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 30^0C, 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 Agro wastes collection

Orange peels, spoilt mango fruits and groundnut shell were collected from Fruit market at Eleme junction while Beans hull was obtained from Woji in Rivers State, Nigeria in labeled polythene bags and transported to the Industrial Microbiology laboratory at the University of Port Harcourt, Nigeria for processing.

2.2 Preparation of agro waste as substrates for lipase production

2.2.1 Proximate analysis of the agro waste substrates

The agro wastes (orange peel, mango seeds, beans hull and groundnut shell) were processed and pulverized. Proximate analysis of the substrates were carried out to ascertain their moisture, ash content, protein content, crude fibre, crude lipid and total carbohydrate contents according to AOAC method [19].

2.2.2 Pretreatment of Agro wastes

The pulverized agro wastes (Mango kernel and Orange peel) were 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.

2.2.2.1 Hydrolysis of Agro waste substrate as carbon source

2.2.2.1.1 Acid Hydrolysis of Substrates

Acid hydrolysis was carried out using a standard technique recommended by Ucuncu *et al*. [20], 1g of the substrate into 100ml of 1% H_2SO_4 solution and allowed to dissolve. The solution was autoclaved at 121^0C for 40minutes. Then the solution was allowed to cool and filtered.

2.2.2.1.2 Alkaline Hydrolysis

Alkaline hydrolysis was carried out by weighing 1g of the substrate into 100ml of 1% 1M NaOH solution and allowed to dissolve, the solution was autoclaved at 121^0C for 40minutes,allowed to cool and filtered.

2.2.2.1.3 Hot Water treatment

The method recommended by Ucuncu *et al*. [20] was modified by weighing 1g of the substrate into 100ml of distilled water. The mixture was allowed to dissolve and the solution was autoclaved at 121^0C for 40minutes. Then the solution was allowed to cool and filtered.

The clarified solutions were used as carbon sources for lipase production.

2.2.2.2 Extraction of nitrate from agro waste for nitrogen source

Extraction of nitrate was done following the standard method approved by Mishra *et al.*[21],1g of the substrate into 100ml of 1% 0.5M K_2SO_4 and autoclaved at 121^0C for 40minutes to extract nitrate from the substrate. The solution was allowed to cool and filtered. The clarified solutions were used as nitrogen sources for lipase production.

2.3 Inoculum Preparation of lipolytic yeasts

Single colony from the freshly cultured 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] were inoculated into 10ml sterile yeast extract peptone dextrose broth [YEPD],incubated at 30^oC for 24hrs at 120rpm and standardized to 1.5×10^8spores/ml according to [22] .

2.4 Submerged fermentation of yeasts for lipase production

The mineral salt composition based on the modification of [23], [30] and [31] methods; NH_4NO_3 – 1g, KH_2PO_4 -0.2g , $MgSO_4.7H_2O$ - 0.3g, NaNO3 – 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 121^0C 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.

2.5 Enzyme extraction

Samples of the fermented medium were collected on the 96hours 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 2hours 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 2hours 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.

2.6 Quantitative Lipase assay

The olive oil emulsion substrate was prepared based on the modification of the recommended techniques adopted by [23] and [24] by mixing 30ml of olive oil and 70ml of emulsifying reagent [NaCl – 17.9g, K_2HPO_4 – 0.41g, Glycerol – 540ml, Gum Arabic – 10g and were made up to 1litre with sterile distilled water] the mixture was homogenized for 5minutes. 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 37^0C 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.

Lipase activity [µ/ml/min] = $\frac{\Delta V \times C \times 1000}{V \times T\ [min]}$ [µ /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 [µ /ml/min].

2.7 Cell density measurement

One milliliter of the fermented sample was collected and placed in a curvette for cell density determination at 600nm using UV spectrophotometer [Model 721] and compared with a non inoculated medium, using sterile distilled water as blank according to Nur *et al*. [25].

2.8 Single factor Optimization

2.8.1 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.

2.8.2 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.

2.8.3 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.

2.8.4 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.

2.8.5 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.

2.8.6 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.

2.8.7 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 µ/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.

