

Original Research Article

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Bioaccumulation of Heavy Metals and Optimization of Lipase Production by *Lysinibacillus sphaericus* Strain ODE16_EKITI Isolated from Domestic Oil-Rich Wastewater

B.I. Aderiye*, A.A. Adebayo and Bello, Mustapha

Department of Microbiology, Faculty of Science, Ekiti State University, Ado-Ekiti, Nigeria

*Corresponding author

ABSTRACT

In a bid to remediate the menace caused by the presence of lipids in many biological wastewater treatment systems, various researchers have focused on a way to explore lipase production by different microorganisms for this purpose. Some cultural parameters were hence optimized while their influences on the production of lipase by *Lysinibacillus sphaericus* strain ODE16_EKITI in an oil-rich wastewater were enumerated using standard laboratory techniques. Inorganic content of the sample and lipase production were estimated using atomic absorption spectrophotometry and lipase colorimetry respectively. Magnesium (Mg) constituted the highest metal concentration of 54.5mg/L while least concentration of calcium (Ca) (13.4mg/L) in the wastewater sample and iron (Fe) as the only heavy metal detected in high concentration (31.6mg/mL). No significant difference in the accumulation potential of the consortium that took up 77.85% of phosphate and that of the organism, which accumulated 77.38% of phosphates in the sample. The maximum lipase activity (5.111mMol) was recorded after 5 days' incubation, with the production rate of 0.78mM/min daily and the microbial growth rate of 0.292 mg/day. The organism had the highest growth (OD 0.298) and lipase production (6.667mMol) with oil concentration of 2.5ml/L at optimum temperature of 30°C. *L. sphaericus* also showed the highest growth (with OD 0.301) and lipase production of 6.67mMol in the presence of magnesium ion (Mg²⁺). The present work has offered us the chance to explore a lipolytic bacterium, *Lysinibacillus sphaericus* strain ODE16_EKITI that can produce lipase ranging between 5.1mMol to 6mMol at 30°C and 2.5% palm oil concentration after 5 days. This organism also showed potentials for bioaccumulation of some heavy metals. *L. sphaericus* strain ODE16_EKITI is thereby presented as a promising tool for biotechnological exploitation in remediation of domestic oil-rich wastewater.

Keywords

Bioaccumulation, *Lysinibacillus sphaericus* strain ODE16_EKITI, lipase production, remediation, Optimization.

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Introduction

Globally, one of the major environmental pollutions detrimental to the ecosystem, including human communities, is a consequence of either deliberate and sometimes regulated (e.g. Industrial emissions) or accidental and mostly unavoidable (e.g. chemical spills) release of

quantities of oil-rich effluents, resulting from human activities. These pollutants are majorly from industries (i.e. food, chemical, pharmaceutical, cosmetic, leather and detergent), restaurants, slaughter house, and households (Shukia and Desai, 2016). Apparently, all anthropogenic activities end

with wastewater, although cannot be quantified but has been estimated that, globally, about 80% of wastewater from human settlements and industrial sources is discharged to the environment without treatment (UNEP and UN-Habitat, 2010). Wastewater effluents from restaurants have attracted interest because of the high concentration (>100 mg/L) of stable organic compounds; lipids (fats and oils), important component of vegetable oil (Prasad and Majunath, 2010; Odeyemi *et al.*, 2013a), that cause major problems in wastewater treatment process, by forming layers on water surface and decrease oxygen transfer rate in the aerobic process (Facchin *et al.*, 2013).

