) more chronic health foodborne diseases have increased **(Schatzmayr and Streit, 2013)** (iii) impacts of mycotoxins on agro-products is growing with increased number of compounds and agro-foods worldwide **(Wu and Guclu, 2012; Mitchell *et al*., 2016)**. Freundlich, Langmuir and Hill models have formed the major equations used in many mycotoxins modeling studies. **Grant and Phillips (1998)** had applied modification to these models in the analysis of the various mycotoxin adsorbing aids. The applications of models to mycotoxin prediction have shown positive improvements in my articles published **(Battilani and Logrieco, 2014; Battilani and Leggieri, 2013; 2014; 2015a; 2015b; Skelsey and Newton, 2015; Chauhau *et al.*, 2010; Marin *et al.*, 2012; Garcia *et al.*, 2011; 2013; Aldars-Garcia *et al*, 2015; Medina *et al.*, 2014; Ioannidis *et al.*, 2014; Passamani *et al.*, 2014; Nazari *et al.*, 2016)**. However, efforts to model the attendant threats of mycotoxins in foods and feeds are still scanty and limited. The reasons for this lack in the application of model could be (i) low interest or many researchers in this direction (ii) naivety of the support modeling could render to the efforts to decimate the growth of mycotoxins (iii) lack of adequate trusts in the model itself by researchers and (iv) the complication witness during model development.

Most of the current models only considered the prediction of the toxin base of single factor. For instance, modeling has been done based on crops **(Battilani and Logrieco, 2014; Skelsey and Newton, 2015; Fels-Klerk *et al.*, 2012; Landschoot *et al.*, 2012; Froment *et al.*, 2011; Asselt *et al.*, 2012; Battilani *et al.*, 2013; Chauhau *et al.*, 2010; Battilaniand Leggieri, 2015a). Some are modeled without crops (Marin *et al.*, 2012; Garcia *et al.*, 2011; 2013; Aldars-Garcia *et al.*, 2015)**, empirical models based on environmental and ecological factors **(Landschoot *et al.*, 2012; Van der Fels-Klerx *et al.*, 2010; Leffelaar and Ferrari, 1989)**. Based on meteorological data **(Battilani *et al.*, 2016; Vaugham *et al.*, 2016)** and based on pre- and post-harvest conditions **(Battilani *et al.*, 2015b)**. **Laila *et al.* (2016)** employed a probability-stochastic model to for the growth of and production of aflaxtoxin. Combination of two or more factors into the model prediction and analysis of mycotoxin are limited **(Fels-Klerx *et al.*, 2016)**. **Vaugham *et al.* (2016)** developed a model that predicted mycotoxin from the combination of climate, pathogen and host and cropping systems.

Various means of quantifying mycotoxins through experimentations and mathematical modeling for mycotoxin prediction and estimation have been published. The experimentations are majorly of the in-vitro while most of the models are based on the empirical and mechanistic. The models are usually directed to the determination of the effects of the ecological factors on the fungi infection cycle. This in-vitro data are based mainly on the growth of fungi. Few or none of these models consider the effects of preventive methods on the prediction of the growth of mycotoxin producing fungi. This study intends to emphasize the quantification of mycotoxins are juxtaposed in a modular framework to prevent the production of these mycotoxin producing funguses. Therefore, the main contribution of this study is to apply mathematical modeling discuss how an integrated Taguchi-Data envelopment model can be used to determine the best (optimum) practice (procedure) that could substantially lead to the retardation of the growth these mycotoxin producing fungi. Specifically, the following are anticipated; (i) provision of basic information about mycotoxins, factors responsible for occurrence of mycotoxins, and to give insight to the prevention, treatment, and control of mycotoxins in foods and feeds, (ii) review previous modeling techniques and (iii) Taguchi-Data envelopment model could be developed for the prediction of the optimum practice that could lead to the reduction of the rate of mycotoxin production and contamination in foods and feeds.

MODEL CONCEPTUALIZATION

Few modeling has been carried out on the combination of various preventive techniques, on a mechanistic level, to predict mycotoxin. This is the challenge facing the researchers today and would probably be in the future. This review will now examine the efficacy of an integrated Taguchi-Data envelopment model to adequately combine various preventive techniques to select an optimal procedure that can be used to decimate the growth and production of mycotoxin in feed.

Taguchi robust signal-to-Noise ratio

The old, online traditional methods of quality assurance are based solely and primarily on inspecting products as they are discharged from the production line and rejecting those products that fail to meet up with the specified acceptance range. However, it has been pointed out that no amount of inspection can

improve product's quality attributes and that quality must be built into the product right from conception **(Taguchi *et al.*, 2005)**. Robust parameter design is an engineering procedure that utilizes different strategies for improving performance during product and process design so that quality response can be obtained efficiently and optimally. This off-line quality control procedure idea stemmed up due to the need to enhance the dependability of controllable factors to the effects of the variations in the uncontrollable factors so that the overall quality response is insensitive to the effects of the variations **(Taguchi *et al.*, 2005; Al-Refaie and Al-Tahat, 2011; Adesina and Daneshvar, 2018; Danesvar and Adesina, 2018).**

Factors are classified into two distinct classes of those that are controllable and those are uncontrollable (noise). Taguchi therefore aimed at identifying optimum controllable factor settings (level combination) that minimize process variability. There is the need to understand these classes of process factors. Controllable factors (design or control factors) are those factors that can be easily moderated, adjusted, or controlled by the designer. These are not limited to material choice, cycle time, or operating temperature, process route choice, and type of catalysts used, choice of condition. Uncontrollable factors (noise factors) could be described as forces compelling or causing deviations from production or quality target.

