Isolation and Molecular Identification of Novel Lipase Producing Bacterium from Cocoa Processing Plant Effluent in Igba, Ondo, Nigeria

[1*]Adedire, S. A., [1]Awojobi, K.O., [2]Agunbiade, J.O., [3]Omojoyegbe R.T. and [4]Adewale I.O.

[1]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Department of Medical Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.
[3]Department of Biological Sciences, Samuel Adegboyega University, Ogwa, Nigeria
[4]Department of Biochemistry and Molecular Biology Obafemi Awolowo University, Ile-Ife, Nigeria.
Corresponding Author: sadedire@oauife.edu.ng; 08060845580

Abstract: Wastewater samples were collected from cocoa processing plant effluent in Ondo, Nigeria. Lipase-producing bacteria were isolated and screened from the effluent. Four bacterial isolates showed lipolytic activities and were assessed both qualitatively (agar plate cultures) using tributyrin agar and quantitatively (liquid cultures) using olive oil as sole carbon and energy source. The bacterium with the highest lipase activity was selected, identified by biochemical methods and using molecular techniques. The optimization of the production medium for maximum production and lipase activity were carried out. Lipase activity was assayed by a spectrophotometric method. The absorbance of p-Nitrophenol released was measured at 410 nm. The 16S rRNA gene sequencing confirmed the bacteria with the highest lipase activity to be *Brevundimonas diminuta*. Maximum enzyme production was obtained when the medium was incubated for 72 h (35.85± 0.5 U/ml) at temperature of 35°C and maintained at pH 7.5. Olive oil (with 30.00 ± 4.09 U/ml) and peptone (with 47.33 ± 1.26 U/ml) were found to be the most suitable substrate for maximum enzyme production. The organism also utilized the effluent (with 4.78 ± 0.16 U/ml) as carbon source. The study concluded that cocoa processing plant effluent contain substrate that makes it a renewable source for the production of microbial lipase.

Keywords; *Brevundimonas dimunita*; Cocoa; Effluent; Lipase

INTRODUCTION

In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Abu *et al.*, 2005). Therefore, research that focuses to use isolated microorganisms from different environments as well as agro-industrial residues based media composition are urgently needed in achieving high value lipase at low cost, which is a recipe to overcome the impending industrial challenges (Colla *et.al*, 2016) The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. The use of lipases in industries is enormous and increasing. Extensive and persistent screening for new microorganisms and their lipolytic enzyme will open new, simple routes for synthetic processes and consequently new and faster ways to solve environmental problems. Lipolytic microorganisms can be found in a variety of oil-contaminated habitats such as soil contaminated with oil, wastes of vegetable oils, dairy waste and deteriorated food (BurcuBakir and Metin, 2017). Ever since that lipase production, different bacterial species has been extensively studied and reported. There are various documents available on the production of bacterial lipases particularly from *Pseudomonas* (Carrazco-palafox *et.al*.,2018) and *Bacillus* sp.(Jia *et al*.,2012), *P. aeruginosa* (Maytham and Hanna,2016), Mobarak-qamsari *et al*.,2011) *Pseudomonas fluorescens* (Yang *et al*., 2009), *Bacillus pumilus* (Sangeetha *et al*., 2010a) *B. thermocatenulatus* (Quyen *et al*., 2003), *B. subtilis, B. licheniformis* (Sangeetha *et al*., 2010b), *B. coagulans* (Mnisi *et al*., 2005) and *B. cereus* (Dutta and Ray, 2009).Other genera like *Burkholderia* (Wang *et al*., 2009), *S. Marcescen* (Long et al., 2007,Subhajit 2012), *Achromobacter, Arthrobacter, Alcaligenes* and *Chromobacterium* (Riaz *et al*., 2010), *Lactobacillus* (Balakrishman *et.al*.,2011)

Klebsiella sp and *Enterobacter* sp (Jiafulin *et al*.,2012) have also been studied.

Effluent from Cocoa-processing plants constitutes one of the sources of oily waste in the environment. Others are household, restaurants and automobile workshops. However, the oily waste could be a good source of lipolytic bacteria that are useful for the complete degradation of the oil. Because of huge variation in application and emerging need of lipases with specific characteristics has prompted the search for new lipases from newer areas with different characteristics Hence, this study is designed to isolate, screen and identify high yielding lipase producing bacterium from cocoa-processing plant effluent.

MATERIALS AND METHODS

Microorganism, media, and growth conditions

Lipase producing bacteria isolates were isolated from the effluent. The wastewater was serially diluted using sterile maximum recovery diluents (0.1% peptone in 0.85% normal saline). One (1)ml of the serial diluents was plated on a pre-screening medium (Congo red agar) at 30° C for 48 h. Distinct colonies which show pink zone of hydrolysis around it were selected, subcultured and purified on Tributyrin agar and screened for lipase production. At 30° C for 48 h. Isolate(s) showing zone of clearance around were noted and the measurement of the zone was taken and recorded between 24 h – 72 h. These experiments were carried out in triplicate (one control and three replicate /sample) the isolate with good lipolytic activity was processed into agar slant/glycerol stock and stored in the refrigerator for further use.(Olutiola *et.al*., 1991)

