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Production of lipase using *Cucummeropsis Mannii* obtain from south Eastern Nigeria

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Abstract
Lipase has attracted a lot of attention in recent years because of its diverse biotechnological applications, like biopolymer synthesis, biodiesel production, treatment of fat-containing waste effluents, enantiopure synthesis of pharmaceuticals and nutraceutical agents, have been established successfully. Lipase occurs widely in nature, but plant lipase, is significant because of its unique substrate selectivity/specificity. However new lipases with properties amenable for application in specific industrial process are being sought by the researchers. In this work, a penicillium strain was isolated that showed lipase production from endosperm tissues of decaying seeds of Cucummeropsis mannii obtained from the regular Ogige market in Nsukka metropolis, Enugu state. The optimization studies resulted to an increase in enzyme activity: the strain produces lipase in an inexpensive inorganic medium at highest when ammonium sulphate is used as a source of nitrogen when compared with lipase activity on other sources of inorganic nitrogen. Lipase production known to be induced when lipids such as vegetable oils (triacyiglycerides) are included in the medium as carbon sources. The potential to induce lipase production is dependent on the type of oil used. The inclusion of olive oil in medium showed that lipase extract from Cucumeropsis mannii had its highest enzyme activity when it is used as a carbon source when compared to other sources of carbon like palm kernel oil, groundnut oil, soyabean oil and sunflower oil. Further studies on the lipase activity from Cucumeropsis mannii also showed that the lipase produced from it was highest at pH 8.0, which meant that the lipase is best produced at an optimum medium pH of 8.0.

Keywords: lipase, production, cucummeropsis mannii, Nigeria

Introduction
Lipases are a group of enzymes capable of hydrolyzing the ester bonds of water insoluble substrates at the interface between substrate and water. It is understood that the reaction is reversible and this enzyme can catalyze ester synthesis and transesterification. Since lipases can catalyze numerous different reactions they have been widely used in industrial applications, such as in food, chemical, pharmaceutical and detergent industries (Harwood, 1989). Many microorganisms are known as good producers of extracellular lipases (Ratledge and Tan, 1990). Lipases occur widely in bacteria, yeast and fungi (Jaeger *et al*., 2000). Most of the lipase research focuses on the production of extracellular lipases through a wide variety of microorganisms. Studies on the production of extracellular lipases with Bacillus shows variations among different strains. These enzymes are produced during the utilization of certain nutrients such as proteins, lipid and carbohydrate. Lipases have potential applications in detergent, oleo chemical, paper manufacturing, cosmetics, pharmaceuticals, and agrochemical industries. They are also employed in organic chemical processing, biosurfactant synthesis, nutrition and biomedical sciences (Pandey *et al*., 1999).

Most of the commercial lipases produced are utilized for flavour development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk products and beer. Lipases are extensively used in dairy industry for the hydrolysis of milk fat. The dairy industry uses lipases to modify the fatty acid and chain lengths, to enhance the flavours of various cheeses. Lipases enzyme also accelerates cheese ripening and the lipolysis of butter, fat and cream (Oderinde *et al*., 2009).

Cucumeropsis mannii (white melon) is popularly known as "egusi" in West Africa. This is the true indigenous egusi of West Africa (Burkill, 1985) [10]. Melon is a cucurbit crop that belongs to the *Cucurbitaceae* family with fibrous and shallow root system. It is a tendril climber or crawling annual crop, mostly grown as a subsidiary crop interplanted with early maize and yam in some savanna belt of Nigeria (Mabalaha *et al*., 2007). Cucurbit species are among the economically most important vegetable crops and are grown in both temperate and tropical regions (Paris, 2001). As reported by Jacks *et al*. (Jacks *et al*., 1972), the seeds has about 50% lipid. Most of their oil is made of non-saturated fatty acids. Conjugated fatty acids among some *cucurbitaceae* oils make them highly useful as drying oils, that is, they combine readily with oxygen to form elastic, water proof film (Meneghetti *et al*., 1998).

Lipases
Lipases (triacyiglycerol acyihydrolases EC 3.1.1.3) are a class of hydrolases which catalyze the hydrolysis of triacyiglycerides to glycerol and free fatty acids over an oil-water interface. Lipases also catalyze the hydrolysis and transesterification of other esters as well as the synthesis of esters and exhibit enantioselective properties. The ability of

lipases to perform very specific chemical transformation (biotransfo rmation) has made them increasingly popular in the food, detergent, cosmetic, organic synthesis, and pharmaceutical industries (Park *et al*., 2005).

Structure of Lipase

Lipases belong to many different protein families and they have the same architecture.