Biological treatment comprising of bioremediation and manipulation of microbes, has however been and remains the most efficient, cost effective, ecofriendly and easy approach to stop this menace. In view of this, several attempts have been made to isolate lipase-producing bacteria (Dharmusthii and Luchai, 1999; Kambovrova *et al.*, 2003; Trichel *et al.*, 2010; Facchin *et al.*, 2013; Odeyemi *et al.*, 2013a; 2014; Coronado *et al.*, 2015) and fungi (Maier *et al.*, 2003; Odeyemi *et al.*, 2013b). In recent times, the interest of researchers in microbial lipase is heightened, not only for its biodegradative and bioremediating capabilities, but also its industrial applications including in, production of surfactant (Bora and Kalita, 2007), cheese ripening and flavour enhancement (Mukesh Kumar *et al.*, 2012), and production of chiral drugs (Reddy and Pallavi, 2012). Furthermore, microbial lipases which includes important biotechnologically valuable enzymes degrade these wastes, mainly because of their stability, selectivity, broad substrate specificity, versatile properties for application and ease of mass production (Shukia and Desai, 2016). The search for new lipase with different characteristics becomes so eminent in recent time, because their specificity is still a

limiting factor, as different properties could be required for a particular application. More so, the production of lipases by a microorganism is influenced by several extrinsic and intrinsic factors (Pallavi *et al.*, 2014). In a previous study by Odeyemi *et al.*, (2014), *Lysinibacillus sphaericus* strain ODE16_EKITI isolated from a vegetable oil-rich wastewater was reported as an efficient lipase producer. Efforts were therefore made in this present research to optimize and enumerate the influence of some cultural parameters for maximum production of lipase by the bacteria, *Lysinibacillus sphaericus* strain ODE16_EKITI.

Materials and Methods

Source and collection of wastewater sample

Wastewater sample was collected aseptically from different sampling points including wash-hand basin, dish washing and dish rinsing bowls at Falegan restaurant situated along the Ekiti State Secretariat road, Ado-Ekiti, Nigeria. The wastewater contained food remnants and cleaning soap solution. The sample was collected using sterile 250mL Erlenmeyer bottle, transported in ice ($-5^{\circ}\text{C}\pm 1^{\circ}\text{C}$) to the Microbiology laboratory of the Ekiti State University, Ado-Ekiti until when needed.

Preparation of wastewater sample for mineral analysis

About 5ml of concentrated HCl was added to 250mL of wastewater sample and evaporated to 25ml. The concentrate was transferred to a 250mL Erlenmeyer flask and made up to the 50mL with distilled de-ionized water (APHA, 1998; Odeyemi *et al.*, 2013b).

Mineral analysis of wastewater sample

Mineral analysis of wastewater sample was carried out in accordance with AOAC (2006)

description; heavy metals such as lead (Pb), copper (Cu), manganese (Mn), nickel (Ni), iron (Fe) and Zinc (Zn) were analysed using Atomic Absorption Spectrophotometer (AAS) (PYE Unicam Sp 9, Cambridge, UK), while potassium, sodium, magnesium and calcium were analysed using the Flame Photometer (BUCK 2010 VGP).

The amount of active ingredients (silica, soda ash, surfactant and phosphate) in the wastewater was determined with a Spectronic 20 colorimeter (Gallenkamp, UK) as described by AOAC (1990).

Source of bacterial isolate

The bacterial isolate, *Lysinibacillus sphaericus* strain ODE16_EKITI used in this study has been described and molecularly characterized by Odeyemi *et al.*, (2014) and kept on an agar slant in the Department of Microbiology Laboratory, Ekiti State University, Ado Ekiti, Nigeria.

Preparation of inoculum

Bacterial culture was streaked on to freshly prepared nutrient agar (Lab M, UK) plate, a distinct colony was picked and inoculated into agar slant, and was incubated for 24 hours. Slanted culture was stocked at 4°C. A loopful of cells from a freshly grown slant was transferred into a 250 mL Erlenmeyer flask containing 50 mL of minimal media (without agar); 1.5% K₂HPO₄, 0.05% MgSO₄, 1.0% (NH₄)₂SO₄, 0.2% CaCl₂, 0.2% FeSO₄ in 1 litre of distilled water and incubated at 30°C in a shaking incubator at 180 rpm for 24 h (Gupta *et al.*, 2004).

