

**OPTIMIZATION OF SUBMERGED FERMENTATION PARAMETERS FOR ENHANCED LIPASE
PRODUCTION FROM NEWLY ISOLATED LIPOLYTIC YEASTS USING AGRO WASTE
SUBSTRATES**

ABSTRACT

Fungi are currently recognized as the most preferred sources of lipase due to their ability to produce copious amount of extracellular lipase with lower production's and extraction's costs. Lipase production was carried out in agro wastes formulated-liquid fermentation media at 30°C, at 130rpm, at initial pH of 7 for 96 hours using newly isolated *LipA* gene identified yeasts (*Pichia kudriavzevii* ENS1 (Accession No: OL546800), *Candida orthopsilosis* PMS3 (Accession No: OL546803) and *Saccharomyces cerevisiae* PMS8 (Accession No: OL546805) at inoculum size of 2%. Agrowastes such as Mango kernel and orange peel were used as alternative carbon sources; beans hull and groundnut shell as nitrogen sources while lipidic sources such as olive oil, soyabeans oil, palm oil, groundnut oil and coconut oil were used as inducers. Single factor optimization process was carried out to determine the effects of carbon sources, nitrogen sources, inducers, incubation time, inoculum size, pH and carbon: nitrogen ratio on lipase production and cell growth. Extraction by filtration, centrifugation and acetone precipitation; Quantitative lipase assay using titrimetric method and Cell growth by measuring optic density at 600nm were done. Proximate analysis of the agro-waste substrates showed higher percentage of carbohydrate and crude fat in mango kernel powder at 68.05±0.56% and 19.54±0.62% while groundnut shell recorded higher crude protein at 11.63±0.05%. Single factor optimization study showed optimum lipase production and cell growth at 50.83±1.18u/ml/min and 0.69±0.00(OD) with *Candida orthopsilosis* PMS3 (Accession No: OL546803) using hot water treated mango kernel hydrolysate as carbon source. The results from this study showed that mango kernel would be an economical alternative carbon source for industrial-scale lipase production.

Keywords: Agrowaste substrates, Inducers, Lipase, Mango kernel, Optimization, Submerged fermentation.

1.0 INTRODUCTION

The interest in microbial lipase production has increased in the last decade, because of its large potential in manufacturing applications in production of food additives (flavor modification)(1), fine chemicals (synthesis of esters), cosmetics (removal of lipids), pharmaceutical (digestion of oils and fats in drugs), leather (removal of lipids from animal skins) and medicine (blood triglyceride assay), textile, paper, synthesis of biopolymers (2), biofuel production (3) and waste water treatment (4). Although several bacterial and fungal lipase producers have been isolated and studied, fungi are recognized as the best lipase producers and currently the most preferred sources(5) due to their ability to produce copious amounts of extracellular lipases(6), the low cost of extraction, thermal and pH stability as well as activity in organic solvents (7). Soil contaminated with spillage from the products of oil and dairy harbors fungal species which have the potential to secrete lipases to degrade fats and oils (8). Lipases can be produced by submerged and solid state fermentations (9). Submerged fermentation has been defined as fermentation in liquid medium, which contain soluble nutrients, or as fermentation in the presence of excess water (10). Submerged Fermentation utilizes free flowing liquid substrates (11) and this fermentation technique is best suited for microorganisms such as yeasts and bacteria that require high moisture content (12). The cultivated microorganisms metabolize the nutrients in the liquid medium, grow and release the desired enzymes into the medium (1). When the selected strain produces extracellular enzyme, it makes purification and recovery easier than when it is produced intracellular enzyme (13). Most industries employ submerged fermentation for enzyme production due to the ease of handling on a large-scale (14, 1). Different methods of cultivating the microorganisms in the submerged fermentation are batch culture, fed-batch culture and continuous culture (15). Substrates used in submerged fermentation include soluble sugars, molasses, liquid media, fruit and vegetable juices, and sewage/waste water (12). The culture medium for the fermentation process should contain all the necessary nutrients for growth, which consist of carbon, nitrogen and mineral salts (16).

Lipase production is enhanced by the medium pH, temperature, medium composition, inoculation volume, aeration, agitation (17) and many other factors which have been studied in a number of microorganisms (18). The

success of any fermentation process is dependent on the adaptation of microorganisms to the media, the inexpensive nature of the substrate used and the possibility of synthesizing the enzyme in large quantities. This study aimed to produce lipase from some selected lipolytic yeasts through submerged fermentation using agro waste substrate. This will help to enhance low cost of production and promote waste management culture.

2.0 MATERIALS AND METHODS

2.1 Preparation of agro waste as substrates for lipase production

2.1.1 Proximate analysis of the agro waste substrates

Proximate analysis of the agro waste substrates (orange peel, mango kernel powder, beans hull and groundnut peel) was carried out to ascertain their moisture, ash content, protein content, crude fibre, crude lipid and total carbohydrate contents.

2.1.2 Pretreatment of Agro wastes

Agro wastes (Mango kernel and Orange peel) were processed, pulverized, hydrolyzed (using acid, alkaline and hot water hydrolysis) and substituted as carbon sources while (groundnut shell and beans hull) were mineralized and substituted as nitrogen sources. The clarified solutions were used as carbon and nitrogen sources for lipase production.

2.2 Inoculum Preparation of lipolytic yeasts

Single colony from the freshly cultured selected lipolytic yeasts were inoculated into 10ml sterile yeast extract peptone dextrose broth (YEPD), incubated at 30°C for 24hrs at 120rpm and standardized to 1.5×10^8 spores/ml.

2.3 Submerged fermentation of yeasts for lipase production

The mineral salt composition of NH_4NO_3 – 1g, KH_2PO_4 -0.2g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.3g, NaNO_3 – 0.6g, CaCO_3 - 0.3g, NaCl - 0.1g, K_2HPO_4 -0.1g and 1%(v/v) of olive oil were added to 100ml of agro waste hydrolysates (mango kernel and orange peel) and the pH was adjusted to 7 with the addition of 1M NaOH and sterilized at 121°C for 15minutes. Then 2% (v/v) of the standardized yeast suspension was inoculated into the sterilized fermentation medium. Each flask was incubated at 30°C in an incubator for 96hours on a rotary shaker at 160rpm.