Ollis *et al*. (1992) defines this structure as theα/ß –hydrolase fold. Generally lipase activity has been shown to rely on triad usually formed by serine, histidine and aspartate residues (Arpigny and Jaeger, 1999) [3]. In amino acid sequence of α/ß hydrolases, the three residues follow the order Ser-Asp His. Lipases also have consensus sequence of Gly—Xaa-Ser-Xaa-Gly where X could be any amino acid residue (Kanaya *et al*., 1998). According to Abdelmonaem *et al*. (2011), the modeled enzyme is a monomer folded into α/ß domain consisting of eight central stranded ß- sheet flanked by twenty two α-helices. The number of α-helices and ß-sheets differ from one specie to another. The figure below shows the ß -dimensional structure of lipase.

Figure 1: Three-dimensional (3-D) structure of lipase (adapted from Abdelmonaem et al., 2011)

Sources of Lipase enzyme

Canola Lipases (Brassica napus L.)

Sana *et al*. (2004) evaluated the physical-chemical properties of germinated canola seed lipase (Brassica napus L.) purified by chromatography in a column packed with Sephadex G-50, DEAE and CM-cellulose. Using triolein as the substrate, the highest activity was found at pH 7 with a temperature of 37°C.

Coconut Lipases (*Cocos nucifera linn*)

Ejedegba *et al.* (2007) studied the physical- chemical characteristics of semi-purified coconut lipases extracted with acetone. Four substrates were used to evaluate the enzymatic activity: triolein, tripalmitin, olive oil and coconut oil. I' he lipase showed a greater affinity for coconut oil, with an optimum pH of 8.5 and optimum temperature range of 35-40°C in triolein.

Cereal Seed Lipases

Cereal grains contain from 2 to 10% of lipids, depending on the species and variety, and about 80 to 90% of the triacyiglycerol fatty acids are oleic and linoleic (Hilditch and Williams, 1964). Lipids are normally located in the embryo (germ) and aleurone layer (the bran, which includes the pericarp, testa and part of the endosperm). Lipolytic activity is present in different parts of cereal grains. Many researchers have focused their studies on these enzymes due to the process of rancidification that can occur during grain storage. The physiological function and exact location of these enzymes in the tissues are still important aspects to be discovered (Borgston and Borckman, 1984).

Rice Lipases (*Oryza sativa*)

Most of the lipolytic activity in rice is found in the bran (Borgston and Borckman, 1984). A heat stable lipase was identified in rice and purified by chromatography using Octyl-Sepharose. The enzyme is a glycoprotein with a molecular weight of 9.4 kDa and has shown optimum activity at 80°C and pH 11.0, using triolein as the substrate. The enzyme shows both phopholipase and hydrolysis activities, preferably at position sn-2 of phosphatidyl choline, although apparently it does not show positional specificity with triacylglycerols. Diisopropyl fluoro phospháte inhibited both the lipase and phospholipase activities (Bhardwaj *et al*., 2001).

Factors that affect lipase activity

Inhibitors of lipases

Lipase enzymes are inhibited by heavy metals such as Cu^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Fe^{2+} (Barros *et al*., 2010). Oxidants such as Hydrogen peroxide, atmospheric oxygen and alloxan inhibit lipase probably due to their oxidation of the sulfyhydryl (SH) groups of amino acid side chain of the lipase.

Temperature

Moisture content

pH

Nutritional Value of *Cucumeropsis mannii*

Cucumeropsis mannii mainly consist of fats 44.4%, protein 36.1% and carbohydrate 13.2% (Badifu and Ogunsua, 1991). Minerals and water amount to 3.7 and 5.9% respectively. From this composition a caloric value of 2190 KJ/l00g is calculated (Mbuli-Lingundi *et al*., 1983). The major component of the oil is linoleic acid and constitutes 57.9% of its oil content. Short chain fatty acids are absent in *Cucumeropsis mannii*. Important macro and micro nutrients are present it sufficient amount for human nutrition. Consumption of 100g of dehulled seeds covers the daily requirement of essential fatty acids, vitamin E and amino acids (Adeleke, 2010).

