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Research Article

IMMOBILIZATION AND CHARACTERIZATION OF LIPASE LOADED ON FE_3O_4 NANOPARTICLES AND PRODUCED FROM HALOALKALOPHILIS *KOCURIA POLARIS*-WRS3

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ABSTRACT

A Gram-positive, cocci, thermoduric, haloalkalotolerant, and lipolytic bacterium designated strainWRS3, was isolated from salty soda soil sample collected from Wadi El-Ryan, Egypt. It is extremetolerant , pH10 and NaCl 22%. This isolate was identified as avariety of *Kocuria polria*.Strain WRS3 grows at different ranges of temperatures 15-50°C, pH 7.0-12.5 and salinity 3.0–30% NaCl (w/v).Euclidean distance was 97% between isolate WRS3 variety under *Kocuriapolria*.Over all phenetic relationship of isolate WRS3 was considered as Gram positive Phylum,variety of halo alkalo tolerant thermoduric of *Kocuria*, domain Eubacteria. Olive oil was the most suitable substrate for maximum lipase production by *Kocuria polria*-WRS3.Iron oxide (Fe_3O_4) magnetic nanoparticles and magnetite silica-coated (MSI) nanoparticles were used as immobilization material.Lipase was covalently bound to the silica-coated -functionalized magnetic nanoparticles with excellent properties have been successfully prepared using the chemical co-precipitation. The transmission electron microscope images revealed the size of the uncoated magnetite particles to be 200–500 nm and those of the coated particles about 300 nm. The best environmental conditions for lipase production by *Kocuri apolria*-WRS3 isolate indicated that the pH and temperature of the immobilized lipase were 8-9.5 and 40°C, respectively and, salt concentration range from 10% to 18% NaCl.Also,high enzymes productivity was obtained at aeration speed 150- 220 rpm between 48h. to 72h. Different types of oils (olive oil, corn oil, sun flower oil, punt oil and cotton oil)were tested for the selection of the best substrate for free lipase. Extracellular lipase produced was stimulated and exhibited the highest productivity bythe addition of peptone + histidine or casoamino acid + histidine to the product media. Highly lipase from *Kocuri apolria*-WRS3 exhibited better resistance to commercial detergents.Lipase activity was strongly resistance to Sodium Dodecyl Sulphate (SDS)and Triton X-100Arial, Tween 80 and Persil, respectively.The remarkable productivity of *Kocuriapolria*-WRS3 lipase in this range has proved to be a potential alkaline-saline lipase.

INTRODUCTION

A soda lake or alkaline lake is a lake on the strongly alkaline side of neutrality. They are characterized by high concentrations of carbonate salts, typically sodium carbonate (and related salt complexes), giving rise to their alkalinity. In addition, many soda lakes also contain high concentrations of sodium chloride and other dissolved salts, making them saline or hypersaline lakes as well. The resulting hypersaline and highly alkaline soda lakes are considered some of the most extreme aquatic (Grant, 2006). One of those environmental niches, which have not been studied in details, is Wadi El-Natrun.

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The features of Wadi El-Natrun Valley created an ecosystem considered as rich sources for isolation of alkaliphilic, haloalkalophilic, and thermo-alkaliphilic microorganisms (Horikoshi, 1999). Alkaline lipase from *P. aeruginosa*, which is capable of growing in a water-restricted medium, has excellent properties and good potential for biotechnological applications in the metal industry. Its marked stability and activity in organic solvents suggest that this lipase is highly suitable as a biotechnological tool in a water-restricted medium with a variety of applications including organosynthetic reactions (Barberán and Casamayor, 2010). The majority of lipases exhibit a high activity toward lipids with fatty acid residues of C_8 to C_{18} chain length.The lipase used in each application is selected based on its substrate specificity such as fatty acid, alcohol, position (regio-) and stereo-specificity, as well as temperature and pH stability (Lee *et al*., 2010).

Microbial lipases have already established their vast potential regarding to their usage in different industries. Lipases from different sources are currently used in enzymatic organic synthesis .The expanding interest in lipases mainly lies on their wide industrial applications, including detergent formulation, oil/fat degradation, pharmaceutical synthesis, cosmetics, paper manufacture, and oleochemistry. However, the biggest market of their use is in the detergent formulation regarding detergents applications.To use lipases more economically and efficiently in aqueous as well as nonaqueous solvents, their activity and operational stability needs to be improved by immobilization (Villeneuve *et al*., 2000; Mostafa and El-Hadi, 2010). Lei *et al*. (2011) reported that, the use of nanophase materials, which offers many advantages because of their unique size and physical properties. Magnetic nanoparticles have become very popular when used in conjunction with biological materials such as proteins, peptides, enzymes, antibodies, and nucleic acids because of their unique properties.This application is mainly based on the magnetic feature of the solid phase that helps to achieve a rapid and easy separation from the reaction medium in a magnetic field. Previous studies reported that magnetic nanoparticles tend to lose their magnetizability when biopolymer-coated nanoparticles are circulated in the body. Duguet *et al*., 2006; Wenlei and Ning, 2009 reported that, magnetic Fe_3O_4 nanoparticles treated with (3-aminopropyl) triethoxy saline were used as immobilization material. Lipase was covalently bound to the amino-functionalized magnetic nanoparticles by using glutaraldehyde as a coupling reagent with the activity recovery up to 70% and the enzyme binding efficiency of 84%. Moreover, the immobilized lipase was found to be able to catalyze the transesterification of soybean oil with methanol to produce fatty acid methyl esters (better known as biodiesel). Further study showed that the immobilized lipase could be used four times without significant decrease of activity. The goal of this study aim to purify and characterizethe bacterial isolate WRS3 as lipase producer, isolated from WadiEl-Ryan soil, Egypt,extreme haloalkalotolerant bacteria and study some phenotypic taxonomical characteristics and identification.Also, study the potential of *Kocuria polria*-WRS3 for production of saline - alkaline lipase enzyme immobilized with magnetic Fe_3O_4nanoparticles for commercial applications.