2.4 Enzyme extraction

Samples of the fermented medium were collected on the 96 hours of incubation for enzyme extraction. 10ml of the fermented medium was collected aseptically using a sterile syringe, filtered through 0.7µm whatmann filter paper. The cell free filtrate was stored at 4°C for 2 hours and centrifuged at 4,000 rpm for 10 minutes. The supernatants were decanted and mixed with chilled acetone at ratio (1:1), the mixture was stored at 4°C for 2 hours and centrifuged at 4,000 rpm for 10 minutes and the precipitate was collected, dried and stored in 0.2M potassium phosphate buffer pH 7 at 4°C.

2.5 Quantitative Lipase assay

The olive oil emulsion substrate was prepared by mixing 30ml of olive oil and 70ml of emulsifying reagent (NaCl – 17.9g, K₂HPO₄ – 0.41g, Glycerol – 540ml, Gum Arabic – 10g and were made up to 1 litre with sterile distilled water) the mixture was homogenized for 5 minutes. Then 1ml of the olive oil emulsion substrate, 0.8ml of 0.2M potassium phosphate buffer at pH 7.0 and 0.2ml of the crude enzyme was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 2ml of acetone-ethanol mixture (1:1) followed by titration against 0.05M NaOH using 3 drops of 1% phenolphthalein as indicator. The appearance of pale pink colour indicated the end point. Blank assay was also conducted.

$$\text{Lipase activity (u/ml/min)} = \frac{\Delta V \times C \times 1000}{V \times T} \quad (\text{u/ml}).$$

ΔV = Difference between V₁ and V₀. V₀ = volume of NaOH used against control flask (Blank).

V₁ = volume of NaOH used against experimental flask. C = concentration or normality of NaOH

V = Volume of extracted enzyme used. T = Time of incubation

One lipase unit was defined as the amount of the enzyme that released one micro molecule of fatty acid per minute. Extracellular lipase activity was measured in units per ml per minutes (U/ml/min).

2.6 Cell density measurement

One milliliter of the fermented sample was collected and placed in a cuvette for cell density determination at 600nm using UV spectrophotometer (Model 721) and compared with a non inoculated medium, using sterile distilled water as blank.

2.7 Single factor Optimization

2.7.1 Effect of carbon sources

The effects of inorganic carbon sources such as glucose, fructose and sucrose at 1% (w/v) concentration on lipase production and cell growth of the lipolytic yeasts were investigated.

2.7.2 Effect of nitrogen sources

Inorganic nitrogen sources such as peptone, yeast extract and urea and organic sources such as beans hull and groundnut shell hydrolysates were investigated to determine their effects on lipase production and cell growth at 1% (w/v) concentration.

2.7.3 Effect of inducers

Lipidic sources such as palm oil, coconut oil, soyabeans oil and groundnut oil were substituted in place of olive oil as inducers at 1% (v/v) and their effects were checked on lipase production and cell growth.

2.7.4 Effect of incubation time

Different incubation time ranging from 24, 48, 72,120,144 to168 hours and their effects were checked on lipase production and cell growth.

2.7.5 Effect of initial pH

The initial pH of the basal medium before sterilization was adjusted to pH 5, 6, 8, 9 using 0.1M NaOH and 0.1M HCl respectively and their effects on lipase production and cell growth were investigated.

2.7.6 Effect of inoculum size

Inoculum size was varied at 1%, 3%, 4%, 5% (v/v) and their effects were determined on lipase production and cell growth

2.7.7 Effect of carbon/nitrogen ratio

The ratio of the carbon and nitrogen source with optimum lipase activity and cell growth were varied at 0.5:1,1.5:1,2:1,2.5:1,3:1 to determine the ratio with optimum lipase production and cell growth.

3.0 RESULTS

3.1 Proximate analysis of agro waste substrates

Table1: Proximate composition of agro waste substrates used as alternative carbon and nitrogen sources for lipase production.

Compositions (% dry weight)	Mango kernel	Orange peel	Beans Hull	Groundnut shell
Moisture	2.55±0.01	2.87±0.03	2.38±0.01	2.50±0.12

Crude fat	19.54±0.62	8.59±0.04	0.55±0.07	0.82±0.03
Crude protein	5.55±0.07	2.80±0.00	9.59±0.05	11.63±0.13
Crude fibre	2.31±0.04	21.71±0.13	78.40±0.14	49.10±0.16
Carbohydrate	68.05±0.56	61.01±0.06	6.64±0.11	33.27±0.25
Ash	2.01±0.01	3.03±0.06	2.45±0.07	2.69±0.13

Values are means of duplicate ± Standard deviations values.

3.2 Single Factor Optimization of fermentation parameters and their effects on lipase production and cell growth of lipolytic yeasts

3.2.1 Effect of carbon sources

Basal medium supplemented with hot water treated mango kernel powder showed highest lipase activities of 50.83±1.18, 42.50±0.59 and 39.17±2.35 u/ml/min and biomass population of 0.69±0.00, 0.65±0.05, 0.63±0.01 (OD) for *Candida orthopsilosis* PMS3 (Accession No: OL546803), *Saccharomyces cerevisiae* PMS8 (Accession No: OL546805), and *Pichia kudriavzevii* ENS1 (Accession No: OL546800) respectively and is thereby regarded as the most suitable carbon source for lipase production amongst other sources used in this study, next is Glucose while the lowest lipase activity was recorded with acid hydrolysed orange peel enhanced medium as presented in Figure 1a and figure 1b respectively.