It can be subdivided into three categories namely external, internal, and unit-to-unit noise factors. External noise factors are those that arose due to the exposure or variation in condition of use. Internal noise factors are due to production variations while unit-to-unit are because of deterioration or variation with time of use. Noise factors are difficult or almost impossible to control and could be expensive when attempted to control or eliminate them. Taguchi proposed three steps technique for developing good quality products and processes. These are system design, parameter design and tolerance design. Experiment must be carried out to implement parameter and tolerance designs. Here various mycotoxin preventive activities could be grouped into the orthogonal array and with a response of mycotoxin level, optimum prevention procedure would emerge. Signal-to-Noise ratio of the robust parameter; Larger-The-Better (LTB), Smaller-The-Better (STB), and Nominal-The-Better (NTB) of each orthogonal array would be determined by Equations (1-3).

$$\mathrm{SN} = -10\log\left(\frac{1}{\mathrm{n}}\sum_{\mathrm{i=1}}^{\mathrm{n}}\frac{1}{\mathrm{y_{ij}^2}}\right) \quad (1)$$

$$\mathrm{SN} = -10\log\left(\frac{1}{\mathrm{n}}\sum_{\mathrm{i=1}}^{\mathrm{n}}\mathrm{y_{ij}^2}\right) \quad (2)$$

$$\mathrm{SN} = -10\log\left(\frac{\bar{\mathrm{y}}_{\mathrm{ij}}^2}{\mathrm{s_{ij}^2}}\right) \quad (3)$$

Revamped Data Envelopment Analysis (DEA)

In general, DEA have been referred to as a fractional mathematical programming technique solely responsible for evaluating the efficiency or performance of homogeneous decision making units (DMU) with multiple inputs and outputs system. **Rocha *et al.* (2016)** described data envelopment analysis (DEA) as a linear programming technique used for determining the relative performance of a set of competing DMUs whenever multiple inputs and outputs makes the comparison cumbersome. It is a non-parametric technique for measuring technical efficiency of various systems. By technical efficiency, we mean the degree of industry technology level that the production process of a production unit reaches. This can be determined from two perspectives (i) input and (ii) output. From input aspect under the input condition defined for the system, the technical efficiency is measured by the degree of output maximization and for output perspective under the output condition defined; the technical efficiency is measured by input minimization. In both cases, technical efficiency can be estimated quantitatively as a ratio of output to input. Each set of mycotoxin factor combination would form the DMUs.

There are many models in DEA, variable return to scale (VRS) model could be adapted into the suggested integrated model. VRS model in Equation (4) and (5) below would be leveraged to determine the optimum mycotoxin preventive procedure.

$$\begin{aligned} &Max \ \textstyle\sum_{r=1}^{s} u_r y_{ro} + u_o \\ &S.t. \ \textstyle\sum_{i=1}^{m} v_i x_{io} = 1 \\ &\textstyle\sum_{r=1}^{s} u_r y_{rj} - \sum_{i=1}^{m} v_i x_{ij} + u_o \le 0 \\ &u_r \ge 0 \quad r = 1, \dots s \\ &v_i \ge 0 \quad i = 1, \dots m \\ &u_o \ free \end{aligned} \quad (4)$$

$$\begin{aligned} &Min \ \textstyle\sum_{i=1}^{m} v_i x_{io} + v_o \\ &S.t. \ \textstyle\sum_{r=1}^{s} u_r y_{ro} = 1 \\ &\textstyle\sum_{r=1}^{s} u_r y_{rj} - \sum_{i=1}^{m} v_i x_{ij} + u_o \le 0 \\ &u_r \ge 0 \quad r = 1, \dots s \\ &v_i \ge 0 \quad i = 1, \dots m \\ &u_o \ free \end{aligned} \quad (5)$$

Taguchi-data envelopment modeling approach

This robust parameter procedure could be achieved in four phases: data collection and generation, responses evaluation by any of the experiments mentioned before, efficiency determination using DEA model, optimization to determine and select optimum preventive level combination that can reduce the growth and contamination of mycotoxin in foods and feeds.

Phase A (Data generation and collection)

The major aim of this phase is to gather data for signal-to-noise ratio estimation using the orthogonal array. This phase would consist of five steps:

- Step 1 (identifying controllable factors):
- Step 2 (selecting adequate orthogonal array):
- Step 3: Conducting the experiment, literature data (neural network could be used to predict some factor levels as well)
- Step 4: estimation signal-to-noise ratios for responses from experimental data
- Step 5: Normalized signal-to-noise-ratio estimation NSNs

Phase B (Data prediction using BP-NN)

This phase is necessary when all the data needed for the prediction and estimation could not be obtained from the experiment carried. BP-NN neural network can be used to predict the values of the factors levels combinations beyond those obtained through the experimented in phase A. This phase could also be achieved in three steps as follow:

- Step 1 (neural network topology and architecture selection).
- Step 2 (selection of the training and the testing data sets).
- Step 3 (factor levels and corresponding signal-to-noise ratio prediction).

Phase C (determination of efficiency of DMUs using modified DEA)

An analysis will be done to evaluate the efficiency frontier of each factor level combination.

Phase D Optimization to select optimum DMU

To optimize and select optimum DMU, DEA penalization model of the efficient DMUs obtained at Phase C above is estimated.

This integrated procedure is schematically presented in Figure 4. It is believed that this integrated model has high propensity to interrogate all the analytical methods with other mathematical model to optimally determine a way that would lead to the decimation of mycotoxins production and contamination. The inclusion of a perceptron neural network model is for the purpose of predicting from experimental or literature results, the factors combination, and responses. This would save the researcher enormous time and resources that usually dissipated and wasted on experimentations. However, this model is not intended as a substitute to experimentation but rather a better complement to optimizing the search for the optimum result. Furthermore time, money and resources are intended to be saved with the utilization of this model.