Scientific classification of the plant:

Kingdom: Plantae-plants
Division: Magoliophyta-flowering plant
Class: Magnoliopsida – Dicotyledons
Order: Cucurbitales
Family: Cucurbitaceue – Cucumber family
Genus: *Cucumeropsis Naudin* – Cucumeropsis
Specie: *Cucumeropsis mannii Naudin* – Mann's *cucumeropsis*
Binomial name: *Cucumeropsis manii Naudin*.
(http://zipcode200.com/plant/c/cucumeropsismannii.retrieved9/10/2010)

Uses of *Cucumeropsis mannii*

Cucumeropsis mannii is grown for its oily seed. The seeds are sold almost three times more expensive than cocoa and about seven times more expensive than coffee (Zoro-Bi *et al*., 2003). The kernels are milled into a whitish paste which is consumed as thickeners of a traditional soup called "egusi soup" in Nigeria, Cameroon and Benin, and pistachio soup in Cole d'lvoire (Loukou *et al*., 2007 and Koffi *et al*., 2008). The seeds of *C. mannii* are used to prepare. dough or a sauce and soup flavour when fermented (ogiri) (Fomekong *et al*., 2008). It has an important value in the African traditional society (Ponka *et al*., 2005). The flesh of the fruit, though edible is not commonly eaten. In Ghana the fruit juice mixed with other ingredients is applied to the navel of new born babies to accelerate the healing process until the cord-relics drops off. Maccrated leaves are used in Gabon for purging constipated suckling babies. In Sierra Leone cattle boys traditionally use the dried fruit-shell of *C. mannii* as a warning horn (Chweya and Ezaguirre, 1999) [12]. *Penicillium species Penicillium* belongs to the phylum

Ascomycota, however its taxonomic characterization is still a matter of discussion (Grimm *et al.*, 2005) and the difficulties in identifying most *Penicillium species* requires multidisciplinary approaches. Clarification of species concepts in the genus *Penicillium* was supported mainly by morphological characteristics. Raper and Thom, for example, based Penicillium taxonomy classification on the combination of macroscopical
(such as colony texture and color) with micro-morphological features (Raper and Thorn, 1949). The various species of *Penicillium* can colonize many different environments. They are common in soils, in foods, in drinks and in indoor air (Banke *et al*., 1997) [6]. There are several reasons why the use of microorganisms (*Penicillium spp*.) for enzyme production is of great importance to researchers and practitioners. *Penicillium spp*, are usually slow growing and often require substrates for metabolism. The morphology and growth characteristics of *Penicillium spp.* are responsible for the rapid colonization of substrates. Structural heterogeneity is considered to be one of the most important characteristics of *Penicillium spp*. The ability of most *Penicillium spp*. to produce extracellular enzymes for the assimilation of complex carbohydrates without prior hydrolysis makes possible the degradation of a wide range of pollutants. They also have the advantage of being relatively easy to grow in fermenters, thus being suited for large scale production of enzymes. Another advantage is the easy separation of fungal biomass by filtration due to its filamentous structure. During the last decade, Penicillium spp. have been used in the production of different enzymes (Sing, 2006).

Materials and Methods

Cucumeropsis manni (white melon)

Cucumeropsis manni was obtained from a local *Cucumeropsis manni* dealer at Ogige main market in Nsukka metropolis, Enugu State in a clean and sterile container.

Rhodamine 6G reagent

25mg of Rhodamine 6G was dissolved in 25m1 of 0.2M phosphate buffer (PH 10) and immediately extracted with 500m1 of benzene. The clear orange-yellow coloured benzene layer was transferred into an amber coloured glass bottle and preserved over solid caustic soda in the dark.

Hydrochloric Acid (IM HC1)

Molecular weight of HC1 is 36.46, Density equals Mass/Volume (36.46/1.19) equals 30.63m1
(1.19 as given in label). Purity is 30.63/37% multiply by 100% equals 82.78m1. 82.78m1 of HCI make up to I L with water.

Sodium Hydroxide (1M NaOH)

40g of solid Sodium hydroxide was weighed and dissolve with water in 1L measuring cylinder made up to mark with water.

Phosphate Buffer (O.2M)

Molecular weight of phosphate buffer is 96g, to prepare 02M of the solution, 1.92g was weighed and dissolve in 100ml measuring cylinder and make up to mark. 1M HCL and I M NaOH was used to vary the pH ranges

Deoxycholate Solution (10.0mM)

10.0mM is equivalent to 0.0IM, so to prepare 0.01M of deoxycholate solution with molecular weight 414.56g/mol. 0.41456g was weighed and dissolve in l00mI measuring cylinder and make up to mark with water.

Triethanolamine Buffer (IM, PH 7.5)

Molecular weight of triethanolamine buffer is 149.19g., Density is 1.12g/L, To prepare 1M of the solution, 3.32m1 was measured out using measuring cylinder and made up to mark in 100ml measuring cylinder. The pH was adjusted to 7.5 using 1M NaOH.