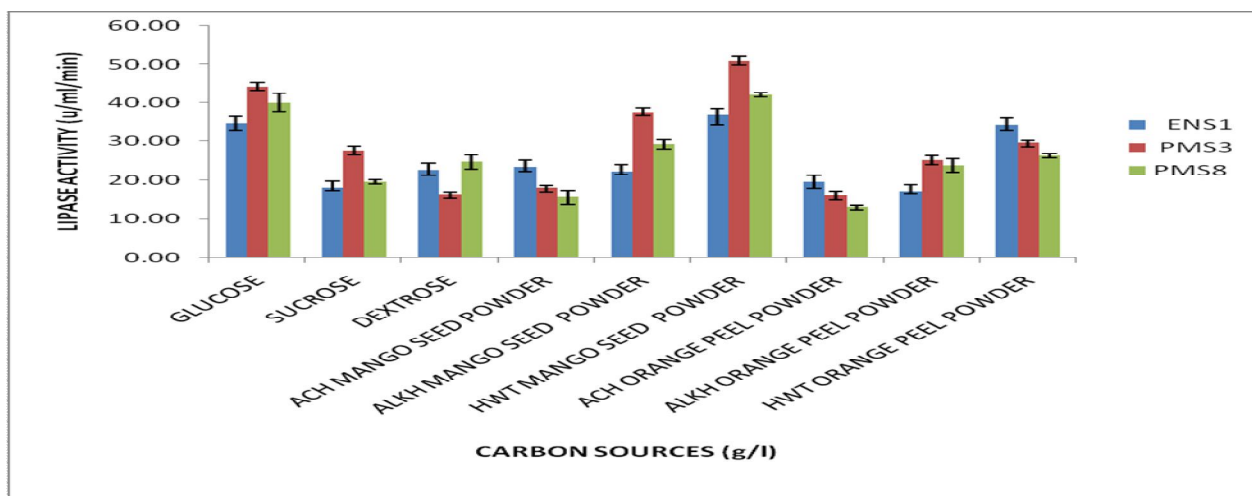


Figure 1a: Effect of carbon sources on lipase production of lipolytic yeasts

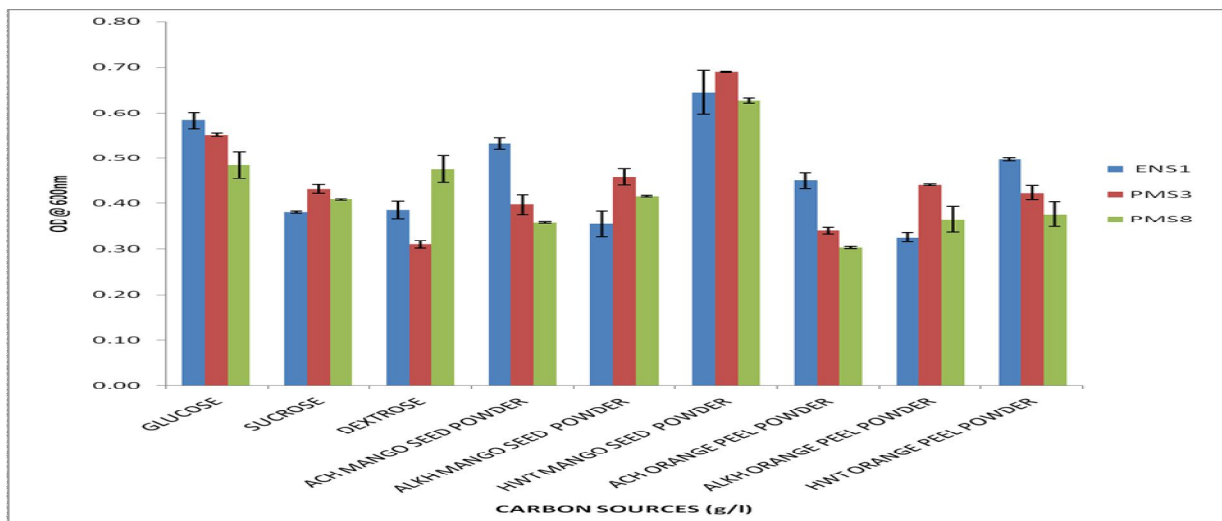


Figure 1b: Effect of carbon sources on cell growth of lipolytic yeasts

3.2.2 Effects of nitrogen sources

The result showed that 1% (w/v) ammonium nitrate exhibited the highest lipase activity with significant level of cell growth, followed by peptone with reasonably high lipase activity of 43.75 ± 1.77 to 32.92 ± 0.59 u/ml/min and high biomass population of 0.72 ± 0.01 to 0.64 ± 0.00 (OD) while the lowest lipase activity of 28.75 ± 1.77 to 20.00 ± 2.35 u/ml/min and cell growth of 0.55 ± 0.03 to 0.46 ± 0.02 (OD) was recorded with the medium supplemented with urea as presented in Figure 2a and Figure 2b.