Identification of Bacteria strain: isolates were identified by sequencing of 16s rRNA gene. genomic DNA of the isolates were extracted by using qiagen DNA protocol. Genomic DNA was determined by agarose gel electrophoresis (Sambrook and Russell, 2001).the 16sr RNA gene from the genomic DNA was amplified by PCR . The highly purified DNA was then amplified in a thermocycler in a thermocycler with program comprised intial denaturation at 92°C for 2 min. followed by 31 cycles each of 94°C for 30 sec.,51.8^{o}C for 45 sec.,72°C for 1.5 min. and 72°C for 5 min. Strands of PCR product was detected by agarose gel electrophresis,5µl of PCR product was transfered into the wells of agarose gel and in the first well was for (10 µl)DNA Ladder(1Kb).

These sequences were read in Genbank databases (BLAST) and compared with the other sequences to analyze the bacterial class. The identity of the sequence obtained was established by comparing with the gene sequences in the database using BLAST software provided by the National Center for Biotechnology Information Service (NCBI) http://www.ncbi.nlm.nih.gov after treatment and recorrection (Kerbauy *et al*., 2011).

Standardization of inoculum for lipase production

A loopful of bacteria culture was taken from 24 h old culture and aseptically inoculated into 50 ml (250 ml Erlenmeyer's flask) of medium, consisting of 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.3% NaCl, 0.2% K_2HPO_4, and incubated at 30° C for 24-72 h on a rotary shaker at 150 rpm. Samples were taken at 12 h interval until 72 h for cell density measurement by taking the optical density at 600 nm against the cell-free control.(Stevenson *et.al*., 2016)

Lipase production medium

The enzyme production was carried out in a 250 ml Erlenmeyer's flask containing 50 ml medium. The production medium was made up of 0.3% peptone, 0.1% yeast extract, 0.05% NaCl, 0.05% $CaCl_2.2H_2O$, 0.1% gum acacia and 1% olive oil. The pH of the medium was adjusted to 7.5 and the medium was sterilized at 121° C and 1.05kg/cm^3 for 15 min. The medium was inoculated with 10^4 cell/ml of the 24 h old cultures and incubated at 30° C for 72 h on a rotary shaker at 150 rpm.

At the end of the fermentation the culture broth was centrifuged at 10,000 rpm at 4° C for 20 min. The cell free supernatant was used as crude enzyme.(Wang *et.al*., 2012).The enzyme activity and the protein concentration were determined as described below for lipase activity measurement.

Assay of Lipase Activity

Lipase activity was determined by spectrophometric analysis at 30° C using Para-Nitrophenol palmitate (pNPP) as substrate (Vorderwiibecke *et. al*., 1992). At the end of the fermentation the culture broth was centrifuged at 10,000 rpm at 4° C for 20 min. The supernatant was used as crude enzyme for lipase activity measurement. The reaction mixtures consist of 700 µl *p*NPP solution and 300 µl of lipase solution. The *p*NPP solution was prepared by adding the solution A (0.001 g *p*NPP in 1ml isopropanol) into solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50 µl Triton X-100 and 9 ml of 50 mM Tris-HCl buffer, pH 8) with stirring until all was fully dissolved. Then the progress of the reaction was monitored by the change of the absorbance at 410 nm at an interval of 15 seconds over a 3-min period at 30° C using a spectrophotometer. Change in absorbance was used in calculating the enzyme activity. A non enzymatic reaction was taken as the control for this experiment replacing the enzyme with distilled water. One unit (1U) was defined as that amount of enzyme that liberated 1µmol equivalent of *p*NPP per ml per min (ε: 15000 cm^2/mol) under the standard assay conditions.

Estimation of Protein Concentration

Protein concentration was measured routinely according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein.

Optimization of culture conditions for production of lipase

The optimum pH for the production of lipase was determined by varying the pH of the basal medium from 6.0 to 9.0.This was sterilized at 121° C for 15 min at a pressure of 1.05 kg/cm^3 after which it was inoculated with a standardized inoculums and incubated at 30° C for 72 h with agitation at 150 rpm in an incubator shaker. The optimum temperature of lipase production was determined by inoculating the isolated lipolytic strain into the lipase production medium of the optimised pH and incubated at different temperatures such as 25°C-55°C. for 72 h with agitation at 150 rpm in an incubator shaker. The effect of carbon source on the production of lipase, different carbon sources including olive oil, palm oil, coconut oil, vegetable oil and effluent from cocoa processing plant at 1% (v/v) concentration were added to the medium. Different nitrogen sources which included ammonium sulphate, ammonium chloride, ammonium ortho phosphate, urea and yeast extract were supplemented in the production medium (50ml). All the media were previously adjusted to pH 7.5 and sterilized at 121° C and 1.05 kg/cm^3 for 15 min. (Olutiola *et.al*., 1991).