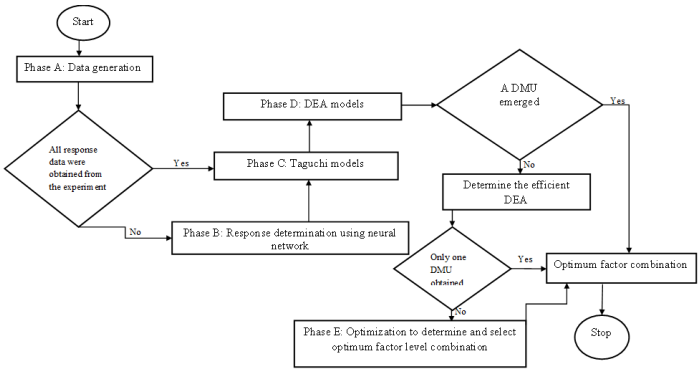

Figure 3 Mycotoxin Taguchi-Data Envelopment prediction model framework

CONCLUSION

Numerous valuable habits for the prevention and management of harmful fungi and the hazardous mycotoxins in feeds and foods have been discussed. A lot of issues responsible for the formation of mycotoxins in feed and food in different ways have been revealed. They are planting crops that are a not resistant to fungi invasion, fitness of fungal substrate, comfort of the temperature climate, moisture for mycotoxins, injured product seeds of food and feed due the activities of small invertebrate and arthropod animals, poor farming systems and

agricultural techniques, pre- and post-harvesting techniques, storage methods transportation conditions and food processing.

Mycotoxin treatments and control in feed and food can be done through any of primary, secondary, and tertiary actions, good agronomic and agricultural practices, and detoxification. Currently, biological-breed plants of fungal resistant hybrids are selected for planting to avoid the production and contamination of the produce by mycotoxin of fungi or other microbial origins. Producers and suppliers now must understand how to handle pre- and post-harvest issues. They should be aware and be knowledgeable of the causes of mycotoxin production and contamination. Drying of commodities of food and feed after post-harvest is the most important preventive and corrective actions for mycotoxin contamination. The use of chemical is an appropriate dosage which does not pose dangers to health has been advocated and another way of controlling the growth of mycotoxin in products. The over-all anticipated outcome of the integrated model explained is the selection of an optimum preventive procedure for reducing mycotoxins in crops, foods, and feeds.

The reviews therefore recommend the prediction of the mycotoxin and its control from the combination of all the preventive strategies (primary, secondary, and tertiary). More so all the means of quantifying mycotoxin should be incorporated into an optimum procedure for predicting, quantifying, preventing and detoxifying mycotoxins in foods and feeds. The suggested integrated Taguchi-data envelopment model has a great propensity through the integration of various robust steps, to produce results adequately and optimally.

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REGULAR ARTICLE

PHYSICOCHEMICAL, MICROBIOLOGICAL, ANTINUTRITIONAL AND PHYTOCHEMICAL CHARACTERIZATION OF HONEY PRODUCED AND CONSUMED AT FOUR DIFFERENT LOCATIONS

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ABSTRACT

The floral source, physicochemical, microbiological, anti-nutritional and phytochemical characterization of four different honey samples obtained from different locations in Ondo State were determined. This was with the view of authenticating their nutritional makeup as was as determine their potential to pose health hazards amongst the consumers. Physicochemical analysis gave moisture (13.5± 0.02-15.0±0.03%), Ash (0.5±0.02%-1.5±0.01%), pH (4.33±0.03-5.28±0.02) Titratable acidity (0.19±0.01-0.47±0.02%), total sugar (0.21±0.01-0.03±0.03%), dextrose (0.21±0.01-0.29±0.02%), fructose (0.22±0.03-0.31±0.01%), hydrated lactose(0.28±0.1-0.04±0.02%), hydrated maltose (0.34±0.03-0.48±0.01%), anhydrous lactose (0.27±0.01-0.48±0.01%), electrical conductivity (0.39x 0^2± 0.02-5.21x 10^2±0.02ms/cm). Total phenol content from (1.45 to 1.66 mg GAE/100g), Total flavonoid content from (0.04-0.07mg QE/100g) and not detected in samples EF3 and GH4. Alkaloid content ranged from (0.06-0.24%), Phytate content ranged from (2.06-3.91mg/100g), Tannin content ranged from (1.37-1.76 mg/100g), Oxalate content ranged from (0.18-0.27mg/100g), Saponin content ranged from (0.06-0.225) and the Ferric Reducing Antioxidant Power ranged from (0.04-0.05mg/100g) and not detected in sample CD2 and GH4. Also, the microbial analysis results revealed that the honey samples had bacterial count between 2.0 x 10^3- 9.0 x 10^3 CFU/ml, the total yeast/mold count had a count of 1.0 x 10^3, 3.0 x 10^3, 10.0 x 10^3 and 5.0 x 10^3 CFU/ml for the four samples respectively. The coliform count indicates the absence of coliform bacteria in all the samples. Isolates from the honey samples were seven different genera of bacteria (*Streptococcus*, *Enterococcus*, *Pseudomonas*, *Bacillus*, *Klebsiella*, *Micrococcus* and a trace of *Clostridium* spp) which could be hazardous to health if not properly handled.