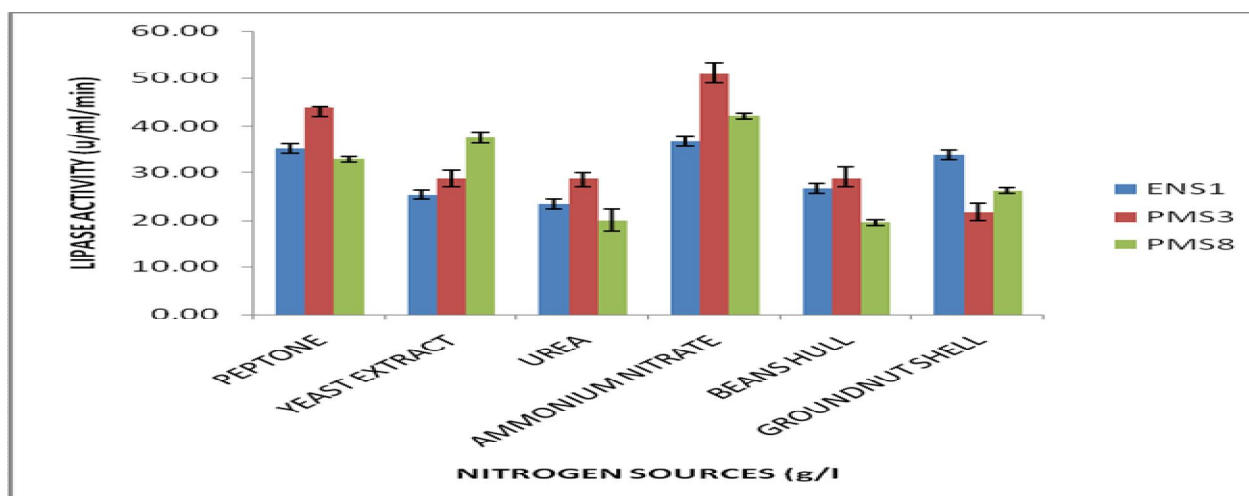


Figure 2a: Effect of nitrogen sources on lipase production from lipolytic yeasts

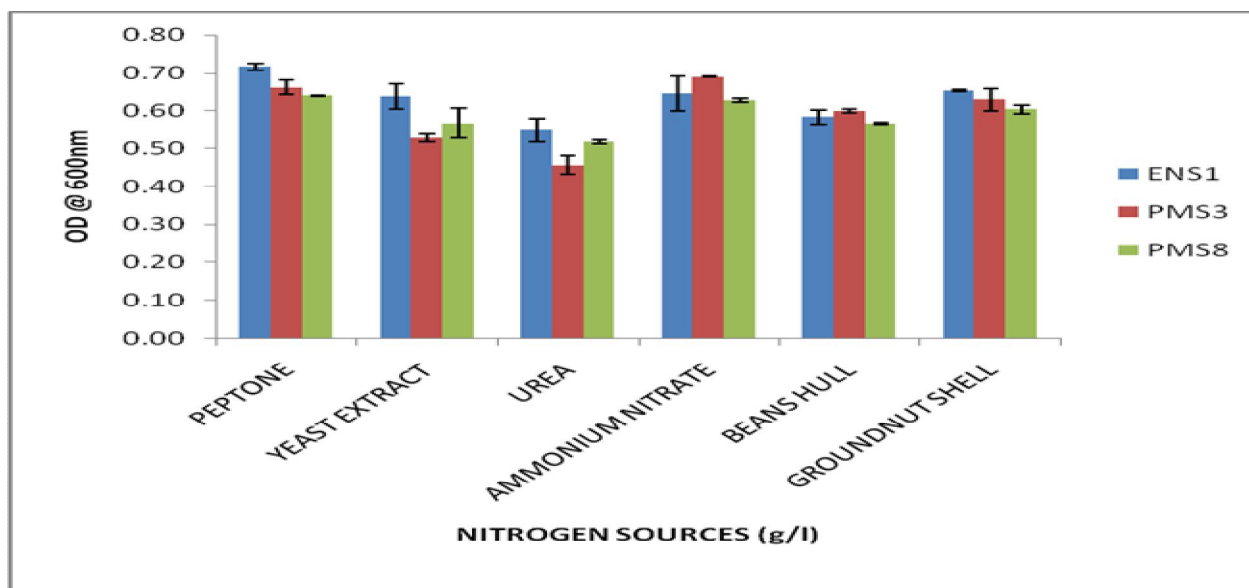


Figure 2b: Effect of nitrogen sources on cell growth of lipolytic yeasts

3.2.3 Effect of inducers

Highest lipase activity was recorded in medium induced with olive oil from 50.84 ± 1.18 to 36.67 ± 2.35 u/ml/min while the highest biomass population was recorded with palm oil induced basal medium from 0.77 ± 0.02 to 0.72 ± 0.00 (OD). Medium supplemented with coconut oil recorded the lowest lipase activity from 23.75 ± 0.59 to 12.75 ± 1.53

u/ml/min and soybeans' oil fortified medium recorded the lowest cell growth from 0.54 ± 0.02 to 0.41 ± 0.01 (OD) as shown in Figure 3a and Figure 3b respectively.