Lipase assay medium (0.2% v/v palm oil, 1.5% K₂HPO₄, 0.05% MgSO₄, 1.0% (NH₄)₂SO₄, 0.2% CaCl₂, 0.2% FeSO₄ and 0.5% yeast extract) at pH 7 was used for enzyme assay.

Estimation of microbial growth

Aliquots of the culture suspension were withdrawn at specific conditions and the cells were harvested after centrifugation (10,000 rpm at 4°C for 30 mins). The cells were macerated with 1 mL normal saline and the absorbance was measured at 600 nm with Atomic Absorption Spectrophotometer (PYE Unicam Sp 9, Cambridge, UK) to determine the optimum growth (Gupta *et al.*, 2004).

Estimation of lipase activity

Crude enzyme (1 mL) obtained as cell free supernatant after centrifugation at 10,000g for 30 min (usually stored at 4°C until needed) was assayed for lipase activity using colorimetric method described by Lotrakul and Dharmsthiti (1997).

Lipase activity was determined based on the cleavage of p-nitrophenylpalmitate (p-NPP) at pH 8.0. The reaction mixture contained 180 µL of solution A (0.062 g of p-NPP in 10 mL of 2-propanol, sonicated for 2 min before use), 1620 µL of solution B (0.4% triton X-100 and 0.1 % Arabic gum in 50 mM Tris-HCl, pH 8.0) and 200 µL of properly diluted enzyme sample. Optical density of the solution was measured at 410 nm wavelength after incubation for 15 min at 37°C. One unit of lipase activity was defined as the amount of lipase releasing 1 µmol of p-nitrophenol (p-NP) per minute by 1 mL of enzyme (Cho *et al.*, 2000; Shukia and Desai, 2016).

Optimization of conditions for microbial growth and lipase production

Various cultural parameters affecting enzyme production were independently optimized. In all cases, 1 mL of 1.86 x 10⁶ CFU/mL bacterial suspension was added to the medium (Gupta *et al.*, 2004).

Incubation periods

Microbial load and lipase production were estimated for 6days at 24hours interval of incubation.

Oil concentrations

Palm oil was inculcated into the inoculated minimal medium at different concentrations ranging from 0.50% to 4.0% (v/v) and the microbial growth and lipase production were estimated.

Temperatures

The optimum temperature for optimal growth and lipase activity was determined by incubating the assay solution at different temperatures between 20°C and 50°C with readings taken at 10°C intervals for 24 hours.

Metals

Effect of metals on the growth and lipase production by *L. sphaericus* strain ODE16_EKITI was examined. 1mg of metals such as sodium (Na), potassium (K), magnesium (Mg) and iron (Fe) were inculcated into the assay suspension and incubated for 1hour at 30°C before spectrophotometric and colorimetric assay (Mukesh Kumar *et al.*, 2012).

Bioaccumulation of inorganic compounds

Lysinibacillus sphaericus strain ODE16_EKITI was used separately in the sterile medium and in combination with indigenous organisms in the wastewater to test their accumulation abilities under non-agitation condition (Ray, 2009). By heating 500ml of wastewater sample (in sterile round bottom flask) in a water bath (Model CS200) at 47°C, fats and oils in the sample were homogenised and uniformly distributed

(Huges and Christy, 2003). The experiment was set in three categories; control, which was sterilized and not inoculated; sample A, which was sterilized and inoculated with *L. sphaericus*; and sample B, which was non-sterile and inoculated. Sterilization of control and sample A was done using UV radiation with 60Hz (Model FG-Y15W).

Inoculated samples were incubated at 37°C for 7days un-agitated after which were centrifuged at 10,000rpm for 30min. The supernatant contained the inorganic compounds that were not accumulated while the residue contained the organisms with accumulated inorganic compounds. The organisms (in the residue) were then lysed immediately using sodium dodecyl sulphate (SDS) so that the contents that have been accumulated over the period will be released. The mineral content of both the supernatant and the residue was analysed (Sulaimon *et al.*, 2014).