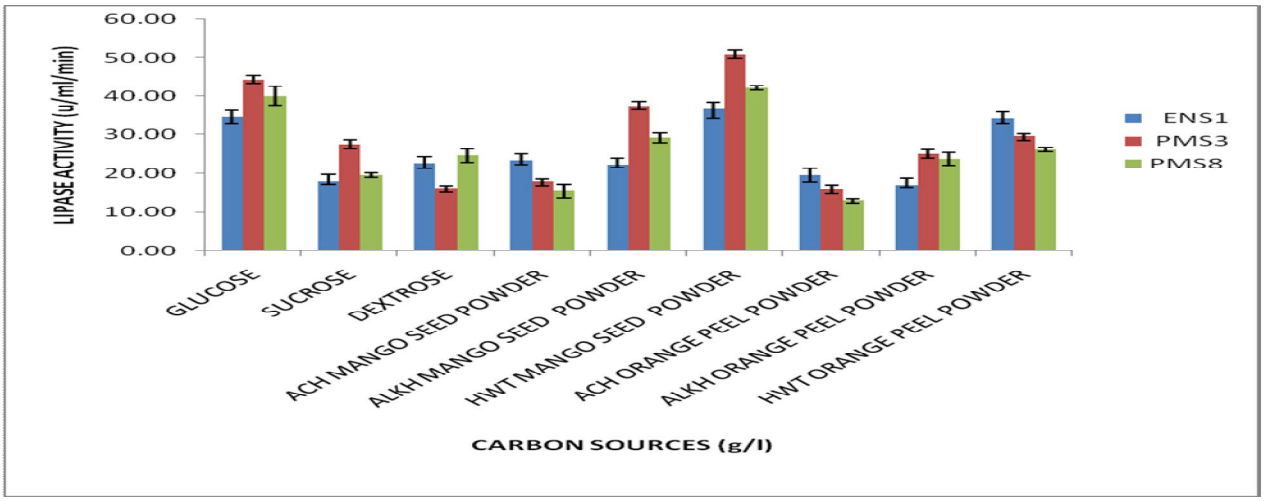

Figure 1a: Effect of carbon sources on lipase production of lipolytic yeasts

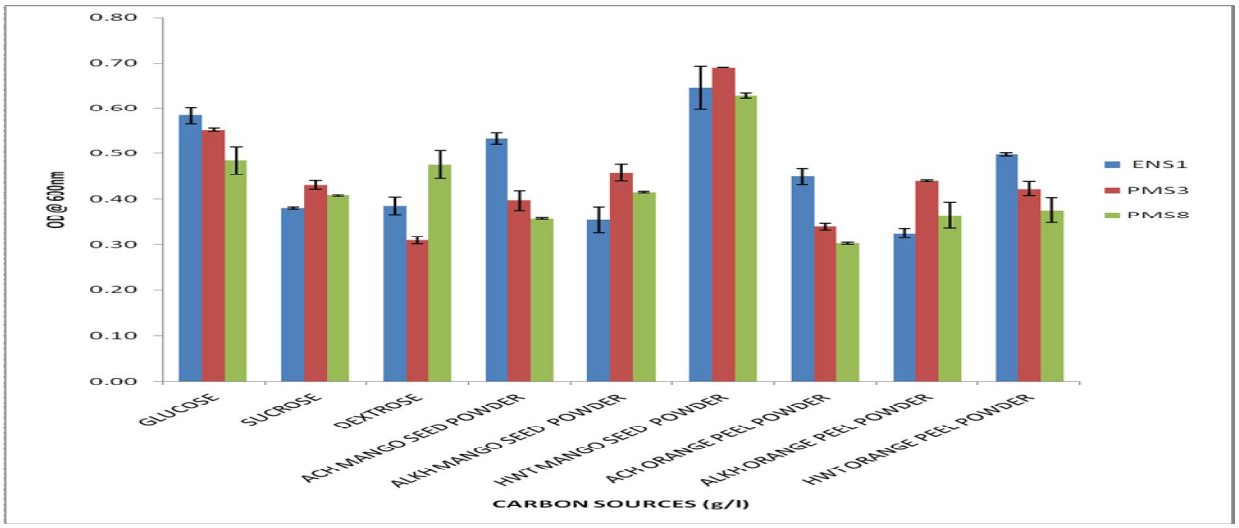

Figure 1b: Effect of carbon sources on cell growth of lipolytic yeasts

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 µ/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.