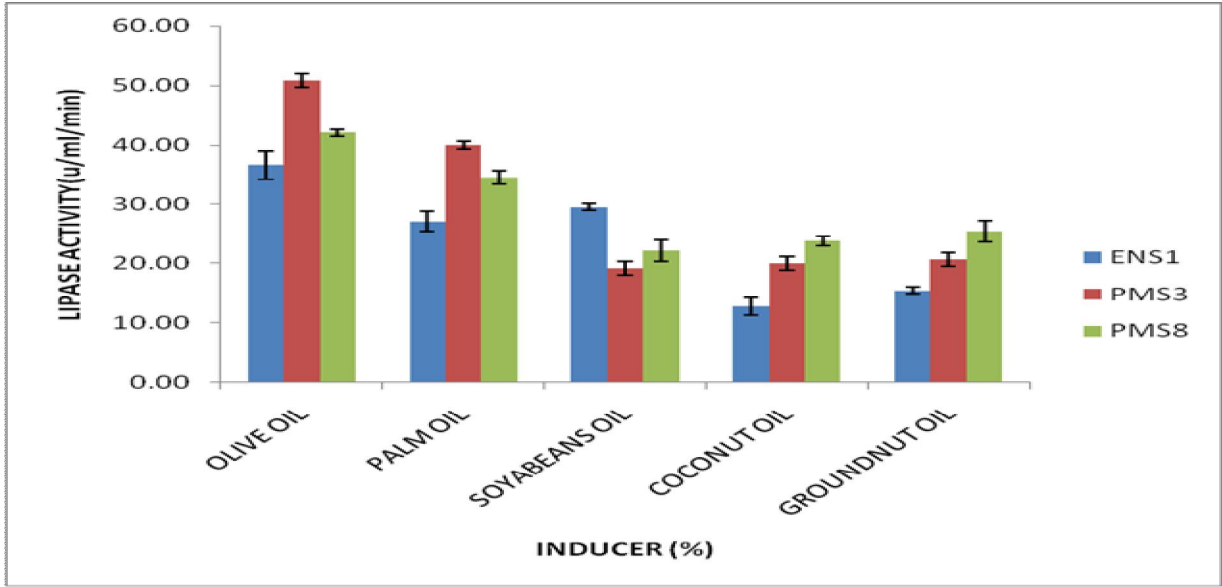


Figure 3a: Effect of Inducers on lipase production of lipolytic yeasts

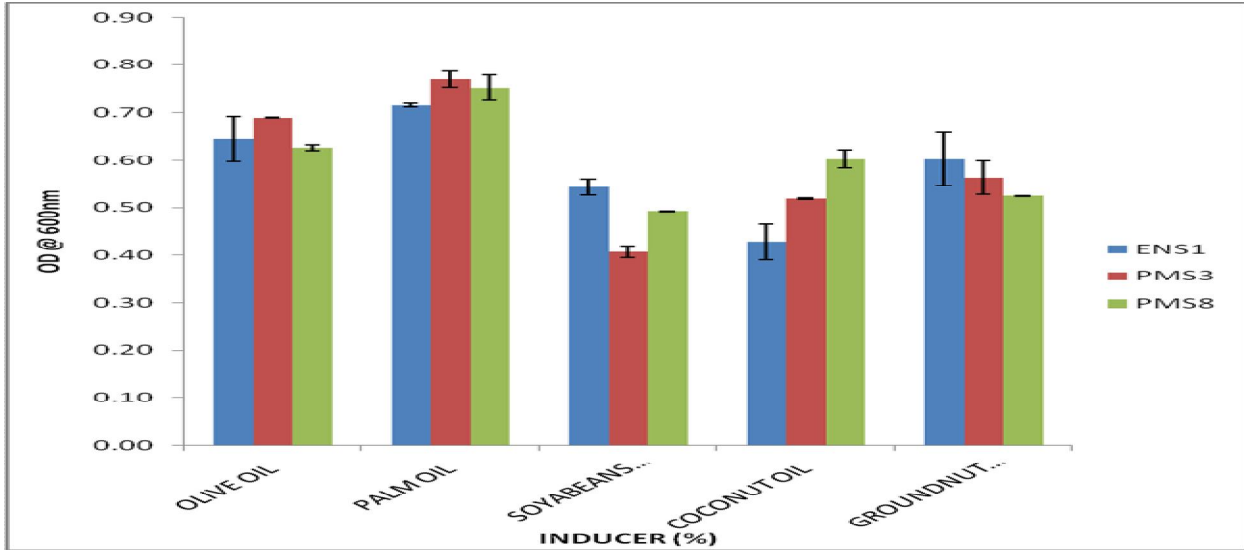


Figure 3b: Effect of Inducers on cell growth of lipolytic yeasts

3.2.4 Effect of initial pH

The result as presented in figure 4a and 4b showed that highest lipase production and cell growth was recorded at pH 7 followed by pH 6 from 43.75±0.59 to 30.00±2.36 u/ml/min and cell growth from 0.67±0.02 to 0.59±0.02OD while the lowest lipase activity from 17.50±1.17 u/ml/min to 11.67±1.18 u/ml/min and cell growth from 0.51±0.02 to 0.47± 0.04OD was recorded at pH 9. However *Pichia kudriavzevii* ENS1 (Accession No: OL546800) exhibited optimum lipase production and cell growth at pH5.

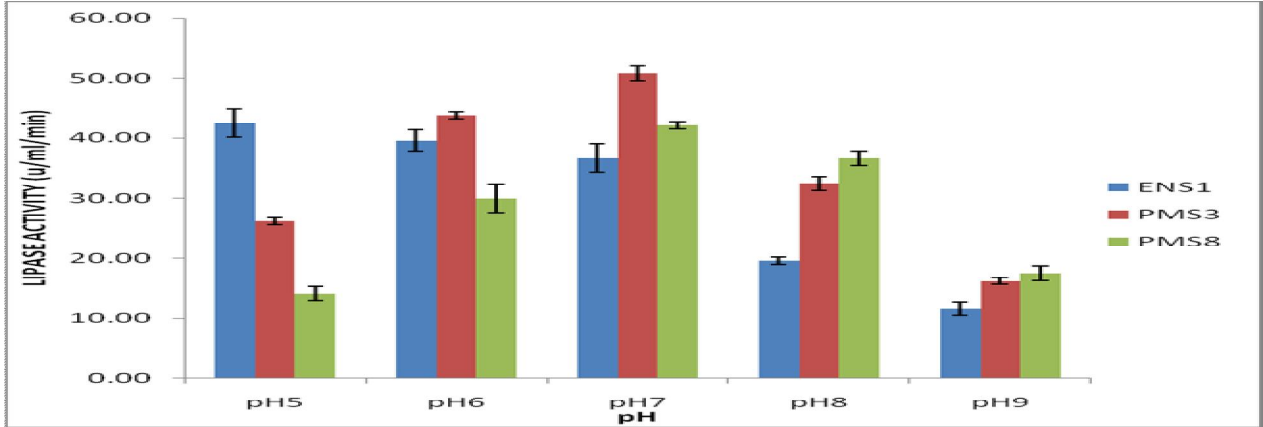


Figure 4a: Effect of initial pH on lipase production of lipolytic yeasts

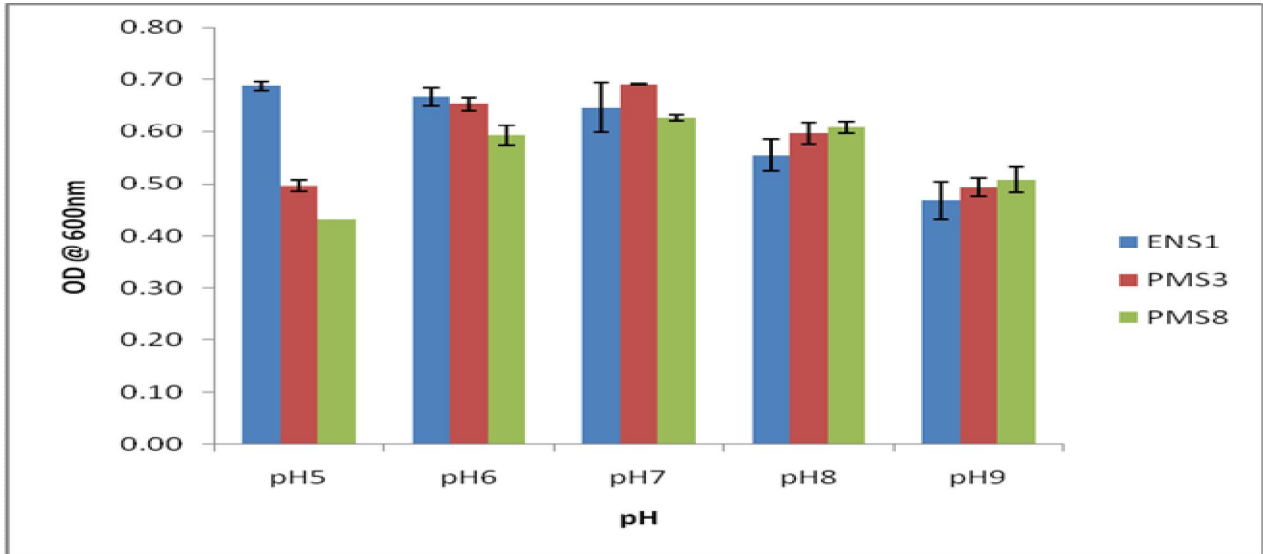


Figure 4b: Effect of initial pH on cell growth of lipolytic yeasts

3.2.5 Effect of Incubation time

Optimal lipase activity was recorded at 96hours while the highest biomass population was obtained at 120 hours from 0.70 ± 0.01 to 0.67 ± 0.04 OD. The lowest lipase activity was recorded at 168hours from 17.50 ± 1.17 to 12.92 ± 0.59 u/ml/min and cell growth at 24hours from 0.48 ± 0.04 to 0.41 ± 0.00 OD as presented in Table 5a and 5b.