Results and Discussion

Inorganic components of wastewater

Inorganic components, which included metal/heavy metal contents and active ingredient of vegetable oil-rich wastewater obtained from Falegan restaurant, Ado-Ekiti, were estimated and depicted in Table 1. Magnesium (Mg) constituted the highest metal concentration of 54.5mg/L in the wastewater sample. Among the heavy metals, only iron (Fe) was significantly high with 31.6mg/L concentration, and a trace amount of copper (Cu) with 0.08mg/L. Heavy metals such as nickel and lead were not detected in any of the samples. The concentrations of each active ingredient in the sample was also presented in Table 1: phosphates (PO₄) 6.41mg/L, sulphates (SO₄) 4.67 mg/L, silica 1.65 mg/L, soda ash 0.82 mg/L and surfactants 0.02 mg/L.

Bioaccumulation of inorganic components of oil-rich wastewater sample by *L. sphaericus*

The trend of accumulation of inorganic components of oil rich wastewater by *Lysinibacillus sphaericus* strain ODE16_EKITI is shown in Figure 1.

About 98% (97.8%) of total calcium (Ca) was accumulated *L. sphaericus*, while the consortium of microbes absorbed 100% of the calcium. Forty-four point six percent of iron in the wastewater sample was accumulated by the isolate. It is therefore obvious that calcium and potassium were very important for the growth of *Lysinibacillus sphaericus* was able to accumulated iron.

There was no significant difference in the accumulation potential of the consortium, which took up 4.99mg/L of phosphate and that of the organism, which accumulated 4.96mg/L of phosphates present in the sample. This showed that the test organism is the major bioaccumulator of phosphate, while it showed a 0% affinity for sulphate as there was no trace of sulphate in their cell.

Effect of Incubation Time on Growth and Lipase Production

The effect of incubation time on growth of *Lysinibacillus sphaericus* strain ODE16_EKITI and its lipase production was determined for a period of six days (Figure 2). It was observed that the highest growth of the isolate (with OD 1.443) was obtained on the fourth day which gradually reduced by 21% on the sixth day (with OD 1.143). Lipase production by *L. sphaericus* increased steadily with growth for the first three days with 2.977mMol of lipase, which reduced to 2.866mMol on the fourth day, and finally recorded the highest production of 5.111mMol on the sixth day.

Effect of Oil Concentration on Growth and Lipase Production

The lipase production was inversely proportional to the growth of *Lysinibacillus sphaericus* strain ODE16_EKITI as shown in Figure 3. At oil concentration of 0.5ml/L, the growth indicated by the optical density was 0.205 while the lipase production was 4.556mMol. The growth and lipase production was fairly constant at oil concentrations of 5ml/L through 15ml/L. There was an increase in the growth (OD 0.245) and lipase production (5.622mMol) at 20ml/L oil concentration. The organism had the highest growth (OD 0.298) and lipase production (6.667mMol) with oil concentration of 2.5ml/L.

Effect of Temperature on the Growth

Figure 4 shows the growth of *Lysinibacillus sphaericus* strain ODE16_EKITI at different temperatures through three different times. The highest growth of the isolate (with OD 2.81) was recorded at 30°C after 24h while the least growth was recorded at 50°C with OD 0.022 after 12h of incubation and OD 0.044 after 24h of incubation. At 20°C, the organism showed a substantial growth after 12 h.

Effects of Metals and Heavy Metals on the Growth and Lipase Production

Varying growths were recorded for *L. sphaericus* strain ODE16_EKITI in the presence of metals and heavy metal as shown in Figure 5.

The organism showed the highest growth (with OD 0.301) and lipase production of 6.67mMol in the presence of magnesium (Mg). In contrast, the least growth of the organism (with OD 0.045) and lipase production of 1.033mMol were recorded in

the presence of iron. There were appreciable growth and lipase production by the isolate in the presence of sodium (Na) and potassium (K).