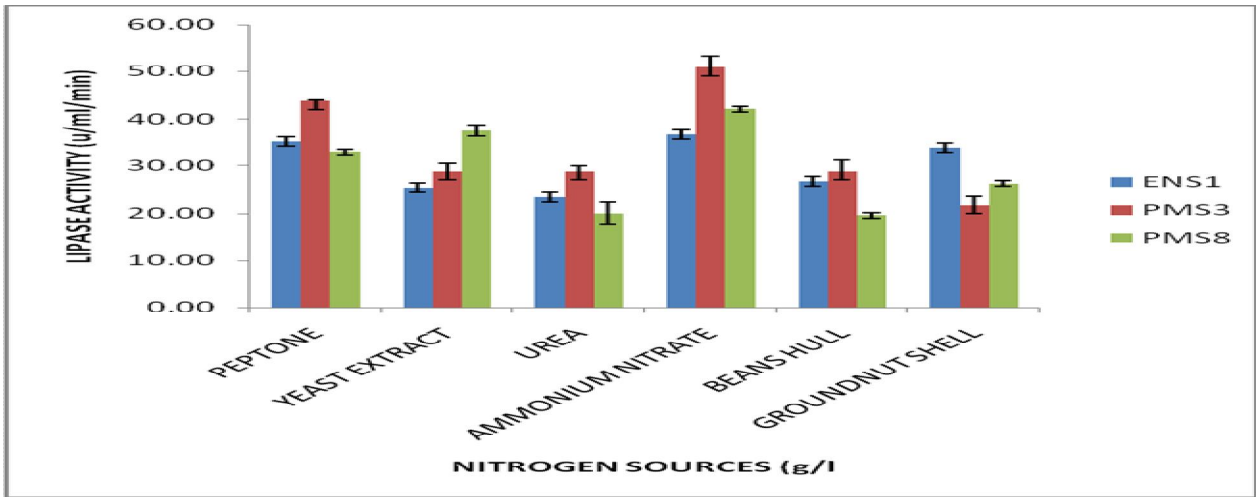

Figure 2a: Effect of nitrogen sources on lipase production from lipolytic yeasts

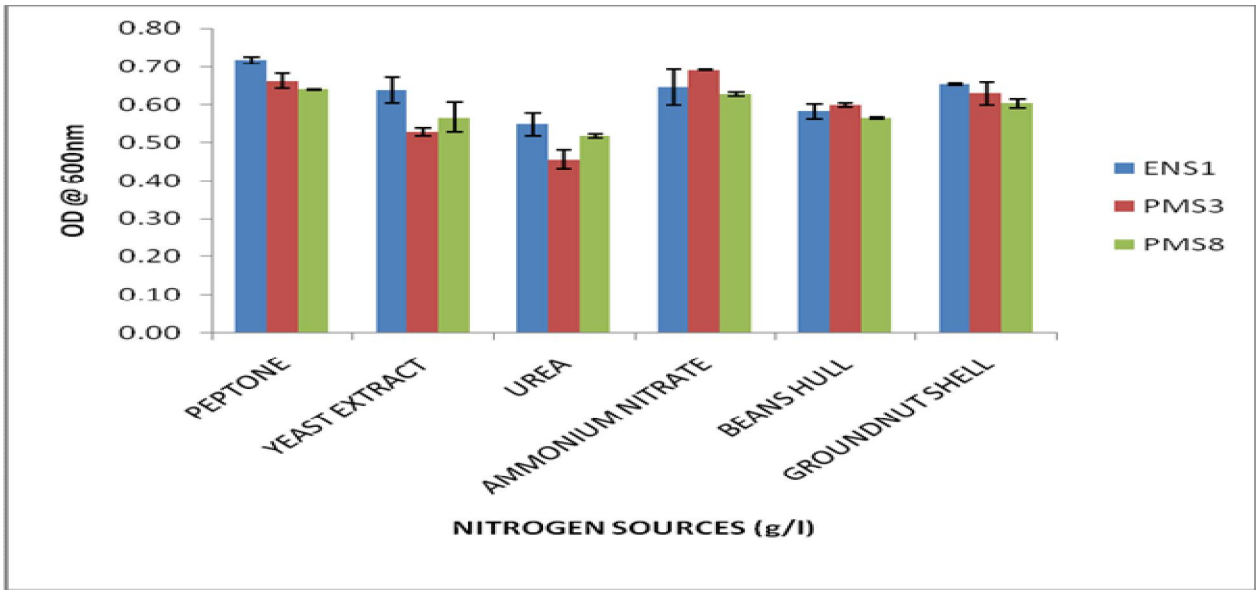

Figure 2b: Effect of nitrogen sources on cell growth of lipolytic yeasts

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 µ/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 µ/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.

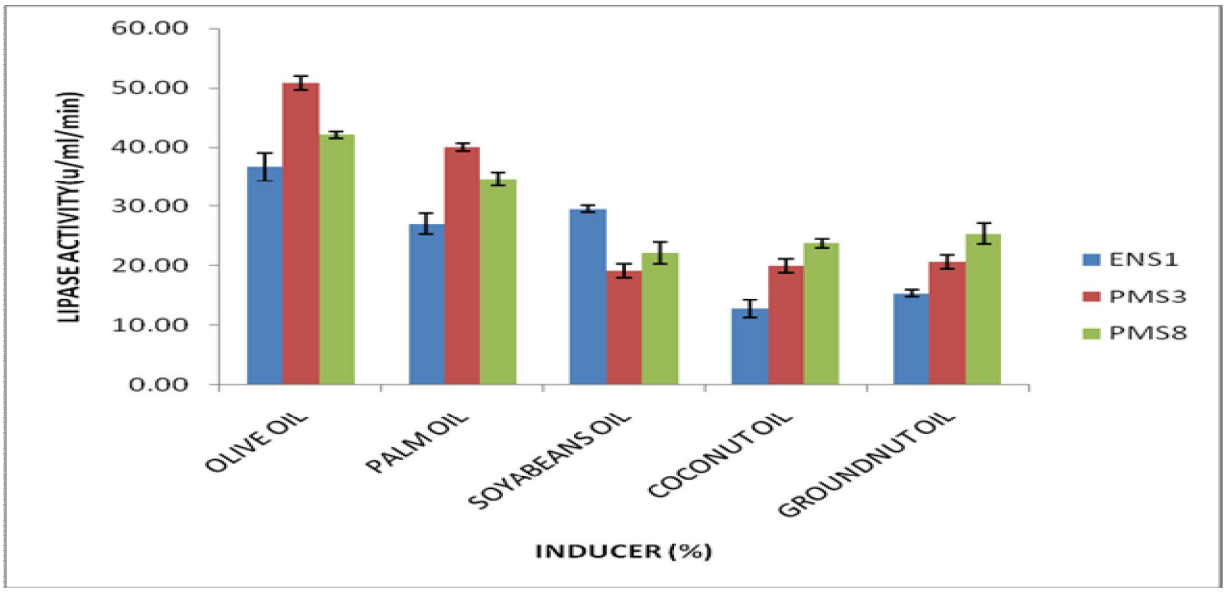

Figure 3a: Effect of Inducers on lipase production of lipolytic yeasts

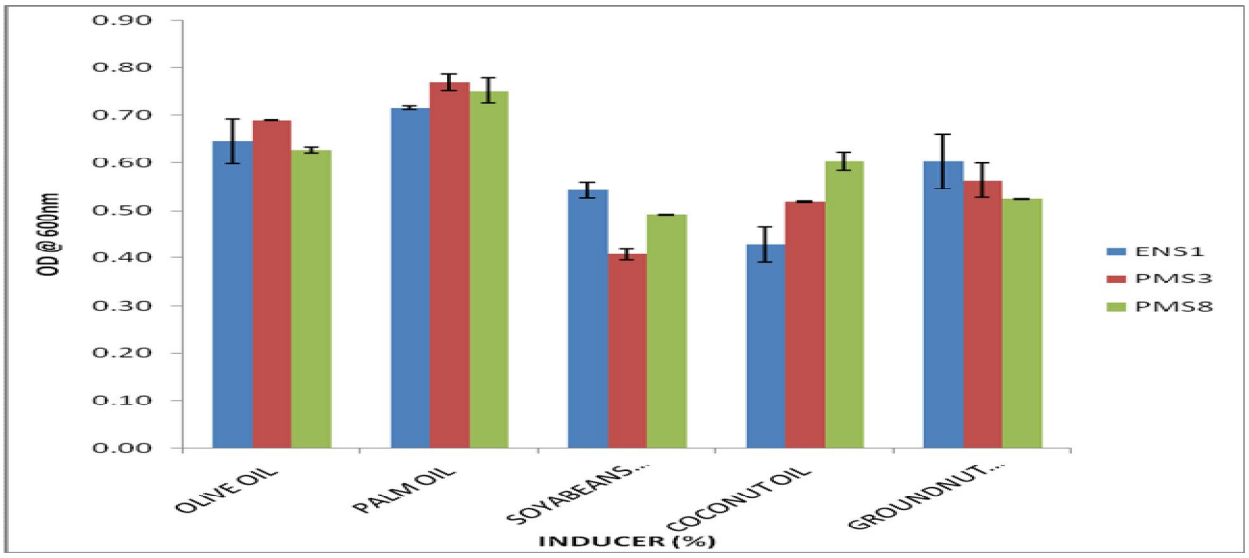

Figure 3b: Effect of Inducers on cell growth of lipolytic yeasts

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 µ/ml/min and cell growth from 0.67±0.02 to 0.59±0.02 [OD] while the lowest lipase activity from 17.50±1.17 µ/ml/min to 11.67±1.18 µ/ml/min and cell growth from 0.51±0.02 to 0.47± 0.04[OD] was recorded at pH 9.However *Pichia kudriavzevii* ENS1 [Accession No: OL546800] exhibited optimum lipase production and cell growth at pH5.