RESULTS

Lipase producers gave positive results on tributyrin agar plate indicated by the lipolytic zone designating free fatty acid release. Four bacteria isolates showed lipolytic activities and were assessed both qualitatively (agar plate cultures) using tributyrin agar and quantitatively (liquid cultures) using olive oil as sole carbon and energy source. The corresponding results are presented in Table 1 and Figure 1. Following the results of this primary screening this research work proceeded by selecting a strain that secreted highest average extracellular lipolytic activity in liquid culture and produced intense clear zone of hydrolysis around it on Tributyrin agar. Isolate SA fulfilled these criterions. From microscopic appearance and the biochemical tests, the isolated strain SA was identified as *Brevundimonas* sp. The almost complete 16S rRNA gene of the lipase producing bacteria that was isolated from cocoa processing plant effluent was sequenced and was identified to be *Brevundimonas diminuta.*

This sequence data has been submitted to the NCBI/GenBank databases under accession No. KX 349741. The 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level.. Other researchers have isolated and identified lipase producing bacteria to be *Bacillus* sp (Mukesh., *et al.,* 2012) *P. aeruginosa* (Madan and Mishra, 2010, Mobarak-qamsari *et al*.,2011) *Pseudomonas fluorescens* (Yang *et al*., 2009),*Staphylococcus* sp (Sahu and Martin, 2011) from various environmental sources. *Brevundimonas dimunita* is a proteobacteria with the old name *Pseudomonas dimunita* (NCBI). Mobarak-Qamsari *et al.,* 2011 has identified lipase producing *Pseudomonas aeruginosa using* 16S rRNA sequence analysis. Akanbi *et al*.,(2010) has identified lipase producing *Bacillus cereus* using 16S rDNA sequence analysis. To our knowledge there are no published reports on lipolytic activities from *Brevundimonas dimunita* isolated from cocoa processing plant effluent. The biomass increased up to 60 h, after that there was a decline in its amount. Extracellular lipase production exhibited a similar increase with biomass and remains in higher amount in medium upto 72 h with the production at stationary phase (Fig.1). The lipase activity appeared to couple with growth. The enzyme production was found to be maximum when the cell population entered into the stationary phase suggesting that enzyme secretion is growth associated. The lipase activity was observed to start soon after beginning of incubation, and the crude enzyme extract obtained after 72 h of growth in the culture medium exhibited highest activity (35.85 ± 0.5 Units/ml) at 72 h (Fig.1). The maximum lipase production was obtained when the initial pH was 7.5 (47.33±1.26 Units/ml). At pH 6.0 the activity of lipase was 0.01± 0.01 Units/ml (Fig.2). The influence of temperature on lipase production was studied in the range of 20-50°C while keeping all the other conditions constant. Like other mesophilic bacteria, *Brevundimonas dimunita* producing lipase was best grown when incubated at 35°C (Fig.3). In most of the organisms, both the organic and inorganic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components (Akcan, 2011).

Various nitrogen sources were used, of which, organic sources such as peptone and yeast extract had profound influence over the enzyme production when compared to inorganic nitrogen sources *Brevundimonas dimunita* grew well in all the nitrogen sources used in this study. Among the different nitrogen sources tested, peptone was found to be the best among all the nitrogen sources tested with a production of 47.33±1.26 Units/ml (Figure 4).

Table 1: Zones of clearance of the isolates on Tributyrin Agar Plate measured in (mm)

Time (h)/Isolate	12	24	36	48	72
RA	0.00	0.00	1.53 ± 0.06	2.00 ±0.1	2.03 ±0.06
RB	0.00	0.00	1.46 ±0.06	2.07 ±0.06	2.56 ±0.12
RC	0.00	0.00	1.76 ±0.06	2.46 ±0.06	2.90 ±0.00
SA	0.00	0.00	1.63 ±0.06	2.17 ±0.06	2.60 ±0.00
CONTROL	0.00	0.00	0.00	0.00	0.00

The values are mean ± SD of three replicates

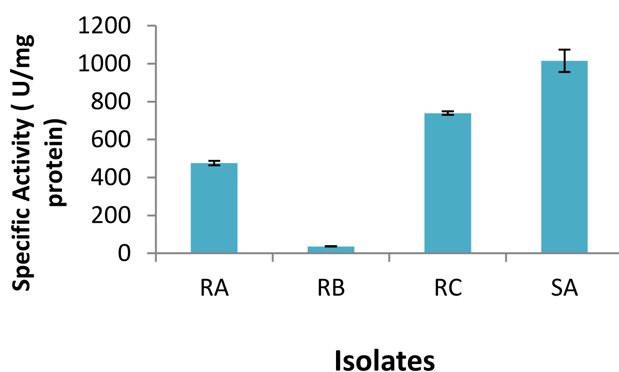

Figure 1: Comparison of the Lipolytic Activities of Bacteria Isolates from Cocoa Processing Plant Effluent

Table 2: Sequences Producing Significant Alignments with *Brevundimonas diminuta*

Description	Max score	Total score	Query cover	E value	Ident	Accession
B.diminuta 16S rRNA gene	1107	1107	97%	0.0	99%	X87288.1
Brevundimonas subvibrioides ATCC 15264, complete genome	1051	2098	97%	0.0	97%	CP002102.1
Brevundimonas subvibrioides ATCC 15264 strain ATCC 15264 23S ribosomal RNA, complete sequence	1046	1046	97%	0.0	97%	NR_076765.1
Caulobacter segnis ATCC 21756 strain ATCC 21756 23S ribosomal RNA, complete sequence	917	917	98%	0.0	93%	NR_076742.1
Caulobacter crescentus CB15 strain CB15 23S ribosomal RNA, complete sequence	917	917	98%	0.0	93%	NR_076169.1