Keywords: Honey characterization, phytochemicals, floral source, microbiological, anti-nutritional factors

INTRODUCTION

Honey is used in the food industry due to its nutritive, therapeutic, and dietetic quality. Honey is the sweetest natural product, obtained by processing flowers nectar or plants manna **(Marghitas, 2008)**. Honey is defined by the Codex Alimentarius Commission as the natural sweet substance produced by honeybees from the nectar of blossoms or from the secretion of living parts of plants or excretion of plant-sucking insects living on the plants, which honeybees collect **(Anonn, 2003)**. In main features depending on the floral origin or the nectar foraged by bees. The composition and quality of honey also depend on several environmental factors. Bacteria spores and less often Clostridium spp. may be present, but honey possesses antibacterial activities that do not support bacterial growth and/or production **(Anonn, 2004)**. However, fungi have been reported to grow and ferment or spoil unprocessed honey **(Anonn, 2003)**. Therefore, the microbiological quality of honey may serve as an indicator of the hygienic conditions under which the product was processed, handled, and stored **(Ramirez, 2000)**.

Honey is a very concentrated sugar solution with a high osmotic pressure, making impossible the growth of any micro-organisms. It contains less micro-organism. It contains fewer micro-organisms than other natural food, especially there no dangerous *Bacillus* species. *Bacillus* bacteria, causing the dangerous bee past, but these are not toxic for humans. That is why to prevent bee pests, honey should not be disposed in open places, where it can easily be accessed by bees. However, several bacteria are present in honey, most of them being harmless to man. Recent extensive reviews covered the main aspect of honey microbiology and the possible risks. To establish the standards that ensure honey quality, microbiological properties must be taken into consideration. A large interest was shown in the last few years for the composition of Romanian honey and its prospective value on the market regarding the U.E standards **(Bratu *et al*., 2001)**. The presence of *C. botulinum* spores in honey was reported for the first time in 1976. Since then, there were many studies in honey all over the world. In some of them no botulinum was found, in others, few kinds of honey were found to contain the spores. Honey does not contain the botulinum toxin, but the spores can theoretically build the toxin after digestion **(Floris 2006)**. The presence of yeast in honey is due to its introduction in the hive by the bees. In a case where the quality of the honey is not affected, it comes from the extraction and manipulation spaces, from the machines etc. and it is dangerous because of its quantity and acidophilus.

Due to its chemical composition, the honey favors the development of osmophilic yeast that produces the fermentation, the decrepit aspect, and the crystallization of the honey **(Clemansa, 2004)**. Also, honey has been incriminated as a source of spores of *Clostridium botulinum* responsible for causing the infant botulism. A low percentage of the contamination with B. cereus and fungi has been proved: yeast, *Mucor* spp., *Penicillium* spp. and other species from the *Aspergillus* type *A. candidus* **(Martins, 2003)**. The microbiological contamination during and after the processing of the honey has been demonstrated through the absence of micro-organisms from the collected samples from the primary sources and the sources and the presence of a type of bacterium (*Bacillus* spp.) and types of fungi (most frequently *Candida*, *Aspergillus*, *Geotrichum* and *Rhizopus* can be collected from samples from local markets. This indicates the contamination from secondary sources while further manipulation and processing. The contamination with fungi and bacteria indicates improper hygiene conditions during the collection, manipulation, processing, and storage **(Tchoumboue *et al*., 2003)**. The aim of the study is to characterize and describe the floral origin of honey obtained from four locations in Ondo state Nigeria using their physicochemical, microbiological, anti-nutritional and phytochemical compositions. This was with a view of authenticating their nutritional makeup as was as their potential for health hazards amongst the consumers at those locations and beyond.

MATERIALS AND METHODS

Collection of Samples

The sample (honey) were bought from four different locations in Ondo State, from Akure (mainly for industrial use), from Owo (Honey from roadside), Oyinnitemi (which is a consumer-ready product) and lastly, Ile-Oluji (gotten from beekeepers). The samples were kept in a dry clean place, safe from dirt and rodent infestation.

Floral Source Determination

This was determined by using the method of **Erdtman (1986)**. 10 g of the honey sample was dissolved in 10 ml of distilled water, the mixture was concentrated by centrifuging at 1500 rpm for 30 minutes. 10ml of acetotycin (9.1) acetic acid (1 ml) and H_2SO_4 (9 ml) was added and it was placed in a water bath at 100ºC for 30minutes. Then it was centrifuged and decanted again the sediment or precipitate was washed with 12 ml of distilled water and 12 ml of 7% KOH was added and stirred thoroughly and decanted. Finally, the sediment was stunk with

basic fuchsin solution and on a glass slide and it was checked under the microscope.

- Predominal and pollen (p, more than 45% of pollen grain counted)
- Secondary pollen (S, 16-45% pollen grain)
- Important minor (L, 3-16% pollen grain)

Physicochemical Characterization of Honey

Physicochemical parameters were determined using the standard method of **AOAC (1990)**.

Determination of Moisture Content

Petri-dishes were washed and dried in an oven at 105 ºC for 1hour; the petri dish was removed and cooled in the desiccator. 2 g of the honey sample was accurately weighed into the Petri-dish and it was spread properly, and the petri-dish was placed in an oven, and it was heated at 105 ºC for 3 hours, they are cooled in the desiccator and weighed.

Ash Content Determination

Ashing crucible was washed and heated in the muffle furnace for 15 minutes and cooled in a desiccator. The crucible was weighed, and 2 g of the honey sample was weighed into the crucible was then heated in the muffle furnace for 3 hours at 550 ºC until a trace of carbon could be seen.

Determination of pH

pH meter was used to determine the pH of honey, at a room temperature, the electrode of the pH meter was dripped into a beaker containing 100 ml of buffer solution (pH 9) in other to calibrate the instrument, after calibration, it was turned on the electrode was immersed in the honey for a period and the reading was taken.