Tris-Hcl Buffer (0.02M)

The molecular weight of Tris-HCl buffer is 157.595g, to prepare 0.02M of the solution, 0.3g was weighed and dissolved in 100mI measuring cylinder and made up to mark using water. 1M HCl and 1M NaOH was used to vary the pH ranges (PH 2-12).

Sodium Acetate Buffer (0.02M)

Sodium acetate buffer has a molar mass of 82g, to prepare 0.02M of the solution 0.1 64g was weighed and dissolved in l00ml measuring cylinder and made up to mark with water. I M I ICI and IM NaOH was used to vary the PH ranges (PH 3-6).

Methods

Isolation and characterization of microorganism from decaying seeds of *Cucumcropsis manni* (white melon).

The endosperm tissues of egusi seeds were carefully removed and washed in a distilled water.
The endosperms were ground using mortar and pestle, the grinded powder were mixed with water, poured into a container and left for 7-8weeks to allow microorganisms grow on it and develop into mature spores. Sabouraud Dextrose Agar (SDA) was prepared and poured into three (3) petri dishes at the department of Microbiology Laboratory, University of Nigeria, Nsukka and the most dominant organisms were isolated and cultured using streaking method. It were characterized and identified to be *Penicillum spp*. The strain was subsequently cultured on the Sabouraud Dextrose Agar (SDA) slants at room temperature for 5-6 days and sub-cultured every week for the period the work lasted.

Preparation of mineral source media

In one litre of water, 3g of $MgSO_4$, $7H_2O$, 3g of $CalCL_2$, 6g of K_2HPO_4, 2g of KH_2PH_2, and 5g of $(NH_4)2SO_4$ were added. 10ml of vegetable oil was added as the main carbon source and/or 0.5g of inorganic nitrogen as the main nitrogen source depending on the parameter that was carried out. 90ml of this mineral source was measured into six different conical flasks. This was sterilized at 121^0C, 15psi for 15 mins. After cooling, the media were inoculated with 10ml of 1.5 x 10^8 spores/ml of suspension from the penicillium strain into the six different conical flasks and were incubated for 7 days in a rotary shaker.

Slant preparation and microorganism Inoculation

SDA slant was prepared by measuring out 6.5g and was dissolved in 100ml of distilled water in a conical flask according to manufacturer's preparation specification

(65g/L), 5mg of chloramphenicol was added and allowed to dissolve in IL of distilled water, 8ml of the resulting solution was poured into bijou bottles and autoclaved at 121°C and 15psi for 15 minutes. After, the bijou bottles were placed at an angle 45^0 for the agar to solidify. After cooling, *penicillum spp*. was inoculated on it and left for 5-6 days for growth.

Lipase Assay

This was done according to the method of Hirayama and Matsuda, 1972 modified by Eze and Chilaka, 2010. A quantity of the enzyme reacted mixture (1.0ml) was incubated for 25 minutes 5ml of n-hexane-ethanol-3NH_2SO_4 (300-200-1, $^V/_V$) was added; the mixture was vigorously mixed and centrifuged at 10.0000 rpm for 4 minutes, 2ml of the resultant n-hexane layer was then transferred into a test tube and 1.0ml of Rhodamine 6G reagent was added. After 20 minutes, the optical density of the solution was read at 514nm against an appropriate blank. (Reaction mixture of 1.0ml contained 0.05ml of 10.0mM dexycholate solution. 0.45ml of 1.0M-triethanolamine buffer, pH 7.5 and 0.5ml of substrate emulsion). One unit of lipase activity is defined as the amount of enzyme releasing 1µmol of palmitic acid per minute under the assay condition.

Determination of the effect of inorganic nitrogen as nitrogen sources on the crude enzyme

The effect of inorganic nitrogen as nitrogen sources on the lipase enzyme activity was determined using NH_4CL, NH_4NO_3, $(NH_4)2SO_4$, $NaNO_3$, KNO_3 and Urea as nitrogen sources to determine the activity of the crude enzyme. The lipase activity was recorded for 7 days.

Determination of Optimum pH of the medium for lipase production

The crude enzyme solutions were mixed with the buffer at the ratio of 0.15ml to 1.35ml respectively in the pH ranges of 2-12 and incubated for 48 hours. The residual lipase activity was detected by the Standard assay method.