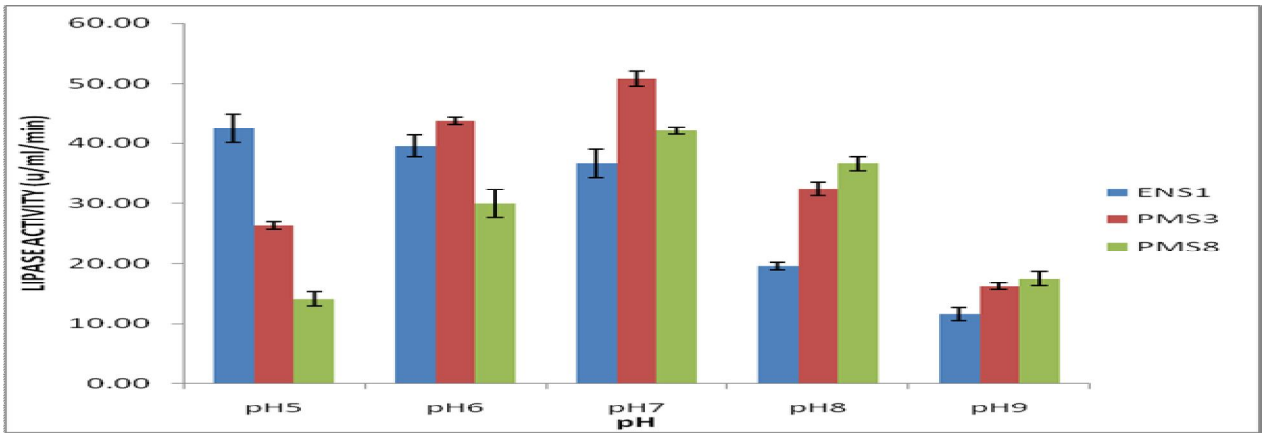

Figure 4a: Effect of initial pH on lipase production of lipolytic yeasts

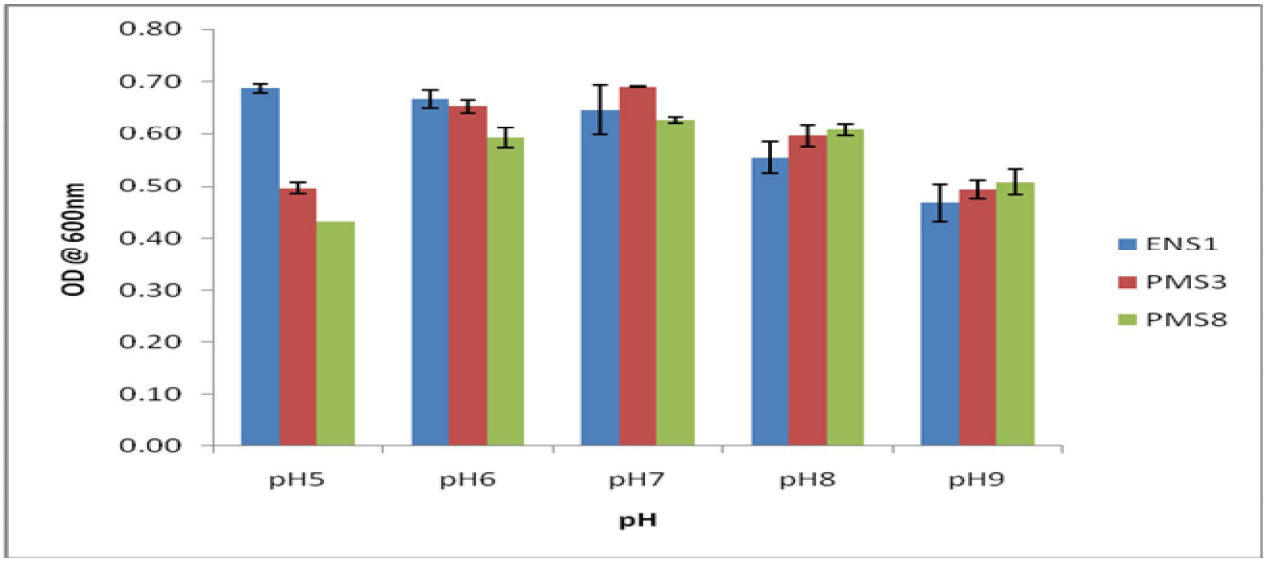

Figure 4b: Effect of initial pH on cell growth of lipolytic yeasts

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 µ /ml/min and cell growth at 24hours from 0.48±0.04 to 0.41± 0.00OD as presented in Table 5a and 5b.