Determination of Reducing Sugar

10 ml of the mixed freshly solution was pipetted into a 250 cm^3 conical flask, the solution was boiled on asbestos covered gauze with a Bunsen burner, and 1ml of the sugar solution was added to the boiling liquid at an interval of 100 of seconds until the blue color was nearly discharged. The 4 dropped of aqueous methylene blue solution (1%) was added and titration continued until the indicator became completely decolorized. The titration was repented adding before boiling almost all the sugar solution, required to effect reduction of the topper, the solution was boiled gently for 2 minutes, and 4 drops of methylene blue indicator was added and titration completely within a total boiling ting of 3 minutes. At the point, the entire blue color gets discharged and the liquid turned the orange-red volume of the sugar solution used was recorded as the liter value and their factors obtain from invert sugar table.

Determination of Non – Reducing Sugar

7 ml of concentrated hydrochloric acid was added to 100 ml of sugar, the mixed sample contained in the flask was immersed in a bath water at 60 ºC FOR 12 minutes, rotating the flask for three minutes. The flask was removed at exactly 12 minutes, and it was immersed in cold water for 15 minutes the content of the flask was neutralized with phenolphthalein with 5N sodium hydroxide.

Determination of Electrical Conductivity

The electrical conductivity was turn on and the probe was calibrated using a stand and solution of known conductivity. The honey sample was collected in a glass container. Enough of the honey sample was collected so that the probe tip can be submerged into the sample, the probe was rinsed with deionized water and blot dry. The probe was submerged into the sample until the electrical conductivity on the water stabilizes the electrical reading is stable.

Phytochemical and anti-nutritional content determination

Extraction of phenolic

For the extraction of phenolics from the honey samples, a modified method **(Yao et al., 2004)** using column chromatography was applied. A 100 g honey sample was totally dissolved in 500 ml acidified distilled water using a magnetic stirrer the PH value was adjusted with HCI to PH to 2.0 at laboratory temperature. The solution obtained was filtered through a lump of cotton wool in a funnel to remove the solid particles. The filtrate was mixed with 150 g of Amberlite XAD 2 (pore size 9 nm, particle size 0.3-1.2 mm) and stirred for 10 min with a magnetic stirrer. This mixture was transferred into a glass column and eluted with 250 ml of acidified distilled water (PH 2.0 adjusted with HCI) followed by 300 ml of distilled water for removing all saccharides. Phenolics absorbed on the solid phase were eluted with 400 ml methanol and the methanolic extract was subsequently evaporated to dryness in a rotary vacuum evaporator at 40 ºC. The solid was dissolved in 5 ml of distilled water and extracted three times with 5 ml of diethyl ether, subsequently, the diethyl etheric extracts were combined, dried with anhydrous sodium sulphate and diethyl ether was removed using a nitrogen flow. The dry extract obtained was stored in a refrigerator (4 ºC for the analyses).

Total phenol content determination

Total phenol content (TP) of the samples was determined according to according to **(Lachman et al., 2006)**. Dry extract of phenolics was dissolved in 5 ml of methanol; 0.5 ml of the sample solution was pipetted into a 10 ml volumetric flask and diluted with distilled water. Subsequently, 0.5 ml Folin ciocalteau reagent was added to the solution and after stirring 1.2 ml 20% sodium carbonate solution was added. After refilling with distilled water to mark and through agitation the reaction mixture was left standing for 20min and was measured on the spectrophotometer at wavelength 725 min against the black. Total phenol was expressed as mg gallic acid equivalent in 100 g of honey (mg GAE 100/g).

Total flavonoid content determination

This was determined according to the method described by **Spilkova *et al.*, (1996)**. Dry extract of phenolics was dissolved in 5ml of methanol; 1ml of the sample solution was pipetted into a 10 ml volumetric flask and diluted with distilled water. Subsequently, 1.2 ml of 0.2 mol/H_2SO_4, 1.2ml of 3 mol/$NaNO_2$ and 1.2 ml of 10% NaOH were added to the solution. After refilling with distilled water to mark and through agitation the reaction mixture was left standing for 15min and was measured on the spectrophotometer at wavelength 395nm against the black. Total phenol was expressed as mg quercetin equivalent in 100g of honey (mgQE/100g).

Alkaloids content determination

Alkaloids of the samples were determined according to the method of **Harborne (1973)**. 5g of each sample was weighed into a separate 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4hrs. This was then filtered, and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was completed. The whole solution was allowed to settle, and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. Residual alkaloid was dried and weighted.

Phytate content determination

4 g of the samples were soaked in 100 cm3 2% HCl for 3 hours and then filtered. 25 cm3 of the filtrate was placed in a 100 cm3 conical flask and 5 cm3 of 0.03% NH_4SCN solution was added as an indicator. 50 cm3 distilled water was then added to give it the proper acidity. This was titrated with ferric chloride solution which contained about 0.05 mg of Fe per cm3 of $FeCl_3$ used, the equivalent was obtained and from this, the phytate content in mg/100 g was estimated.

Tannin content determination

Method of **Makkar and Goodchild (1996)** was adopted. 200 mg of the sample was weighed into a 5 ml sample bottle. 10 ml of 70% aqueous acetone was added and properly covered. The bottle was put in an ice bath shaker and shaken for 2 hours at 30 oC. Each solution was then centrifuged, and the supernatant stored in ice. 0.2 ml of each solution was pipetted into test tubes and 0.8 ml of distilled water was added. A standard tannic acid solution was prepared from a 0.5 mg/ml stock and the solution made up to 1ml with distilled water. 0.5 ml Folin reagent was added to both sample and standard followed by 2.5 ml of 20% Na_2CO_3, the solutions were then vortexed and allowed to incubate for 40 minutes at room temperature after which absorbance was read at 725 nm against a reagent blank concentration of the samples from a standard tannic acid curve.