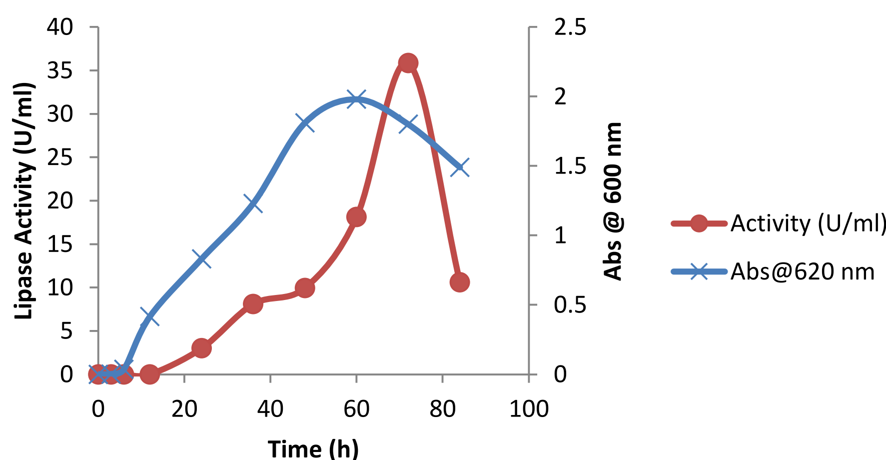

Figure 2: Growth and Lipase Production Curve at 30ºC and pH 7.5

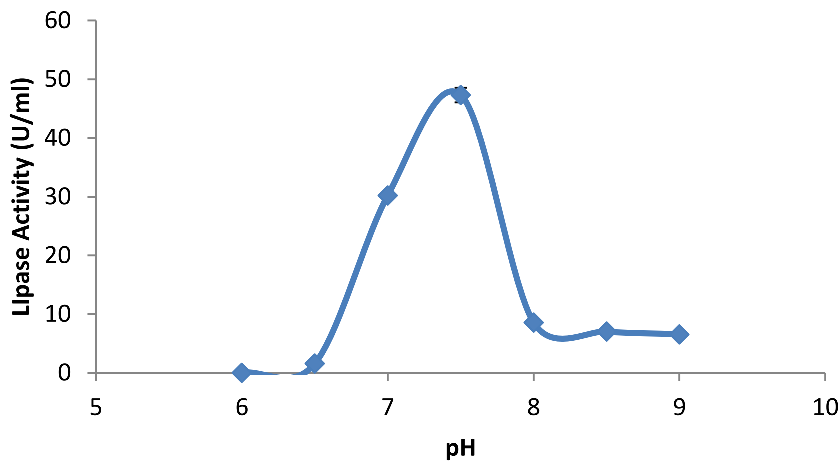

Figure 3: Effect of pH on the Production of *Brevundimonas diminuta* Crude Lipase

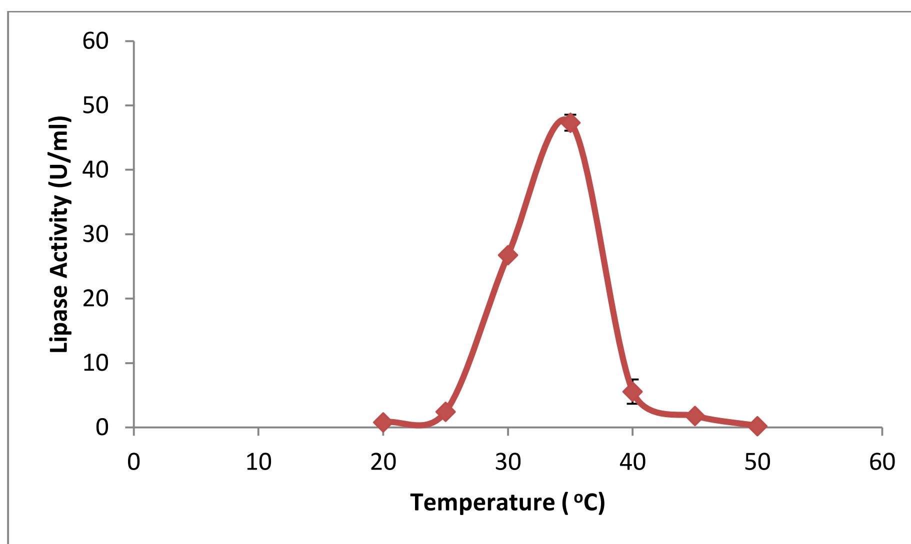

Figure 4: Effect of Temperature on the production of *Brevundimonas dimunita* crude Lipase.