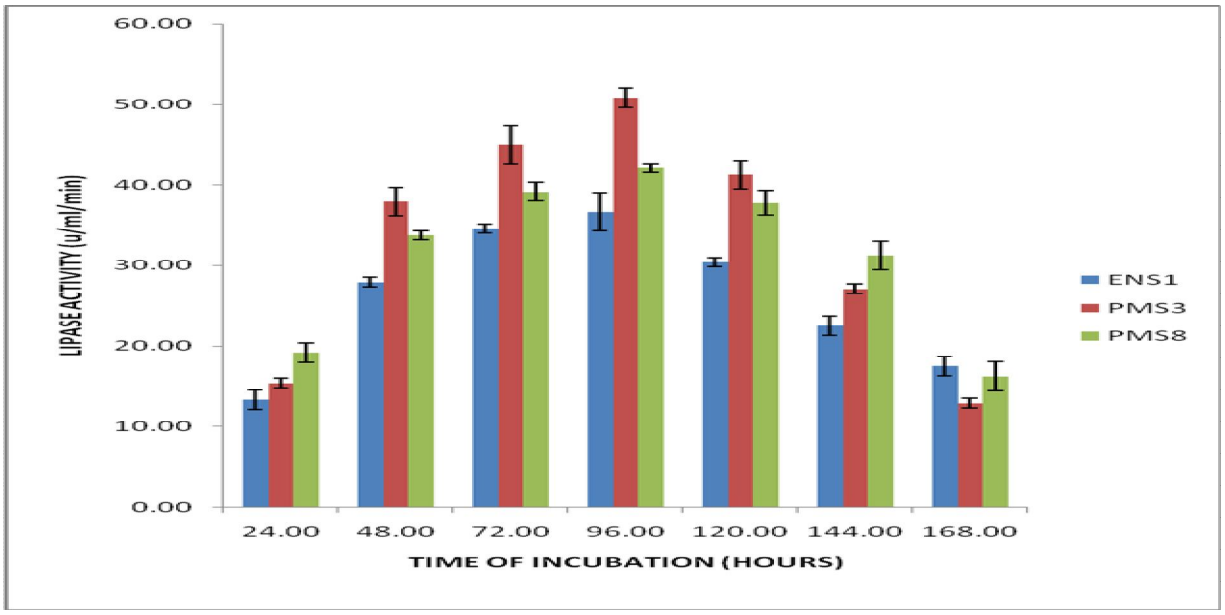

Table 5a: Effect of incubation time on lipase production of lipolytic yeasts

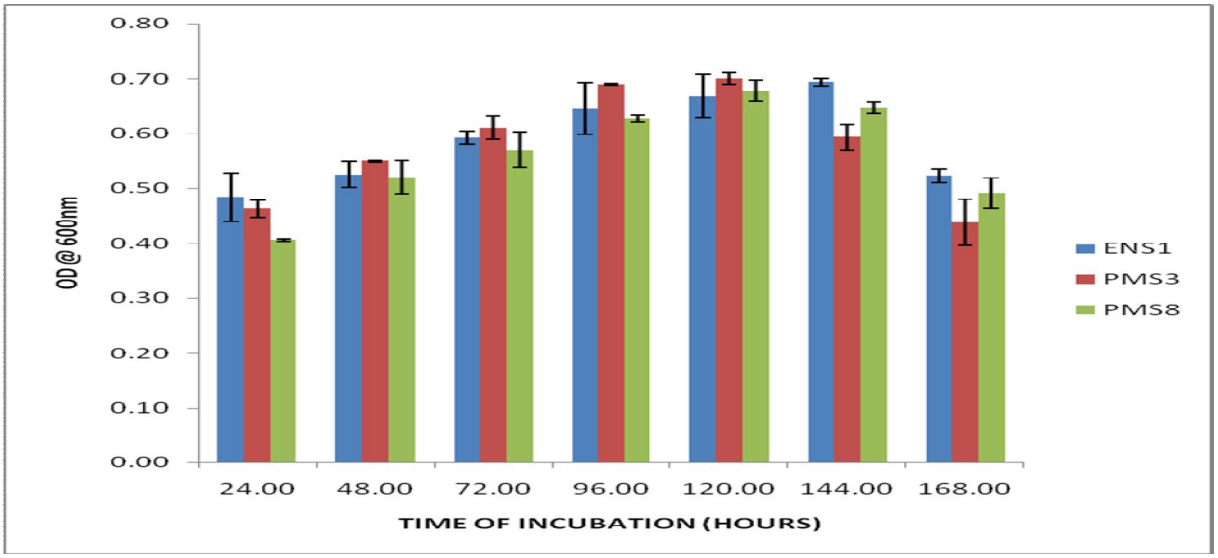

Figure 5b: Effect of incubation time on cell growth of lipolytic yeasts

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 µ/ml/min and 0.53± 0.05 to 0.49±0.02 OD as shown in Figure 6a and 6b.