Oxalate content determination

Oxalate was determined according to the method of Day and Underwood (1986) as taken 1 g of the sample into a 100 ml conical flask of 4 5ml and 1.5N H_2SO_4 was added. The solution was carefully stirred intermittently with a magnetic stirrer for about 1 hour and then filtered using Whatman No. 1 filter paper. 25 ml of sample filtrate was collected and titrated hot (80 °C– 90 °C) against the 0.1N $KMnO_4$ solution to the point when a faint pink color appeared that persisted for at least 30 seconds.

Saponin content determination

Obadoni and Ochuko (2001) method were used to estimate the saponin value. 5g of the sample was measured into a conical flask and 100 cm^3 of 20% aqueous ethanol was added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55 °C. The mixture was then filtered, and the residue re-extracted with another 200 ml 20% ethanol. The combined extract was reduced to 40 ml over the water bath at about 90 °C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the other layer

was discarded. The purification process was repeated, 60 ml of n-butanol was added, and the combine n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath and after evaporation, the samples were dried in the oven to a constant weight and the saponin content was calculated in percentage.

Ferric Reducing Antioxidant Power (FRAP) content determination
1 ml of each dilution of phenolic extract were dispensed into different test tubes and 2.5 ml phosphate buffer was added followed by 2.5 ml FeCN solution and incubated at 50 °C for 20 minutes. 2.5 ml trichloroacetic acid (TCA) solution was added to stop the reaction. The reaction mixture was separated into 2.5 ml, and each was diluted with 2.5 ml of distilled water. 0.5 ml of ferric chloride ($FeCl_3$) solution was added into each tube and allowed to stand in the dark for 30 minutes for color development and the absorbance was read at 700 nm against a reagent blank.

Pollen Analysis- Determination of the floral Source of the Honey Samples
The samples were subjected to qualitative pollen analysis as per Erdtman's acetolysis method **(Erdtman, 1986)**. 10 ml of each of the samples were dissolved in distilled water and the sediment was concentrated by repeated centrifuging for 30 minutes at 1500 rpm. About 10 ml of acetolysis mixture (9:1 $C_2H_3O)_2O$: H_2SO_4) was added and the tubes were incubated in a water bath (100 ºC for 30 mins). It was stirred vigorously and centrifuged and then decanted. About 12 ml of water-free acetic acid was added. Stirred thoroughly, centrifuged, and decanted. The precipitate was washed in about 12 ml of distilled water, Centrifuged and decanted. About 12 ml of 7% KOH was added, stirred thoroughly centrifuged and decanted. Finally, the pollen grains were stained with a solution of Basic Fuchsin and mixed with glycerin. The examination of the pollen slides was carried out with an optical microscope at 400X and 1000X to count the pollen grains.

Microbiological characterization

Preparation of Culture Media
The media employed in this project work includes nutrients agar, MacConkey Agar, and Potato Dextrose Agar. Appropriate gram measurement of the medium to be used were obtained and dissolved in the appropriate volume of distilled water. All culture media was autoclaved at 121ºC for 15 minutes.

Preparation of Serial Dilution
A series of test tube each containing 9 ml of sterile distilled water was set up, then a sterile pipette was used to transfer 1ml from the stock sample, then it was introduced to the first test tube labelled 10^{-2} from the first test tube and mixed, the same procedure was repeated fill the last test tube **(Cheesbrough et al., 2000)**.

Culturing
The pour plate method was used in culturing bacteria of the honey samples. 1 ml of dilution from the test tube labelled 10^{-3} was pipette into a sterile petri dish labelled accordingly. 15 ml-20 ml of the already prepared agar was poured into each sample dilution. It was allowed to solidify. The plate was incubated at an inverted position at 37 ºC for 24-48 hours.

Isolation of Micro-organisms
After the culture for total plate count has been incubated for 24 hours, the pour plate was examined for the growth of isolated colonies strains of the organism was transferred from growth substrate into another plate using streak method to obtain pure culture of each colony, which is the population of cell from single colony, and it was later isolated and kept on agar slant with the aid of a sterile loop **(Ogbulie *et al*., 1998; Fawole *et al*., 2001)**.

Total Viable Count
The total yeast count of the honey samples was done with a potato dextrose agar using the pour plate. The plates were incubated at 37 ºC. All visible colonies appearing at the end of the incubation period were counted.

Total Coliform Count
Multiple tubes of MacConkey broth were incubated with different volume of the honey sample (10 ml, 1.0 ml and 0.1 ml) respectively. The number of coliforms in 100 ml can be computed based on the various combinations of positive and negative results. Standard tables for computing these values were used to determine the number of coliforms per 100 ml of the honey sample.

Biochemical Test -Identification and Characterization of the Isolates
The pure cultures of the isolates were identified using biochemical tests, as described by **Cheesbrough (2003)**.

Catalase Test
A smear of the test organism was made on a slide and 3 drops of 3% hydrogen peroxide solution was added to release oxygen bubbles as effervescence which indicates a positive reaction which signifies the production of catalase enzymes which break down the peroxide to release oxygen.

Coagulase Test
Three separate loops of saline were placed on a clean slide and a loopful of test colony was suspended in two of these and a loopful of control organism in the third one. A drop of citrated rabbit plasma was added to one test with a sterile loop and the controlled suspension clumped occur in some which indicate a positive result **(Ogbulie *et al*., 1998)**.