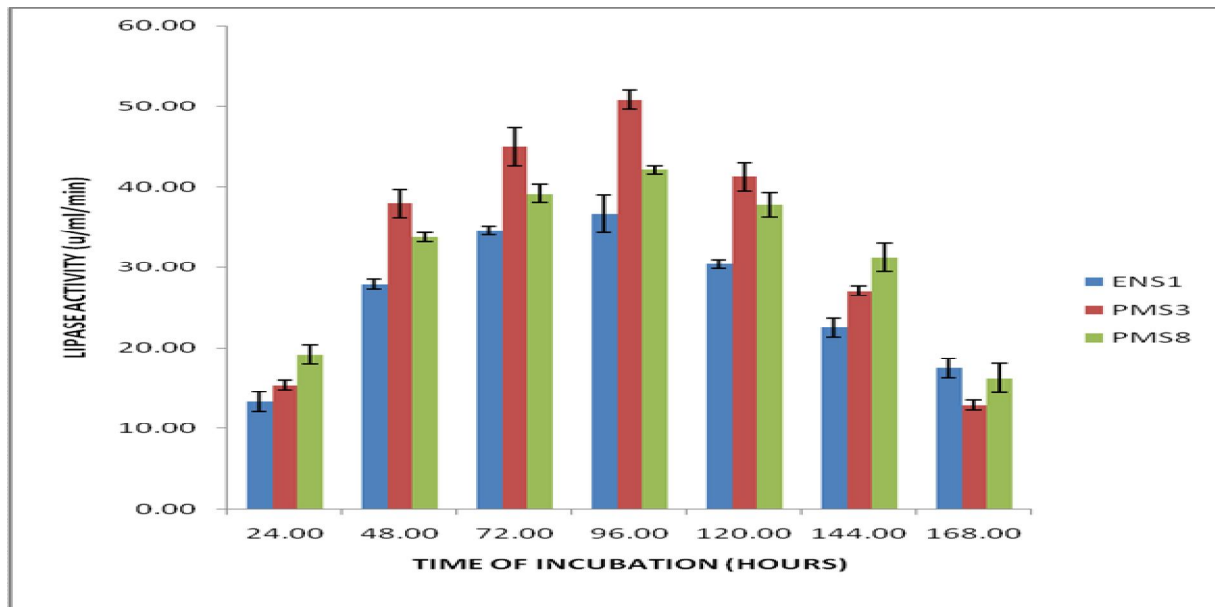


Table 5a: Effect of incubation time on lipase production of lipolytic yeasts

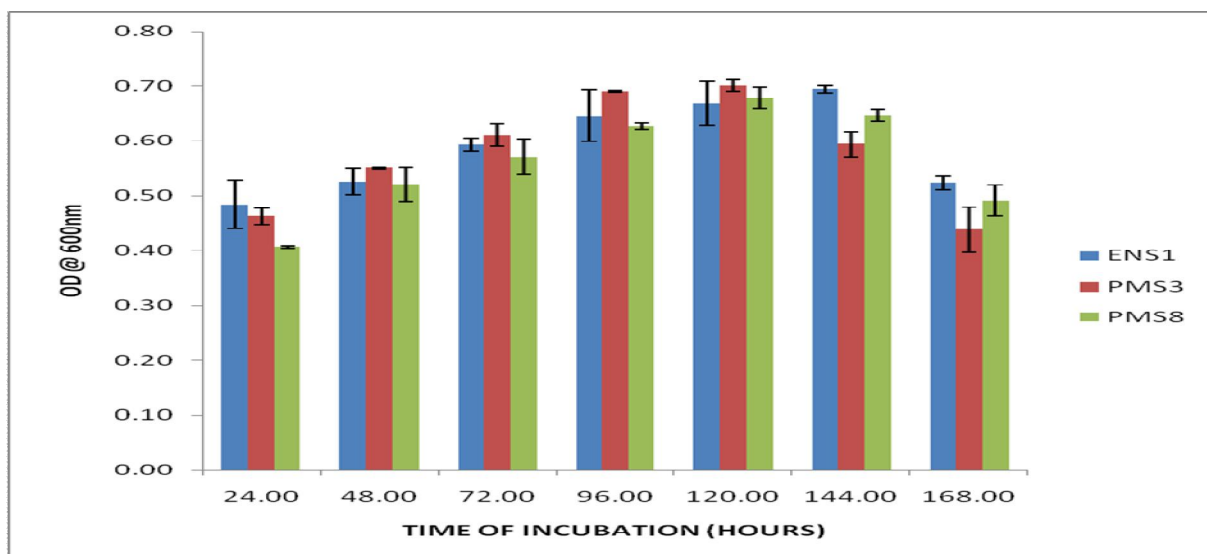


Figure 5b: Effect of incubation time on cell growth of lipolytic yeasts

3.2.6 Effect of Inoculum size

The inoculum size of 2% (v/v) of the yeast suspension inoculated into the basal medium was adjusted to 1%, 3%, 4% and 5% (v/v) to determine their impact on lipase production and cell growth. The result showed that the highest lipase production was recorded at 2% (v/v) while the highest biomass population was recorded at 3% (v/v) from 0.78 ± 0.02 to 0.74 ± 0.00 OD. The lowest lipase activity and cell growth was recorded at 5% (v/v) from 37.92 ± 0.59 to 24.58 ± 1.77 u/ml/min and 0.53 ± 0.05 to 0.49 ± 0.02 OD as shown in Figure 6a and 6b.