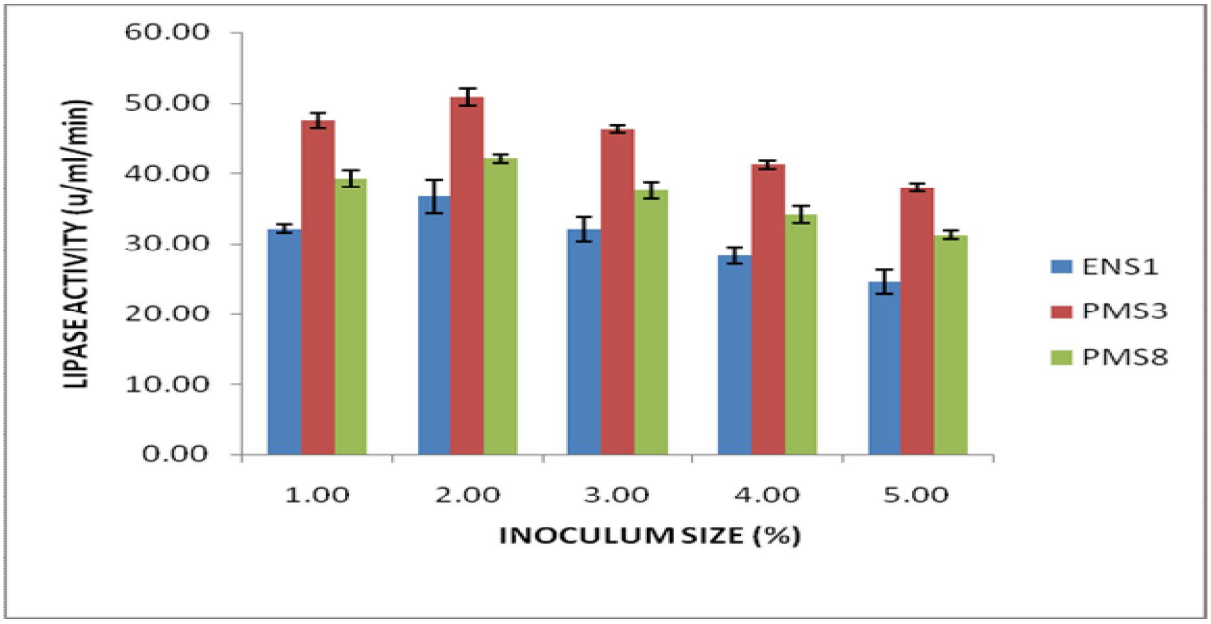

Figure 6a: Effect of inoculum size on lipase production of lipolytic yeasts

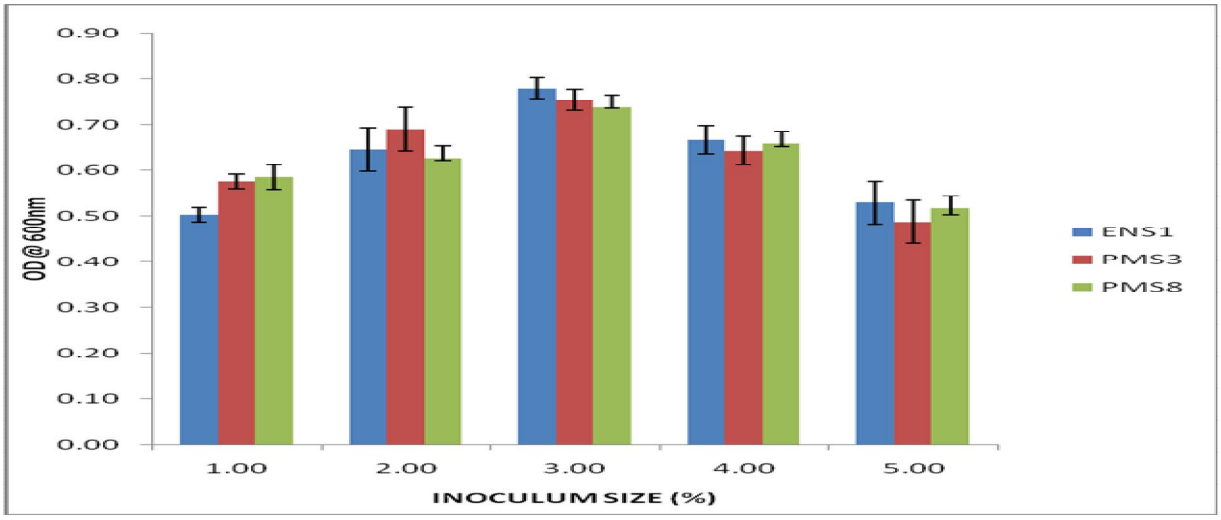

Figure 6b: Effect of inoculum size on cell growth of lipolytic yeasts

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 µ/ml/min and cell growth at 0.5:1 from 0.58± 0.02 to 0.53± 0.01OD as shown in figure 7a and 7b respectively.

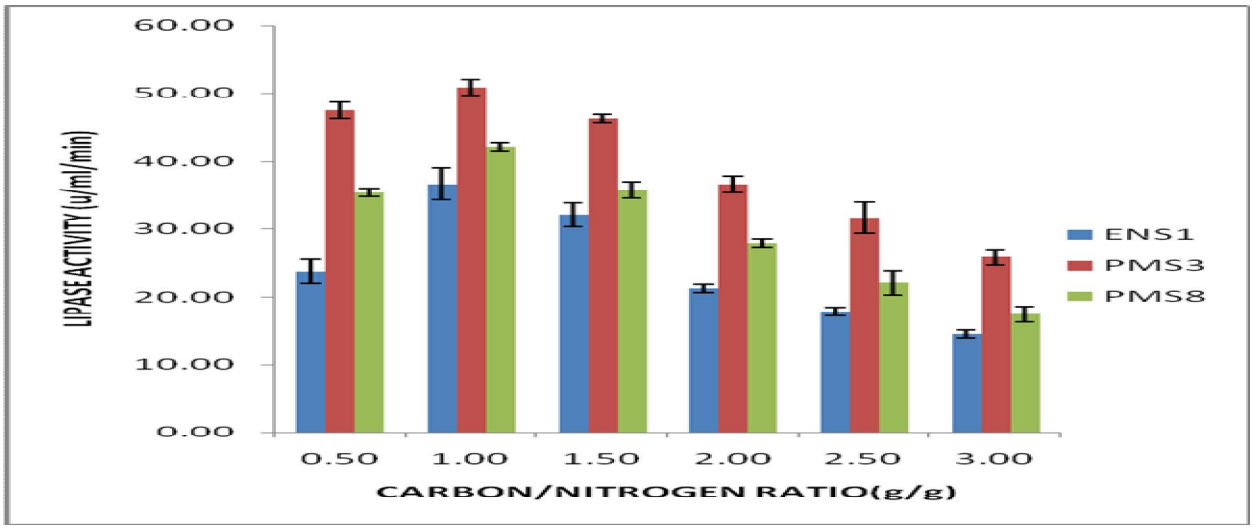

Figure 7a: Effect of carbon-nitrogen ratio on lipase production of lipolytic yeasts