Motility Test
This test was carried out using young culture (24 hours old) of the bacterial isolates which were placed on a coverslip of petroleum jelly. The cover slide was then inverted and placed on the Vaseline container.

RESULTS AND DISCUSSION

Summarize the results obtained from the physicochemical analysis of the honey sample is presented in Table 1.
The amount of moisture content in the honey is an important factor in determining the quality of honey in the world honey trade. It is also an important factor in determining its storage quality. Moisture content varied from 13.5±0.02% to 15.0± 0.03%. Honey sample OP3 has the highest value, honey collected from Akure II honey (MN2), Owo (QR4) and Ile-Oluji (ST5) have the same value of 13.5 ± 0.02%. This is an indicator of premature extraction of honey which was obtained by Bogdanov, (1999). The ash content varied from 0.5± 0.02% to 1.5± 0.01%, the highest ash value was observed and lowest was observed in the sample collected from (OP3) and Owo (QR4), the mean value along four samples. It was observed in this analysis that light colors honeys usually have low ash content compared to dark color which usually has higher ash content. This also corroborates the same results reported by **Bodganor (1999)**.

Table 1 Physicochemical properties of the honey sample

Sample	Physicochemical Properties										
	Moisture %	Ash (%)	pH	Titrable acidity (%)	Total Sugar (%)	Dextrose	Fructose (%)	Hydrate Lactose (%)	Hydrate Maltose (%)	Anhydrous Lactose (%)	Electrical Conductivity (ms/cm)
MN2	13.5±0.02	1.00±0.02	4.55±0.03	0.47±0.02	0.21±0.01	0.21±0.01	0.22±0.03	0.28±0.01	0.34±0.03	0.27±0.01	3.95X 10^2±0.01
OP3	15.0±0.03	0.5±0.02	4.33±0.03	0.32±0.02	0.30±0.03	0.29±0.02	0.31±0.01	0.40±0.01	0.48±0.01	0.38±0.01	5.21X10^2±0.02
QR4	13.5±0.02	0.5±0.02	4.58±0.03	0.19±0.01	0.28±0.03	0.27±0.03	0.29±0.02	0.38±0.03	0.45±0.03	0.48±0.01	2.64x10^2±0.03
ST5	13.5±0.02	1.5±0.01	5.28±0.02	0.26±0.01	0.30±0.01	0.29±0.03	0.31±0.02	0.40±0.02	0.36±0.01	0.38±0.01	0.39X10^2±0.02

Note: Results are expressed as mean values+standard deviation

The pH values of honey are of great importance during extraction and storage, since acidity can influence the texture, stability, and shelf life of honey. All kinds of honey are acidic, the pH of the honey sample range from 4.33± 0.03 to 5.28± 0.02 honey sample collected from ST5 has the highest value compared to honey collected from Akure 1 honey which has the lowest value. Sample OP3 and QR4 have the same mean value 0.02 which mean they are not significantly different.

The acidity of honey is due to the presence of organic acid and inorganic ion. Honey, in general, is acidic in nature irrespective of its variable geographical origin. The titratable acidity of honey samples was observed in the study 0.19±0.01% to 0.47±0.02%, it was observed in the study that the honey samples collected from different locations are varied in value. The honey sample was collected from Akure II honey (MN2) has the highest value and sample at QR4

has the lowest value. The variation of this factor is the source of nectar and climatic condition of this is related to the result of **Asit *et al.* (2002)**.

Total sugar is the main constituent of honey, the sugar spectrum of honey depends on the sugar present in nectar and enzyme present in bees. Sample OP3 and ST5 have the highest value to be 0.30±0.03% while sample MN2 has the lowest value to be 0.21±0.01%. Dextrose ranges from 0.21±0.01% to 0.29±0.02%. 0.29±0.02% is the highest value and it was the sample. ST5 and sample OP3 and ST5 have the same value of 0.29±0.02%. The fructose varied in value except OP3 and ST5 with the same value 0.31±0.01% which is the highest value while 0.22±0.03% is the lowest value within the range of 0.22±0.03%-0.31±0.01%. The hydrated lactose ranges from 0.28±0.01% to 0.40±0.02% and it was obtained from (MN2). Among the value discussed above, it was observed that the highest value of hydrated lactose was obtained from sample OP3 and ST5 and the lowest dextrose was obtained from sample MN2 from a range of 0.21±0.01%-0.40±0.02%. hydrated maltose and Anhydrous lactose of OP3 and QR4 range from 0.27±0.01% to 0.48±0.01% respectively with MN2 0.27±0.01% having the lowest value. The hydrate lactose ranges from 0.34±0.03% to 0.48±0.01, anhydrous range from 0.27±0.01% to 0.48±0.01%. Electrical Conductivity of OP3 has the highest electrical conductivity $5.21x10^2$ ± 0.02ms/cm, ST5 has the lowest value $0.39x10^2$. The values of the electrical conductivity depend on the mineral organic acid, polyol content and vary with botanical origin.

Table 2 summarizes the outcome of the phytochemicals and antinutritional screening of the honey samples.

Table 2 Phytochemicals and antinutritional composition of the honey samples

Sample	Phytochemicals and antinutritional							
	Total Phenol (mgGAE/100g)	Total Favonoid (mgQE/100g)	Alkloid (%)	Phytate (mg/100g)	Tannin (mg/100g)	Oxalate (mg/100g)	Saponin (mg/100g)	FRAP (mg/100g)
MN2	1.57±0.07	0.04±0.01	0.06±0.01	3.71±0.58	1.76±0.07	0.18±0.01	0.13±0.03	0.04±0.01
OP3	1.57±0.07	0.07±0.01	0.13±0.01	3.91±0.29	1.43±0.07	0.23±0.06	0.22±0.02	ND
QR4	1.45±0.04	ND	0.24±0.01	3.71±0.58	1.37±0.01	0.18±0.01	0.19±0.01	0.05±0.01
ST5	1.66±0.04	ND	0.13±0.01	2.06±0.58	1.58±0.07	0.27±0.01	0.06±0.01	ND

ND-Not Detected, GAE-Gallic Acid Equivalent, QE-Quercetin Equivalent. Note: Results are expressed as mean values+standard deviation.