In a bid to remediate the menace caused by the presence of lipids in many biological wastewater treatment systems, various researchers have focused on a way to explore lipase production by different microorganisms (Prasad and Manjunath, 2010; Trichel *et al.*, 2010; Odeyemi *et al.*, 2011; Facchin *et al.*, 2013; Odeyemi *et al.*, 2013a; 2013b; Odeyemi *et al.*, 2014). The ability of *Lysinibacillus sphaericus* strain ODE16_EKITI to produce lipase has been reported by Odeyemi *et al.*, (2014), but it has to be stressed that lipases with different characteristics became so prominent in recent times. The bioremediation capability of the organism on various components of the domestic oil-rich wastewater is hence further investigated.

From the inorganic components analysed, it was revealed that metals such as Mg, K, Na, Ca and heavy metals such as Fe were found in high amount (31.6mg/L). Manganese, Zinc and Copper were present in minimal amounts, which goes in line with Ghorbani *et al.*, (2002) who reported that heavy metals such as Zn, Cu and Pb are mainly present in industrial wastes and if present, can reduce the amount of microorganisms, especially at high concentrations. Magnesium and copper exhibited the highest and least concentrations respectively. This observation is in agreement with that of Malik and Jaiswal (2004) who reported that magnesium, potassium, sodium, iron and calcium are found in high concentrations in the wastewater because they are not usually bioavailable for microorganisms when present in low amounts. As expected, Lead and Nickel were not detected in the oil-rich wastewater since they are not biologically redox reactive and

thus not usable for microbial respiration (Berg *et al.*, 2001).

Lysinibacillus sphaericus strain ODE16_EKITI in this work, bioaccumulated high amounts of some of the inorganic content of oil-rich wastewater; indicating that the organism needed them for their metabolic process. Little amount of iron was accumulated, which may be because the organism only needs little amount of the heavy metal for its biological processes. Roane (2001) explained that the ability of some organisms to thrive in the wastewater may be attributed to their accumulation capability and therefore necessary for enzymatic functions and bacterial growth. An uptake mechanism exists that allows for the entrance of the metal ions into the cell (Nies, 1999). Considerable amount of phosphate was accumulated by the organism, showing that the organism had the ability to bio-remediate some active ingredients. The organism showed no accumulation ability whatsoever for sulphates, which may be because sulphate had an inhibitory effect on the growth of the microorganism (Surjawidjaja *et al.*, 2004).

Lipase production was shown to be affected by incubation period, as it was recorded that the growth of the organism progressively reduced after the fourth day and the enzyme production also reduced after the fifth day of incubation. The maximum lipase activity (5.111mMol) was observed after 5 days' incubation. The organism grew in the medium at the rate of 0.292 mg/day while it produced the lipase enzyme at the rate of 0.78mM/min every day. This agrees with the work of Chipasa and Medrzycka (2007) in which the highest growth was observed on the fifth day and the oil content reduced due to the production of increasing level of lipase enzyme with maximal enzyme production was recorded on the fifth day.

Lipases are generally produced in the presence of lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, bile salts and glycerol (Sharma *et al.*, 2009). Lipidic carbon sources serve as inducers, vegetable oils have high

contents of oleic and palmitic acid and has been reported as a good inducer for lipase production by many bacterial and fungal strains (Wang *et al.*, 2008; Odeyemi *et al.*, 2014).

Table.1 Inorganic components of domestic oil-rich wastewater sample

Mineral	Element	Concentration (mg/L)
Metals		
	Calcium (Ca)	13.4
	Magnesium (Mg)	54.4
	Potassium (K)	43.2
	Sodium (Na)	39.2
Heavy metals		
	Copper (Cu)	0.08
	Iron (Fe)	31.6
	Lead (Pb)	ND
	Manganese (Mn)	0.40
	Nickel (Ni)	ND
	Zinc (Zn)	0.61
Active ingredients		
	Phosphate (PO ₄)	6.41
	Silica	1.65
	Soda ash	0.82
	Sulphate (SO ₄)	4.67
	Surfactant	0.02