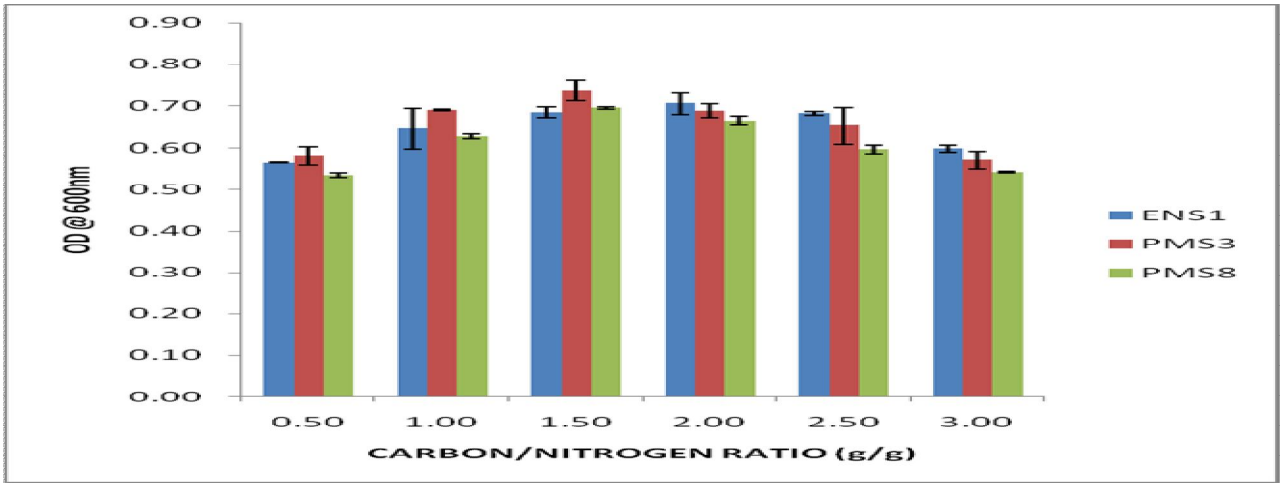

Figure 7b: Effect of carbon –nitrogen ratio on cell growth of lipolytic yeasts

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±0.56% and crude fat at 19.54±0.62 % in mango kernel , orange peel has an appreciable level of carbohydrate at 61.01±0.06 % and crude fat at 8.59±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±0.13% while beans hull recorded percentage crude protein of 9.59±0.05% respectively as shown in Table 1. [26] reported 71.99±0.06% carbohydrate and 4.29±0.01% crude fat from mango kernel powder[27] reported 45.05% carbohydrate and 3.75% crude fat from sweet orange peel; [28] recorded 12.14±0.92% of crude protein in groundnut shell; 7.71% of crude protein was recorded by [29] 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], [30] and [31]. 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 [32] in which ammonium nitrate and olive oil stimulated both higher lipase and cell productivity at 58.50 µ/ml/min and 19.50g/l respectively in the production of lipase by newly isolated *Candida viswanathii* strain. Similar results was also obtained from [33] in which maximum lipolytic activity was reported with ammonium nitrate at 15.6±0.036µ/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 innoculum 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. This would also enhance the commercialization of lipase due to the inexpensive and readily available nature of mango kernel powder as feedstock for lipase production. *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 .

ACKNOWLEDGEMENT

The authors are grateful to United Nations Educational, Scientific and Cultural Organization-International Centre for Biotechnology [UNESCO-ICB] for their financial support towards this research.

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.

REFERENCES

1. Qais AA, Waleed A, Amer AM. Microbial enzymes produced by fermentation and their applications in the food industry. *International Journal of Agriculture, Innovations and Research*. 2019; 8[1]:62-82.ISSN:2319-1473.
2. Imandi SB, Garapati H. Lipase production by *Yarrowia lipolytica* NCIM 3589 in Solid state Fermentation using mixed substrate. *Research Journal of Microbiology* .2007; 2[5]:469 – 474; DOI: 10. 17311/jm.2007. 469.474.
3. Bernardo DR, Aline MC, Maria AZC, Denise MGF. Production and Use of lipases in Bioenergy: A review from the feed stocks to biodiesel production. SAGE- Hindawi Access to Enzyme Research. 2011;615803: 1-16;doi:10.4601/2011/615803.
4. Vikay ND, Amit B. A review on the utility of microbial lipase in waste water treatment. *Journal of Water Process Engineering.*2022*;* 46:102591;doi.org/10.1016/j.jwpe.2022.102.
5. Salvatierrra HN, Regner EL, Baigori MD, Pera LM. Orchestration an extracellular lipase production from *Aspergillus niger* MYA 135: Biomass morphology and fungal physiology. 2021; 11[42]: 1-11; doi.org/10.1186/s13568-021-01202-y.
6. Cesario LM, Pires GP, Pereira RFS, Fantuzzi E, Xavier AD, Cassini STA, de Oliveira JP.Optimization of lipase production using fungal isolates from oily residues. *BMC Biotechnology*. 2021; 21[65]:1-13; doi.org/10.1186/s12896-021-00724-4.
7. Nasaruddin RR, Zahangir MA, Mohammed SJ.Enzymatic biodiesel production from Sludge Palm oil (SPO) using locally produced *Candida cylindracea* lipase*, African journal of Biotechnology.*2013*;* 12[31]:4966-4974; doi:10.5897/AJB2013.12075.
8. Akshita M, Urgyn B, Reena G. Fungal lipases: a review. *Journal of Biotech Research.2017;* 8:58-77.ISSN: 1944-3285.
9. Demir BS, Tukel SS. Purification and characterization of lipase from *Spirulina platensis*. Journal of Molecular Catalysis. 2010; 64:123-128; doi.org/10.1016/j.molcatb. 2009.09.011.
10. Lima LGR, Gonclave MMM, Couri S, Melo VF, Sant'Ana GCF, da Costa ACA. Lipase Production by *Aspergillus niger* C by Submerged fermentation. Brazillian Archives of Biology and Technology.2019; 62[3]:1-14; doi.org/10.1590/1678-4324-2019181013.
11. Vassileva M, Malusa E, Sas- Paszt L, Trzcinski P, Galvez A, Flor- Peregrin E, Shilev S, Canfora L, Mocali S, Vassilev N. Fermentation Strategies to improve soil bio-inoculants production and quality. Microorganisms. 2021;9 [1254]:1-18;doi.org/10.3390/microorganisms9061254.
12. Subramaniyamn R, Vimala R. Solid state and Submerged fermentation for the production of Bioactive substances: a comparative study. *International Journal of Science and Nature;* 3[3]:480-486.ISSN 2229-6441.