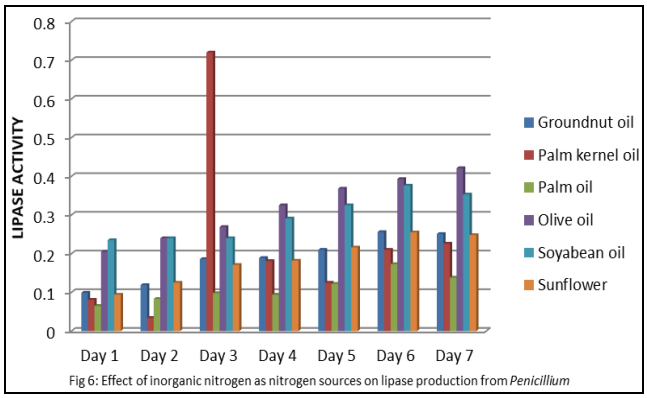

Fig 1: Effect of Triacyiglycerols as carbon sources on the activity of lipase from *C. mannii*

The effect of Triacylglycerols as carbon sources were studied and the result obtained showed that lipase enzyme extracted from *C. manni* had more activity when olive oil was used as the main source of carbon when compared to other carbon sources such as palm kernel oil, palm oil, sunflower oil and groundnut oil. However soyabean oil also showed high enzyme activity

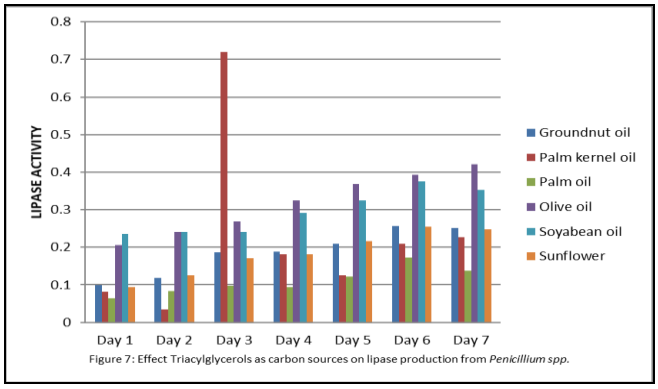

Fig 2: Effect of medium pH on the activity of lipase from *C. manni*

The effect of pH on the activity of lipase from *C. manni* was studied and it was found that at pH8.0 of the medium the enzyme had the highest activity. From the result of the graph below it can be obtained that the activity of the lipase enzyme from *C. manni* decreased as the pH increased above pH 8.0 and had less activity at pH values from 2.0 - 6.0.

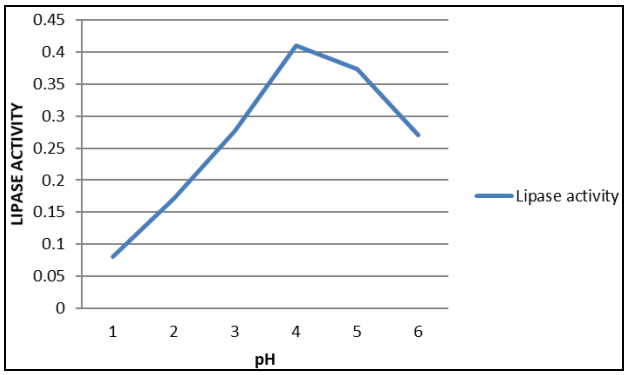

Fig 3: Effect of medium pH on lipase production from *Penicillium spp* using Olive Oil as the most preferred carbon source.

Discussion

Lipases are currently valued as industrial biocatalysts. A great number of research has been performed and articles have been published, especially concerning synthesis reactions, emphasizing the importance of lipases. The search for new lipases must be continuous and interesting seed sources must be well explored, since they may present different biochemical properties with respect to the reactions of hydrolysis and synthesis. There are a huge number of new seed sources for possible lipase exploitation. Lipases from castor bean, sorghum seed and soybean have been exploited for different reactions which resulted to very promising result. Seed lipase sources can represent a cheaper and faster way to produce these important biocatalysts.

Conclusion

In summary, the results obtained demonstrate that the seeds of *Cucumeropsis mannii*, which is widely grown in tropical West Africa and consumed largely as food (soup thickener) contains lipases. The research result shows that when inorganic nitrogen sources were used as the nitrogen sources, ammonium sulphate was found to give the highest lipase activity. When triacylglycerols were used as the carbon sources, it was found that Olive oil had the highest Iipa.se activity as compared to other triacylglycerols.

Further result also shows that lipase activity was high at pH 8.0 and low as the pH increased or decreased above or below pH 8.0 respectively in the medium. Therefore it can be concluded that lipase has an optimum pH of 8.0 in the medium when olive oil is used as the carbon source. It can be concluded that *Cucumeropsis mannii* seeds would he useful and important source of lipase for industrial biocatalytic processes, pharmaceuticals, medicine and in food industries

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