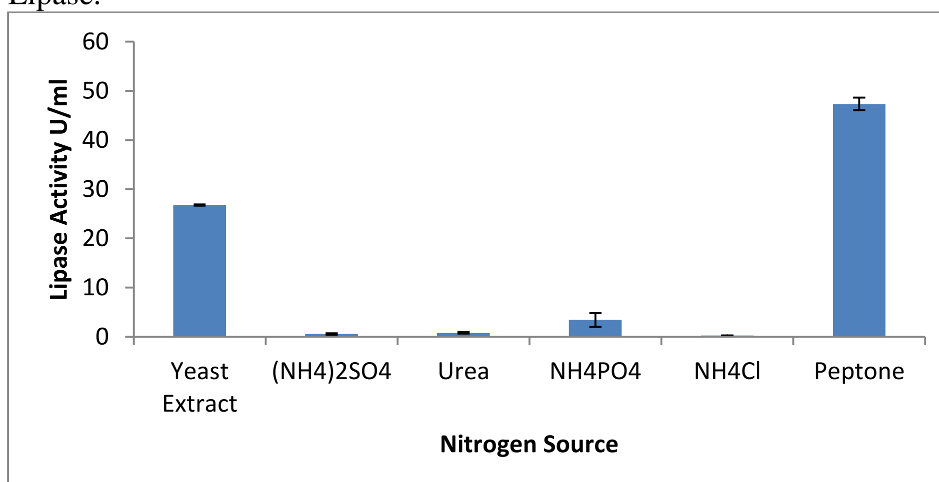

Figure 5: Effect of Nitrogen Source on the production of *Brevundimonas dimunita* crude Lipase.

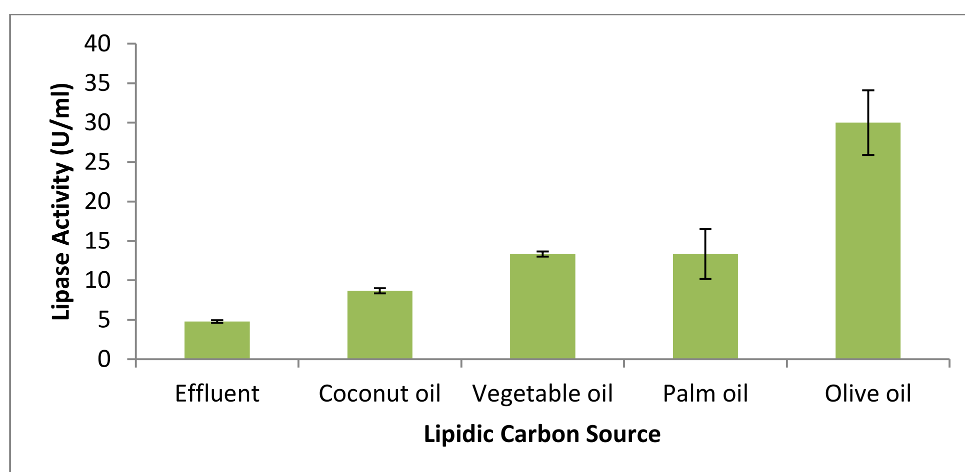

Figure 6: Effect of Lipidic Carbon Source on production of *Brevundimonas dimunita* crude lipase.

DISCUSSION

Nigeria is the world's 4th largest producer and exporter of cocoa. Paradoxically, over 90 per cent of the cocoa produced is exported One of the on-going debates in the sector is the attitude towards processing of cocoa into products for export as opposed to the mere export of raw beans. This value addition of processing cocoa into its derivates such as cocoa butter and liquor for the international and local markets has encouraged the springing up of cocoa processing company in south western part of the country and the oily effluent being discharge from this plant could be source of lipase producing microorganisms. The isolation of lipase producing bacteria is a confirmation that the effluent contains substrate for the microorganisms to flourish. From microscopic appearance and the biochemical tests, the isolated strain SA was identified as *Brevundimonas* sp. Other researchers have isolated and identified lipase producing bacteria to be *Bacillus* sp.(Mohan *et al*.,2008, Mukesh., *et al.*, 2012) *P. aeruginosa* (Madan and Mishra, 2010, Mobarak-qamsari *et al*.,2011) *Pseudomonas fluorescens* (Yang *et al*., 2009),*Staphylococcus* sp (Sahu and Martin, 2011) from various environmental sources. When compared to morphological and biochemical characterization methods,16S rRNA analysis is found to be the novel and accurate method for identifying unknown species The BLASTN program analysis clearly demonstrated that strain SA was a member of the genus *Brevundimonas* and exhibited maximum similarity with the 16S rRNA sequence of *Brevundimonas dimunita* 16S rRNA (Accession No X87288.1) (99% sequence similarity). 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level. *Brevundimonas dimunita* is a proteobacteria with the old name *Pseudomonas dimunita* (NCBI). Mobarak-Qamsari *et al.,* 2011 has identified lipase producing *Pseudomonas aeruginosa using*16S rRNA sequence analysis. Akanbi *et al*.,(2010) has identified lipase producing *Bacillus cereus* using 16S rDNA sequence analysis. To our knowledge there are no published reports on lipolytic activities from *Brevundimonas dimunita* isolated from cocoa processing plant effluent.