13. Szymczak T, Justyna C, Marcin P. Various Perspectives on Microbial lipase production using agri-food waste and renewable products. *Agriculture*. 2021; 11 [6]:540; doi.org /10.3390/agriculture11060540.
14. Talukdar S, Talukdar M, Manorama B, Archana Y, Yadav RNS, Bora TC. Enhanced candicidal compound production by a new soil isolate *Penicillium verruculosum* MKH7 under submerged fermentation.*BMC Microbiology*. 2016; 16 [288]:1-12: DOI: 10.1186/s12866-016-0713-8.
15. Singhania RR, Sukumaran RK, Patel AK. Advancement and comparative profiles in the production technologies using solid state and submerged fermentation of cellulases. Enzyme and Microbial Technology.2010; 46 [7]:541-549; DOI: 10.1016/ j.enzmictec.2010.03.010.
16. Campestrini LH, Salles-Campos C. Aspects of mushroom cultivation to obtain polysaccharides' in submerged cultivation. *African Journal of Biotechnology*, 2021;0[2] :100-107; DOI:10.5897/ AJB2020.17265.
17. Saloon L. Raja Bad Raman RNZ, Salleh AB, Mohd Sheriff F, Convey P, Pearce D, Ali MSM. Isolation, Characterization and Lipase Production of a cold adapted bacterial strain *Pseudomonas* sp. LSK25 isolated from Signy Island, Antarctica. *Molecules*. 2019; 24[7&15]:1-14; doi: 10.3390/molecules24040715.
18. Singh A, Mukhopadhyay M. Overview of fungal lipase: A review. *Applied Biochemistry and Biotechnology. 2012;* 166[2]:486-520; DOI: 10.1007/s12010-011-9444-3.
19. AOAC.AOAC Official Methods of Analysis [18th edition].2010.Association of Official Analytical Chemists, Washington, DC.
20. Ucuncu C, Tari C, Demir HG, Buyukkileci AO. Dilute –acid hydrolysis of Apple, orange, apricot and peach pomaces as potential candidates for bioethanol production. *Journal of Biobased materials and Bioenergy*. 2013; 7[3]:376-389.DOI:10.1166/jbmb.2013.1361.
21. Mishra A, Kumar N, Kumar R, Tomar D. Mineralization of carbon, nitrogen, phosphorus and sulphur from different organic waste in silty clay loam soils. Journal of Applied and Natural Science. 2016; 8[1]:16-22.
22. Abu ML, Nooh HM, Oslan SN, Salleh AB. Optimization of physical conditions for the production of thermostable T1 lipase in *Pichia guilliermondii* strain SO using response surface methodology. *BMC Biotechnology* .2017; 17:78.DOI 10.1186/s12896-017-0397-7.
23. Ire FS, Ike VC Screening and Optimization of process parameters for the Production of lipase in Submerged fermentation by *Aspergillus carbonarius* [Bailer] IMI366159. *Annual Research and Review in Biology.* 2014*;* 4[16]:2587-2602.
24. Sushma K, Sumathy J. Isolation , production and purification of lipase from *Aspergillus niger* using gingerly oil cake as substrate. *FS Journal of Pharmaceutical Research*. 2021; 1[2]:11-14.ISSN 2279-0586.
25. Nur HA, Liziyatin N, Rurin LAU, Nengah DK, Enny Z, Maya S.Lipase production in lipolytic yeast from Wonorejo mangrove area. AIP Conference Proceedings 1854, 020001 .2017; doi.org/10.1063/1.4985392.

26. Egbuonu ACC, Atasie OC, Ezeala CC, Ejike GA. Mango [Mangifera indica L.] seed kernel: Proximate properties and effect on Normal and monosodium glutamate hepta-compromised wistar rats. *International Journal of Biochemistry Research and review .2*017;20[4]:1-9;DOI:10.9734/IJBCRR/2017/38639.
27. Okpanachi U, Luka J, Sabo, Danjuma RA.Proximate composition, anti-nutrients and crude fiber fractions of selected peels fed to ruminants. *Agricultural Studies.2021;* 5 [3]:1-8:DOI:1010.31058/j.as2021.53001.
28. Omogbai BA, Obazenu EI, Iduoriyekemwen AS, Ubaka LN, Imoni AA.Proximate composition of agro shell wastes and their utilization for bioprotein production with *Candida tropicalis* MYA-3404. *BIU Journal of Basic and Applied sciences.*2019; 4[1]:101-114. ISSN: 2563-6424.
29. Tona GO, Ogunbosoye DO, Ayano MO. Nutritive value assessment of flour crop residues by proximate composition and in vitro rumen fermentation techniques. *International Journal of Applied agricultural and apicultural Research*.2015; 11[1&2]: 123-129.
30. Soleymani S, Houri A, Hossein M, Emmad R, Fatemeh M, Hamid MS, Ziaedin SS, Zahra E, Mohammed R. Efficient media for high lipase production: One variable at a time approach. *Avicenna Journal of medical Biotechnology.*2017*;* 9[2]:1-6
31. Asma A, Badar R, Khalique N. Screening and optimization of submerged fermentation of lipolytic *Aspergillus oryzae*. *Bioresources*.2019; 14[4]: 7664-7674.
32. de Almeida AF, Dias KB, Da Silva ACC, Terrasan CRF, Tauk-Tornisielo SM, Carmona CE. Agro- industrial wastes as alternative for lipase production by *Candida viswanathii* under Solid state cultivation: purification, biochemical properties and its potential for poultry fat hydrolysis. *Enzyme Research.*2013*;* 2016:1-14:doi.org/10.1155/2016/1353497.
33. Praveen D, Sharmishtha P. Isolation, screening and production of extracellular alkaline lipase from a newly isolated *Bacillus* sp. PD-12. *Journal of Biological Sciences.*2011; 11[5]: 381-387; DOI: 10.3923/jbs.2011.381.387.