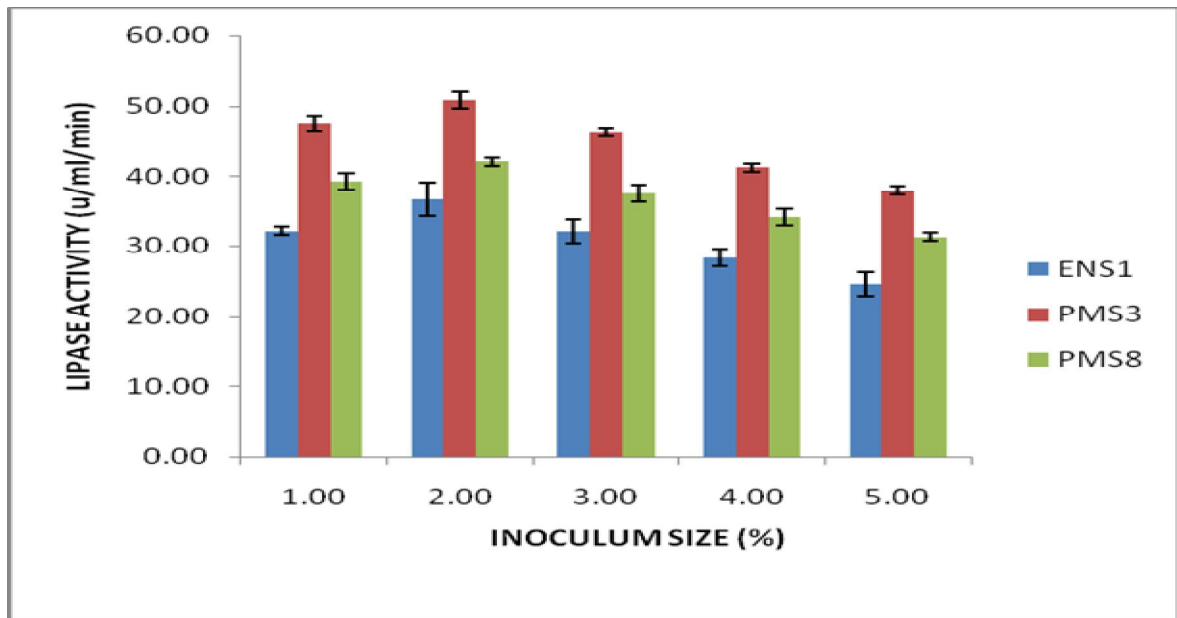


Figure 6a: Effect of inoculum size on lipase production of lipolytic yeasts

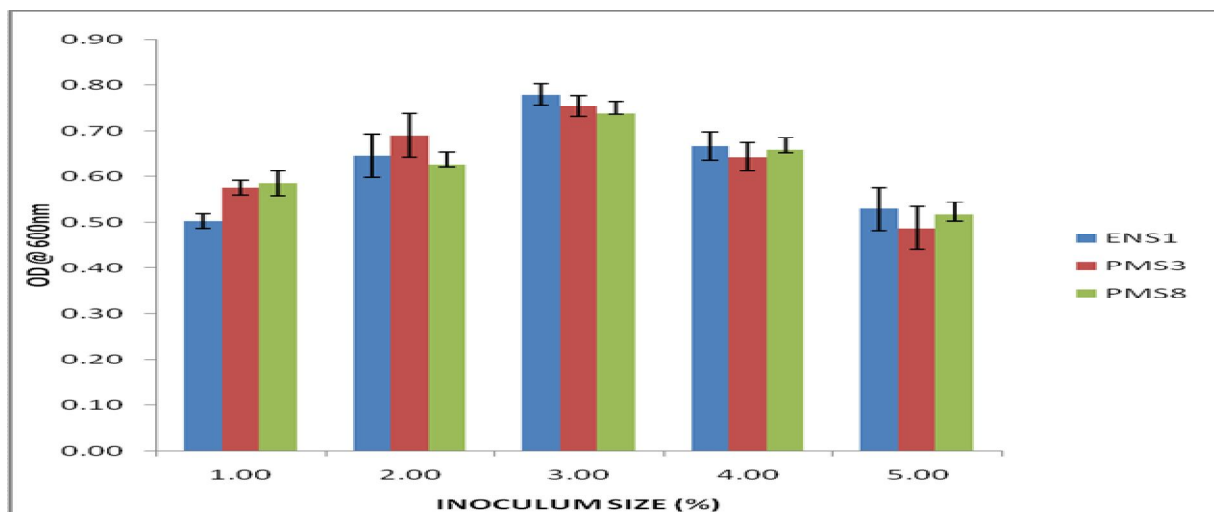


Figure 6b: Effect of inoculum size on cell growth of lipolytic yeasts

3.2.7 Effect of carbon – nitrogen ratio

The initial carbon – nitrogen ratio of the basal medium was at 1:1(w/w) and was later adjusted to ratio 0.5:1, 1.5:1, 2:1, 2.5:1 and 3:1 to determine the influence of carbon-nitrogen ratio on lipase production and biomass population. Highest lipase activity was recorded at 1:1, while optimal cell growth was recorded at 2:1 from 0.71 ± 0.03 to 0.66 ± 0.01 OD. The lowest lipase activity was at ratio 3:1 from 25.83 ± 1.18 to 14.59 ± 0.59 u/ml/min and cell growth at 0.5:1 from 0.58 ± 0.02 to 0.53 ± 0.01 OD as shown in figure 7a and 7b respectively.

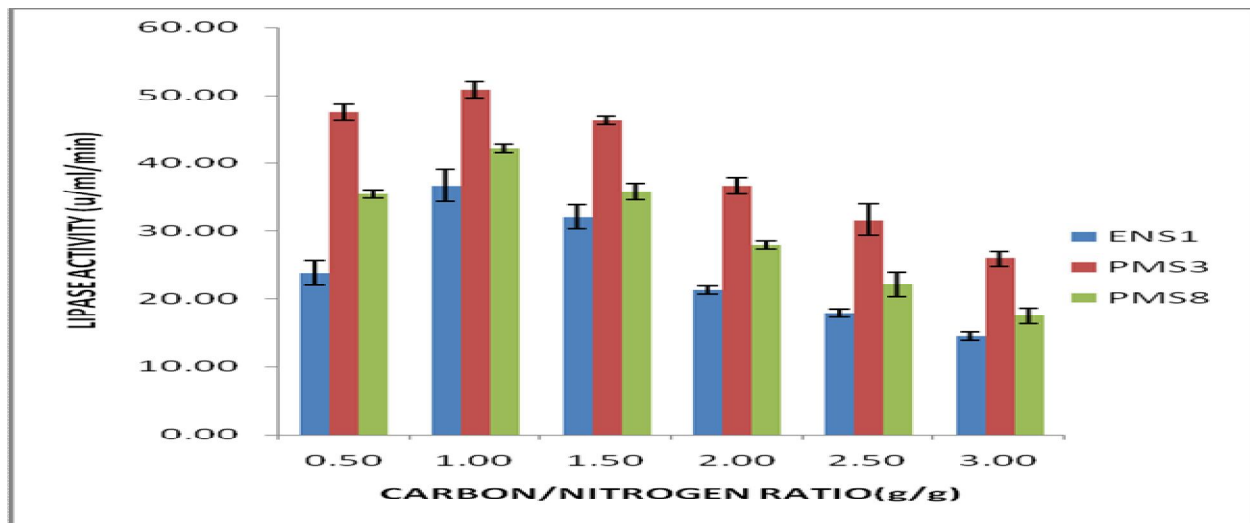


Figure 7a: Effect of carbon-nitrogen ratio on lipase production of lipolytic yeasts