Some environmental factors also influenced the growth of the organisms as well as maximum production of enzymes which may be at certain optimum temperature, pH, time and so on (Immanuel *et al*., 2006). Growth rate and enzyme production is a function of incubation time (Akcan, 2011). The production of lipase by *Brevundimonas dimunita* was correlated with growth curve/ biomass. Similar results were obtained by Mukesh-kumar *et al*., 2012 while working on the production of lipase from *Bacillus* Sp. Maximum lipase production was also

reported at 72 h by Bora and Bora (2012). A decrease in lipase activity was apparent during the late stationary phase presumably due to the decrease in the amount of nutrients in the medium, denaturation of the enzyme and the presence of proteases in the culture medium. (Bora and Bora, 2012), it could also be due to enhanced biomass, which would result in a decrease in metabolic activity (kashyap, 2002). A balance between the proliferating biomass and available substrate materials would yield maximum enzyme (Pandey *et al*., 2000). A comprehensive review of all bacterial lipase done by Gupta, *et al*. (2004), states that maximum activity of lipases at pH values higher than 7 has been observed in many cases. Bacterial lipases have a neutral or alkaline optimum pH. Medium pH is very important in nutrients absorption and growth of bacteria, stimulation of enzyme production via signaling pathways and release of extra cellular enzymes based on proteolytic mechanism of signal peptidases (Palekar *et al*., 2000). So maintaining the pH value is very important.

Mukesh-kumar *et al.*, 2012 showed that lipase was produced at maximum level when maintained at temperatures of 35°C.The optimum temperature for lipase production corresponds with the growth temperature for lipase production (Gupta *et al* 2004). The temperature regulates the enzyme synthesis at mRNA transcription level and probably translation levels of proteins, thus increases the stability of the proteins and also the production. Bora and Bora (2012). Among the nitrogen sources used peptone gave the best yield, the reason to this effect is the release of NH_4^+ ions from peptone which stimulates the growth and at the same time increase the enzyme yield because of its protease inhibiting nature at low concentration. Bora *et al.*, 2012. The results obtained are in agreement with that reported by Gulati *et al.* (2005) where peptone was found to be the best nitrogenous source for lipase production. Similar findings were also reported in *Pseudomonas* sp (Mobarak-Qamsari *et al* 2011). The lipase production was found to be low with inorganic nitrogen sources. Carrazoco-palafox *et.al*.,2018 reported lipase require co-factor such as Calcium ion for its expression

The major factor for the expression of lipase activity has always been the carbon source, since lipases are by and large inducible enzymes (Lotti *et al*., 1998). The enzyme production level markedly increased in the presence of lipid carbon substrates and represents the key mechanism for making a fatty acid carbon source available to the cell during growth on such compounds. The enzyme is probably induced by these substrates (Abdel-Fattah,2002:Kaushik *et al.,*2006) as fatty acid esters are efficient effectors leading to a significant increase in the enzyme level relative to that obtained with nonlipid substrates such as glucose. Carbon source may have either repressing or inducing effect on enzyme production (Gupta *et al*., 2010).The carbon source is very essential for the effective production of the enzyme. Olive oil (30.00±4.09 Units/ml) (Figure 5) was found to be the most effective carbon source for maximum production of enzyme. This is correlated with the findings of Mobarak-Qamsari *et al.,* 2011. Most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase production (Abdel-Fattah, 2002: He and Tan, 2006: Kaushik *et al*., 2006). In this study, it was found that *Brevundimonas dimunita* was able to utilize a number of other vegetable oils as well as effluent from cocoa processing plant as carbon sources. (Figure 5).This effluent was rich in oil and suspended solids which may contain organic and inorganic nutrients. The substrate that provides the nutrients needed by the microorganism is considered as an ideal substrate. However, some of the nutrients may be available in sub optimal concentrations or even absent in the substrates. In such case, it would become necessary to supplement them externally with other nutrients such as carbon and nitrogen (Padey *et al*., 1995)

The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production cost (Olaniyi *et al*., 2010). The carbon sources accounted for 50% of the production cost in lipase production (Miranda *et al*., 1999). Other researchers have used various cheap substrates as carbon source Peanut oil cake (Annamalai *et al*., 2011), Groundnut oil cake (Manoj *et al*., 2010), Palm oil effluent (Geoffry and Achur 2018, Salihu *et al*., 2011), oil mill effluent (Mukesh-kumar *et al*., 2012).Agro-industrial by products are available in large amounts and they have been used for the production of several enzymes (EL-Helow and EL-Ahwany,1999; Howard *et al*.,2003).

Huge tonnage of agriculture wastes and agro-industrial products are generated annually all over the world. From production, processing and even consumption there are great varieties of left over which creates problems of disposal, use of effluent from cocoa processing plant to substitute other vegetable oil may be an alternative means of reducing high cost of lipase production, also utilization may be a better way of disposing agro industrial by products to ensure environmental protection.

CONCLUSION

This study revealed that lipase producing bacteria was isolated from cocoa processing plant effluent and this effluent was utilize by the organism as sole carbon source which could serves as a renewable source for microbial growth medium for lipase production. This is an indication that the effluent contains substrate for the growth of these organisms isolated from this waste.