Fig.1 Percentage accumulation of inorganic components of oil-rich wastewater by *Lysinibacillus sphaericus* strain ODE16_EKITI

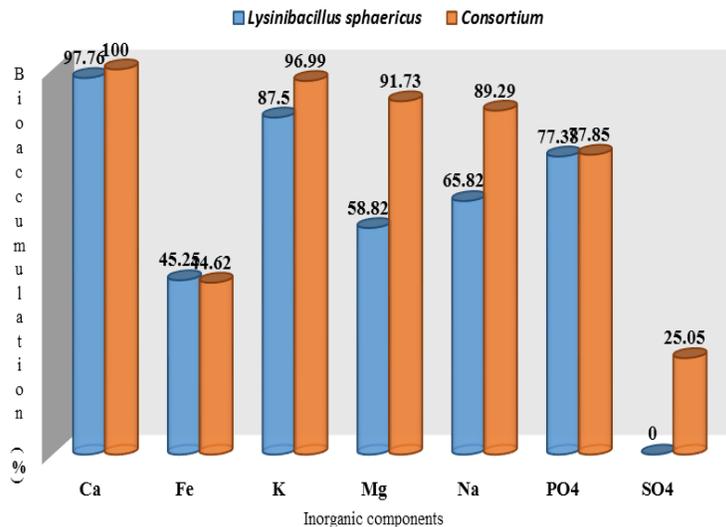


Fig.2 Growth and lipase production by *L. sphaericus* strain ODE16_EKITI at different incubation times

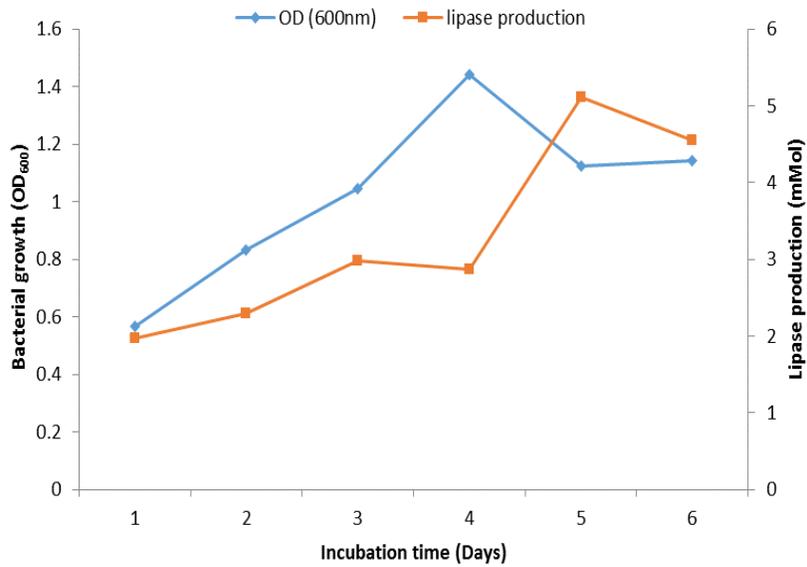


Fig.3 Growth and lipase production by *L. sphaericus* strain ODE16_EKITI at different oil concentrations