Total phenol content ranged from 1.45 mg GAe/100 g to1.66 mg GAE/100 g. This range is like those obtained from the same type- monofloral honey reported by **Beretta *et al.*, (2005)**. Total flavonoid content is 0.04 mg QE/100g to 0.07 mg QE/100 g and was not detected in QRT4 and ST5. **Makawi *et al.*, (2009)** opined that flavonoid has potential to impound metal ions by forming complexes thus preventing the formation of free radicals. Alkaloid ranged between 0.06 t0 0.24, phytate content ranged from 2.06 mg/100 g, tannin content ranged from 1.37 mg/100 g to 1.76 mg/100 g, oxalate content ranged from 0.18 mg/100g to 0.27 mg/100g, saponin ranged from 0.06 to 0.22 and Ferric Reducing Antioxidant Power (FRAP) are 0.04 mg/100g to 0.05 mg/100 g and was not detected in MN2 and ST5. This shows OP3 has both adequate phytochemicals and antinutritional content. Pollen grain counts, kinds of honey are always classified as unfloral, monofloral and multi floral depending on poly frequency. Unfloral kinds of honey were considered those with predominant pollen type over 45%. In this study, the pollen grain count of ST5 was classified as monofloral since it has the value of 1057 and it is extra light Amber in Color. MN2 honey has the value of 1548; OP3 has 1640 and as well has amber color and same with MN2. QR4 has the value of 1221 and it was light amber in color, therefore, the pollen grain count range from 1057 to 1640. Generally, multi-floral honey has mixed flavor and aroma compared to unfloral honey which has unique flavor and aroma. Table 3 shows the result of the floral sources, samples have a pollen count of 1548, 1640, 1221 and 1057respectively. According to Conti, 2000, the bee producing unfloral honey is said to collect its nectar from a single type of flower. On the contrary, polyfloral honey is made from any nectar sources available to bees.

Also, the color of the honey sample gives an indication of the number of pollen grains contained in the honey samples. Samples QRT4, OP3and MN2 have amber, light amber respectively, while ST5 has extra light amber color.

The microbiological assessment of the honey samples was determined. Table 3 showed the total viable count of the samples where aerobic, mesophilic, and thermophilic organisms (bacteria) were counted. Samples QRT4, MN2 and ST5 have between 7.0 x 10^3 CFU/ml, while sample OP3 has the least bacterial count of 2.0 x 10^3 CFU/ml. According to the study, in Table 3, honey sample MN2 contained the highest yeast count of 10.0 x 10^3 CFU/ml. QRT4 has total yeast count of 3.0 x 10^3 CFU/ml, and ST5 has 5.0 x 10^3 CFU/ml count, OP3 has the least count of 1.0 x 10^3 CFU/ml. The bacteria and yeast count indicate improper hygiene conditions during collection and storage, served as the source of contamination and the nutritional composition of the sample which is mainly sugar in which yeast and mould can easily grow. Table 4 showed the characteristics and the biochemical reaction of the homey samples. Probable bacteria isolated from the samples were *Streptococcus* spp., *Bacillus* spp., *Clostridium* spp. of the gram-positive and *Klebsiella* spp., *Pseudomonas* spp., *Enterobacter* of the family *Enterobacteriaceae* of the gram-negative. The presence of *Streptococcus* spp. and *Bacillus* spp. in these samples could lead to infection of the lung, meningitis, and infection of the lower part of the abdomen, as they survive in honey at low temperatures. These submissions have also been reported in the previous work of **Olaitan et al. (2007)**.

Table 4 Cultural characteristics and biological reactions of selected honey samples

Sample	**Morphological Characteristics**	**M.T**	**G.S**	**Catalase**					**Coagulase**					**Probable Organisms**
				A	B	C	D	E	A	B	C	D	E	
MN2	Pinkish strand, opaque cocci colonies	−	+	−	+				+	+				*Streptococcus* spp. *Enterococcus* spp.
OP3	Smooth, creamy translucent	+	+	−	+	+	−	+	+	+	−	+	+	*Bacillus* spp.
QR4	Pink, smooth and slightly convex	−	+	−	+	+	+	+	+	+	+	+	+	*Streptococcus* spp. *Klebsiella* spp
ST5	Smooth, creamy opaque colonies	+	−	−	+	+	+	+	+	+	+	+	+	*Pseudomonas* spp. *Micrococcus* spp. *Clostridium* spp.

MT = Motility test, GS = Gram Staining, A = Isolate from each plate, B= Isolate from each plate, C= Isolate from each plate D= Isolate from each plate, E= Isolate from each plate.

CONCLUSION

The comparison of these honey varieties with published International Standard allowed us to have an idea of the quality of honey produced and consumed in some important locations of Ondo state. The floral source obtained was mostly unfloral. The number of aerobic, mesophilic, and thermophilic bacteria, mold and yeast identified from these different locations were of values greater than the published standard yeast and mold load of 10^3 CFU/ml. Although no standard microbiological loads exist for honey, the results obtained give the degree of wholesomeness and safe quality of the honey samples. By implication these samples must be handled with utmost care for them not to pose any serious potential health hazard especially during sales and handling prior to consumption.

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