Acknowledgement

The Authors are most grateful to Cadbury Nig plc, Igba, Ondo state and Mr, S.A Akinbinu for their contributions towards collecting samples from for this work. We are also grateful to Mrs. Temitope Owoeye Department of Bioscience, International Institute of Tropical Agriculture Ibadan for genome sequencing of the organism.

REFERENCES

Abdel-Fattah Y. R. (2002). Optimization of thermostable lipase production from a thermophilic *Geobacillus sp*. using Box-Behnken experimental design. *Biotechnol. Lett.*; 24: 1217-1222.

Abu, E. A., Ado, S. A. and James, D. B. (2005). Raw starch degrading amylase production by mixed culture of *Saccharomyces cerevisae* grown on sorghum pomase. *African journal of Biotechnology*., 4: 785-790

Akanbi, T. O. Kamaruzaman, A. L. Abu Bakar, F. Sheikh Abdul Hamid, N. Radu, S. Abdul Manap, M.Y. Saari, N. (2010). Highly thermostable extracellular lipase-producing *Bacillus* strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing *International Food Research Journal*, 17,45-53

Akcan, N., (2011). High level production of extracellular galactosidase from *Bacillus licheniformis* ATCC 12759 in submerged fermentation. *African Journal of Microbiology Research*, 5: 4615-4621.

Annamalai, N. Elayaraja, S. Vijayalakshmi, S Balasubramanian, T. (2011). Thermostable, alkaline tolerant lipase from *Bacillus licheniformis* using peanut oil cake as a substrate. *African Journal of Biochemistry Research,* 5(6), 176-181

Balakrishnan, P., Thamaraichelvan, R., Erecelot, N. Dhannya G. and Chidanibaram K. (2011). Production of lipase enzyme from *Lactobacillus* sp. and its application in the degradation of meat. *World Appl. Sci. Journal.* 12(10): 1798-1802.

Bora, L. and Bora, M**.** (2012). Optimization of extracellular thermophilic highly alkaline lipase from thermophilic *Bacillus* sp isolated from hotspring of Arunachal Pradesh, India. *Brazilian Journal of Microbiology*: 30-42

Bradford, M. M., (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein

utilizing the principle of protein-dye binding, *Anal. Biochem*., 72: 248-254.

BurcuBakir, Z.; Metin, K. (2017) Production and characterization of an alkaline lipase from thermophilic Anoxybacillus sp. HBB16. Chem. Biochem. Eng. Q., 31, 303–312.

Dutta, S. And Ray, L. (2009). Production and characteristics of an alkaline thermostable crude Lipase from an isolated strain of *Bacillus cereus* C7 *Appl. Biochem. Biotechnol.,* 159:142-154.

Carrazco-Palafox Jair Rivera-Chavirab Blanca E., Ramírez-Bacac Norma, Manzanares-Papayanopoulos Luisa I., Nevárez-Moorillón Guadalupe V.(2018) Improved method for qualitative screening of lipolytic bacterial strains MethodsX. 5 68–74

Colla LM, Primaz AL, Benedetti S, Loss RA, de Lima M, Reinehr CO, Costa JAV. (2016) Surface response methodology for the optimization of lipase production under submerged fermentation by filamentous fungi. Braz J. Microbiol;47(2):461–7.

EL-Helow, E. R. and EL-Ahwany, A. M. (1999). Lichenase production by catabolic repression-resistant *Bacillus subtilis* mutants: Optimization and formulation of an agro-industrial by-product medium. *Enzyme microbiology and Technology.* 24:325-331.

Geoffry, Kiptoo. Achur Rajeshwara N (2018) Optimization of novel halophilic lipase production by Fusarium solani strain NFCCL 4084 using palm oil mill effluent Journal of Genetical Engineering and Biotechnology. 16 327-334

Gupta, R., Gupta, N. and Rathi, P. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology* 64: 763-781.

He, Y. Q, and Tan. T. W. (2006). Use of response surface methodology 13 to optimize culture medium for production of lipase with *Candida sp. J. Mol. Catal.* B-Enzym., 43, 99-125

Howard, R. L., Abotsi, E. E. Jansen, E. L. and Howard S. S. (2003). Ligno cellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of biotechnology*., 2(12):602-619

Immanuel, G., Dhanusa, R., Prema, P. and Palavesa, A. (2006). Effects of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology*, **3(1):** 25-34.

Jiafulin, Q., Jing L., Zhong A., Xin R., Hui X., Dai, R. and Yicao, L. (2012). Bacterial diversity of lipase producing strains in different Soils in Southwest of China and characteristics of lipase. *Afr. J. Microbiol. Res* 6 (16): 3797-3806.

Kashyap P, Sabu A, Pandey A, Szakacs G.(2002) Extra-cellular Lglutaminase production by *Zygosaccharomyces rouxii* under solidstate fermentation. *Process Biochem.*; 38: 307– 312

Kaushik, R., Saran, S., Isar, J., Saxena, R. K. (2006). Optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus. J. Mol. Catal B-Enzym.,* 40: 121-126.

Kerbaury, G.; Perugini, M.R.E.; Yanauchi, L.M. and Ogatta, S.F.Y.(2011).Vancomycin-Depended *Enterococus faecium* Van A:characterization of the first case isolated in a university Hospital in Brazil. Brazi. J. Med. Biol. Res., 44(3), 253-257.