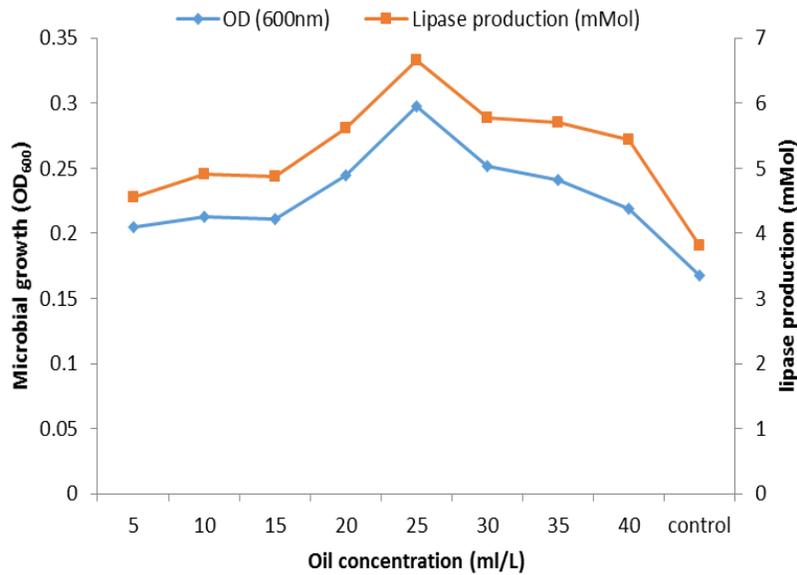


Fig.4 Growth of *Lysinibacillus sphaericus* strain ODE16_EKITI at different temperatures

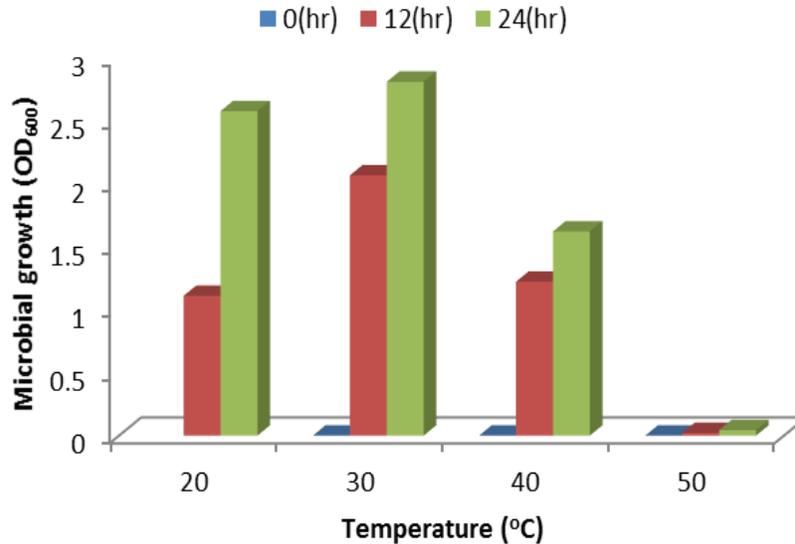
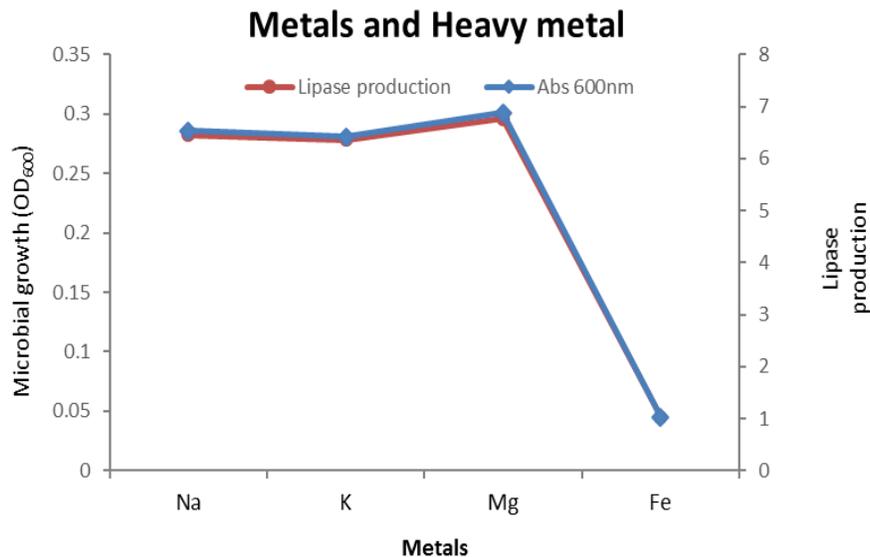


Fig.5 Growth and lipase production by *L. sphaericus* strain ODE16_EKITI in the presence of different metals



During this study, maximum lipase activity was achieved in the presence of 25ml/L, which is in consonance with the observation of Misbah and Haq (2014) who indicated that 20ml/L olive oil served as an inducer with the substrate. An additional amount of lipid was more effective in enhancing lipase production than any other carbon source.