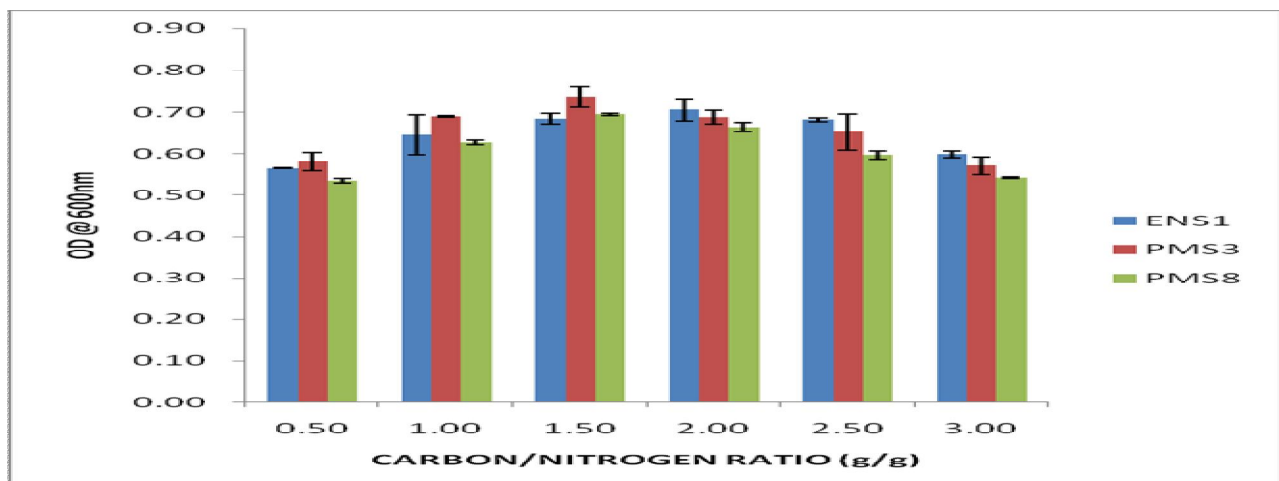


Figure 7b: Effect of carbon –nitrogen ratio on cell growth of lipolytic yeasts

4.0 DISCUSSION

The proximate composition of the agro waste substrates used as alternative carbon sources(mango kernel and orange peel) showed higher percentage of carbohydrate at $68.05\pm 0.56\%$ and crude fat at $19.54\pm 0.62\%$ in mango kernel , orange peel has an appreciable level of carbohydrate at $61.01\pm 0.06\%$ and crude fat at $8.59\pm 0.04\%$, while beans hull and groundnut shell that were used as alternative nitrogen sources showed higher crude protein percentage in which groundnut shell recorded the highest percentage crude protein at $11.63\pm 0.13\%$ while beans hull recorded percentage crude protein of $9.59\pm 0.05\%$ respectively as shown in Table 1. (19) reported $71.99\pm 0.06\%$ carbohydrate and $4.29\pm 0.01\%$ crude fat from mango kernel powder(20) reported 45.05% carbohydrate and 3.75% crude fat from sweet orange peel; (21) recorded $12.14\pm 0.92\%$ of crude protein in groundnut shell; 7.71% of crude protein was recorded by (22) in the study of the nutritive value assessment of beans hull. These are relatively in agreement with the findings of this study.

Optimum lipase production can be achieved based on the composition of the fermentation medium, hence there is need for appropriate carbon and nitrogen sources in the production medium as both energy source and cell forming component with adequate inducer to enhance the synthesis of lipase. In this study, 1% glucose as carbon source and 1% ammonium nitrate as nitrogen source and 1% olive oil as inducer were selected and used for lipase production based on the result obtained from the fermentation media screening with media composition formulated by (23), (24) and (25). However, for higher productivity of lipase producing fungi with lower cost of production, agro wastes were supplemented as organic carbon and nitrogen sources to alternate the inorganic sources. The results indicated that higher lipase activity and optimum cell growth were recorded with hot water treated mango seed hydrolysate which can be attributed to the availability of fermented sugar to the lipase producing fungi without the possible side effects associated with chemically(acid and alkaline) pre-treated hydrolysates. Ammonium nitrate exhibited optimum lipase activity and cell growth amongst other nitrogen sources in addition to olive oil among other inducers that were used for this study. This was in agreement with the findings of (26) in which ammonium nitrate and olive oil stimulated both higher lipase and cell productivity at 58.50u/ml/min and 19.50g/l respectively in the production of lipase by newly isolated *Candida viswanathii* strain. Similar results was also obtained from (27) in which maximum lipolytic activity was reported with ammonium nitrate at $15.6\pm 0.036\text{u/ml}$ in the production of extracellular alkaline lipase from a newly isolated *Bacillus* sp.PD-12.

The experimental single factor optimization was designed to show the key parameters that influence lipase production and cellular population in which the effect of one parameter at a time was investigated while others remain constant. The results obtained have shown that the fermentation parameters such as carbon –nitrogen ration, pH, incubation time, inducer and inoculum size have greatly enhanced the production of lipase and cell growth of the lipolytic yeasts. Report of the two- way analysis of variance (Anova) of the experiment showed that there is significant difference ($p < 0.05$) in the effect of the fermentation parameters on lipase production and cell population. In addition, the post – hoc test using Turkey test of comparison revealed that there is significant difference in the effect of the fermentation parameters on lipase production and cell population. The results of the response values of the fermentation factors that were optimized in the one factor at a time (OFAT) optimization process indicated that there were correlation among the fermentation parameters, lipase production and yeasts cell growth during the production process.

5.0 CONCLUSION

The results obtained from this study has shown that mango kernel powder, a renewable agro-waste substrate was more effective in lipase production than its inorganic counterparts that were used in this study with enhanced lipase production and biomass population. This would be a low cost alternative for large scale production of industrially viable-lipase. *Candida orthopsilosis* PMS3(Accession No: OL546803) was the best producing lipolytic yeast among the newly isolated lipolytic fungal strains used for this study and would be selected for process optimization study using response surface methodology to scale up lipase production for biotechnological application in further study .

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COMPETING INTEREST

The authors declared that no competing interest exist between them.

AUTHOR'S CONTRIBUTION

This study was carried out in collaboration among all the authors. Author AAE designed the study, carried out all the experiments, data analysis and wrote the first draft of the manuscript. Author AOK and AGO supervised the study and contributed to the manuscript's preparation. The final manuscript was read and approved by the authors.

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