Long, Z. D., Xu, J. H., Zhao, L. L., Pan, J., Yang, S. and Hua, L. (2007).Over expression of *Serratia marcescens* lipase in *Escherichia coli* for efficient bioresolution of racemic ketoprofen .J. *Mol.Catal. B. Enzym.,* 27**:**105-110

Manoj S., Kumar S., Neha S., Krishnan K.,(2010) Lipase production by *Bacillus subtilis* OCR4 in solid state fermentation using groundnut oil cakes as substrate *Current Research Journal of Biological Sciences*, 2 (4), 241-245

Maytham A. A. and Hanaa J.A. (2016) Isolation and Identification of Lipase Producing Bacteria From Oil-contaminant Soil Journal of Biology, Agriculture and Healthcare 6(20)

Minisi, S. M.,. Louw M. E and Theron, J. (2005). Cloning and characterization of a carboxyl esterase from *Bacillus coagulans* 81-11 *Curr. Microbiol*., 50: 196-201

Mobarak-Qanisari, E., Kasra-Kermanshahi R, and Moosavi-nejad Z (2011). Isolation and identification of a novel, lipase-producing bacterium, *Pseudomonas aeuginosa* KM 110 *Iran J. Microbol*.3 (2): 92-98.

Mukesh, D. J., Rejitha R., Balakumaran, M. D., Immaculate N. and Kalaichel, P., (2012). Production, optimization and purification of lipase from *Bacillus* sp. MPTK 912 Isolated from oil mill effluent. *Adv. Appl. Sci. Res*., 3(2): 930-938.

Olutiola, P. O., Famurewa, O. and Sonntag, H. G. (1991). An Introduction to General Microbiology; A practical approach. Hygiene-Institut Der Universitat Heidelberg, Germany.

Palekar, A. A. Vasudevan, P. T. and Yan, S. (2000).Purification of lipase: A review. *Biocatal Biotransform.,* 18: 177-200.

Pandey A., Ashakumary, L. and Selvakumar, P. (1995). Copra wastes a novel substrate for solid state fermentation. *Bioresource Technology***.**51:217-220

Pandey, A., Selvakumar, P., Soccol, C. and Nigam, P. (2000).Solid State fermentation for the production of industrial enzymes *.Process biochemistry*.36:1153-1167.

Quyen, D. T. Schmidt-Dannert C. and Schmid, R. D. (2003). High -level expression of a lipase from *Bacillus thermocatenulatus* BTL2 in *Pichia pastoris* and some properties of the recombinant lipase. *Protein Expr. Purif.*, 28:102-110

Sahu, G. K. and Martin M.(2011). Optimization of growth conditions for the production of extracellular lipase by bacterial strains from dairy industry effluents Research Article, *Biotechnol. Bioinf. Bioeng*., 1(3):305-311

Salihu, A. Alam, Z. AbdulKarim, M. Salleh, H. (2011). Suitability of using palm oil mill effluent as a medium for lipase production. *African Journal of Biotechnology*, **10** (81), 18704-18708

Sambrook, J. and Russell,D.W.(2001). Molecular cloning. A Labratory Manual, 3rd edition. cold spring Harbor Laboratory press, cold spring Harbor NY,USA.

Sangeetha, R. Geetha, A. and Arulpandi, I. (2010a). Concomitant production, purification and characterization of protease and lipase by *Bacillus licheniformis* VSGI. *Brazilian J. Microbiol.*, 41: 179 - 185

Sangeetha. R., Geetha A. and Arulipandi I. (2010b). Concomitant production, partial purification and characterization of a serine protease and a proteolysis-resistant Metallolipase from *Bacillus Pumilus* SG2. Z. *Naturfosch.*, 65:61-65

Subhajit R. (2012). Fermentative production of an alkaline extracellular lipase using an Isolated bacterial strain of *Serratia* Sp (C4) *J. Microbiol. Biotech. Res.* 2(4): 545-557.

Stevenson, K., Mcvey A.F., Clark B.N Swain P.S. and Pilizota T. (2016). General calibration of microbial growth in microplate readers. *Sci. Rep.* **6**, 38828; doi: 10.1038/srep38828

Vorderwiilbecke, T., Kieslich, K., Erdmann, H. (1992). Comparison of lipases by different assays. *Enzyme Microb Technol*., 14:631–9.

Wang, X., Yu, X. and Xu, Y. (2009). Homologus expression, purification and characterization of a novel high alkaline and the thermal stable lipase from *Burkholderia cepacia* ATCC 25416. *Enzym Microb. Technol*., 45:94-102

Wang H.; Zhong S.; Ma H.; Wei J(2012).Screening and characterization of a novel alkaline lipase from *acinetobacter calcoaceticus* 1-7 isolated from bohai bay in china for detergent formulation Brazilian Journal of Microbiology : 148-156

Yang, J., Zhang, B. and Yan, Y. (2009).Cloning and expression of *Pseudomonas fluorescens* 26-Z lipase gene in *Pichia pastoris* and characterizing for transesterification *Appl. Biochem. Biotechnol.* 159: 355-365