It was observed that further increase in oil concentration did not favour lipase production in this work, which might be due to poor oxygen transfer as explained by Pereira-Meirelles *et al.*, (2005). Furthermore, they explained that at a constant air flow rate ($Q = 1\text{dm}^3/\text{min}$, 0.8vvm), lipase production was higher at a high stirring speed between

200rpm to 400rpm when most of the lipid was consumed while at lower stirring speed of 100rpm no significant lipid consumption was observed in 24h. The maximum production of lipase at low oil concentration may be due to availability of oxygen for the organism to thrive in the basal medium; this is in agreement with the work of Odeyemi *et al.*, (2011) who also reported that oxygen became much available as an electron acceptor, because *Lysinibacillus sphaericus* strain ODE16_EKITI thrived well wherever oxygen was available.

The optimum temperature for lipase production was observed to be 30°C, concurring with the work of Bhatti *et al.*, (2007) that reported inhibition of microbial growth and enzyme production due to high temperature. An increase in temperature, increases the number of effective collision between the enzyme and substrate to form activated complex and thus the rate of reaction increased. There is limit to increase in the enzyme activity when there is an increase in temperature (Bayoumi *et al.*, 2007). Murray *et al.*, (2003) also related in his work, that when the rate of enzymatic reactions is measured at several temperatures, there was an optimal temperature at which the reaction was most rapid, but when it was above that temperature, the reaction rate decreased sharply mainly due to the denaturation of enzymes by heat. Meanwhile, increase in temperature may also influence the secretion of extracellular enzymes as stated by Pirt (1975) that the temperature influenced the secretion of extracellular enzymes by changing the physical properties of the cell membrane. Going by the work by Palma *et al.*, (2000) which reported that increased production of protease at higher temperatures will lead to deactivation of lipase, the reduction in lipase activity at higher temperature in this study may be due to the deactivation of lipase with increased production of proteases.

Lysinibacillus sphaericus strain ODE16_EKITI lipases showed hydrolytic ability against K⁺, Na⁺ and Mg²⁺ three times higher than against Fe³⁺. Interestingly, Mg²⁺ ions resulted in a maximal increase in lipase activity. This is in agreement with the work of Sharma *et al.*, (2001; 2009) who reported that lipase activity was enhanced in the presence of K⁺ and Ca²⁺ and Mg²⁺ ions, but inhibited by Fe²⁺ and Hg²⁺ ions. Also Annamalai *et al.*, (2011) in their work found that some metal ions, Ni²⁺, Mn²⁺, Hg²⁺, Fe²⁺ and Fe³⁺ slightly inhibited enzyme activity and no effect was found with Cu²⁺. The activity of lipases may be inhibited or stimulated by cofactors. More so, divalent cations such as calcium and magnesium often stimulate enzyme activity due to the formation of calcium salts with long chain fatty acids (Macrae and Hammond, 1985).

In conclusion, as the global need of biodegrading enzymes become alarming, so also the search for a new, highly efficient lipase with different characteristics is rapidly growing and apparently difficult. The present work has offered us the opportunity to investigate a lipolytic bacterium, *Lysinibacillus sphaericus* strain ODE16_EKITI that can produce lipase ranging between 5.1mMol to 6.0mMol at 30°C and 2.5% palm oil concentration after the 5th day and this could be enhanced with magnesium ion (Mg²⁺). This enzyme also showed potentials of bioaccumulation of some heavy metals. *L. sphaericus* strain ODE16_EKITI is thereby presented as a promising tool for biotechnological exploitation in remediation of wastewater.

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