MATERIALS AND METHODS

Sample localities and isolation

A soil sample was collected from WadiEl-Ryan soil, Egypt, salty soda soil. Bacteriological isolation was done by the use of modified Horikoshi medium (Horikoshi, 1999). Dilution plate method was used for the isolation of haloalkalotolerant bacteria. One ml of the dilutions was plated on appropriate sterilized solid modified Horikoshi agar medium, which contains (g/l): Glucose, 5.0; polypeptone, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2; Na_2CO_3, 10.0; agar 25.0, all ingredients were dissolved in 900 ml tap water. Na_2CO_3 and glucose were sterilized separately each in 50 ml water and added to the medium before pouring and incubated for 7 days at 37°C for isolated bacteriaWRS3. The morphological differences of isolated bacterial strain WRS3 has been studied on Horikoshi medium (Horikoshi, 1999). Furthermore, microscopic observations after different chemical treatments that give detailed information on the cell morphology under different extreme conditions, and then they were examined by scan electron microscope at Regional Center for Mycology and Biotechnology(RCMB)Al-Azhar University.

Phenotypic and taxonomical analyses

The bacterial cells cultivation and phenotypic characterization were tested.The physiological conditions werecarried out as growing the bacteria at different pH ranges, temperatures range, different salt concentrations, susceptibility to lyses, nitrogen and carbon sources utilization, and different enzymes detections. Phenotypic methods also include biotyping, and antibiogram according to Cowan and Steel's (1977); Cowan (1992); Horikoshi and Grant (1998) and Horikoshi (1999).

Cluster Analyses

For studying phylogenetic relationships of the investigated isolate WRS3 with other similar reference strains by using statistical cluster analysis with joining (tree clustering) being the clustering method. The phenotypic characters were amalgamated by unweighted pair-group average method analysis (UPGAMA). However, complete linkage was the method for studying character profiles using statistica for windows, release 4.5f, state Soft, Inc.1993 software. Euclidean distances (similarity matrix) were used as the distance metric in both as well as dice coefficient as the calculation method (SIGMA lab.).

Production of lipase metabolites

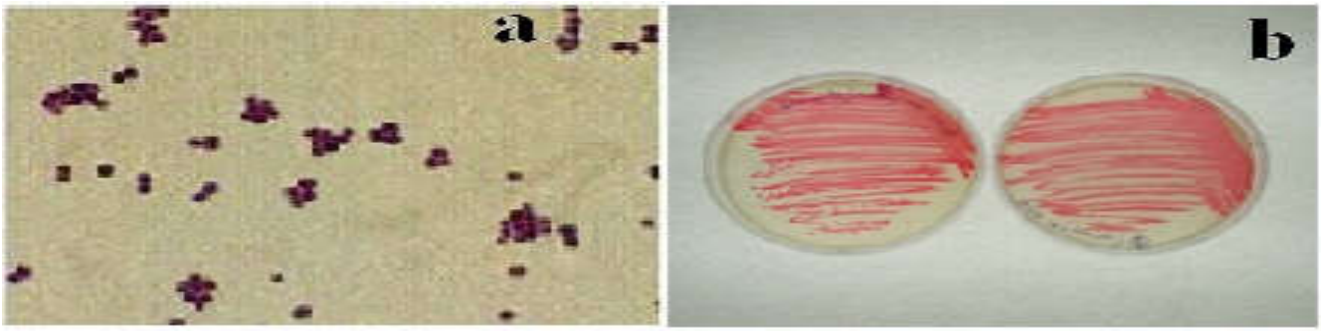
Determination of the production of the active alkaline-saline lipase in the supernatants of WRS3 isolate were held in three replicates and determined according to the method of Horikoshi (1999). Horikoshi liquid medium, supplemented with olive oil and optimal 15%NaCl with pH10 was used. The medium was distributed into 250-ml Erlenmeyer flasks each containing 100 ml .The medium was then autoclaved at 121°C for 15 minutes. The medium (100 ml) was inoculated with 1ml spore of the bacteria isolate. After four days, the metabolic product was adjusted at pH9, suspension of the studied bacteria incubated at 35°C for 21 h. in static conditions for lipase enzyme detection.

Qualitative lipase Assay

A strain was screened for investigating its lipase productivity on agar plates containing Rhodamine B 0.001% (w/v), nutrient broth 0.8% (w/v), NaCl 5.5% (w/v), agar 1% (w/v), and olive oil 3%, in distilled water and the pH was adjusted to be 9. Triplicate plates were incubated at 45°C for 18 hrs and the lipase productivity was identified as an orange halo zone around colonies under UV light at 350nm (Rabbani *et al*., 2013).

Effect of Substrate Concentration

Different olive oil substrate tubes of concentration range from zero up to 20 mml were prepared by dissolving of 113.3 mg of phenyl acetate in 10 ml Na_2CO_3 buffer pH 9, then 0, 2, 4, 6, 8,10,12,14 and 16 mml was taken from the solution and completed up to 20 mml by distilled water, finally 0.1 ml of lipase enzyme was added and the enzyme productivity was measured at the optimized conditions (Dae and Joon, 1986).



Figure(1):(a) IsolateWRS3 Gram positive (b) Dark rose colony

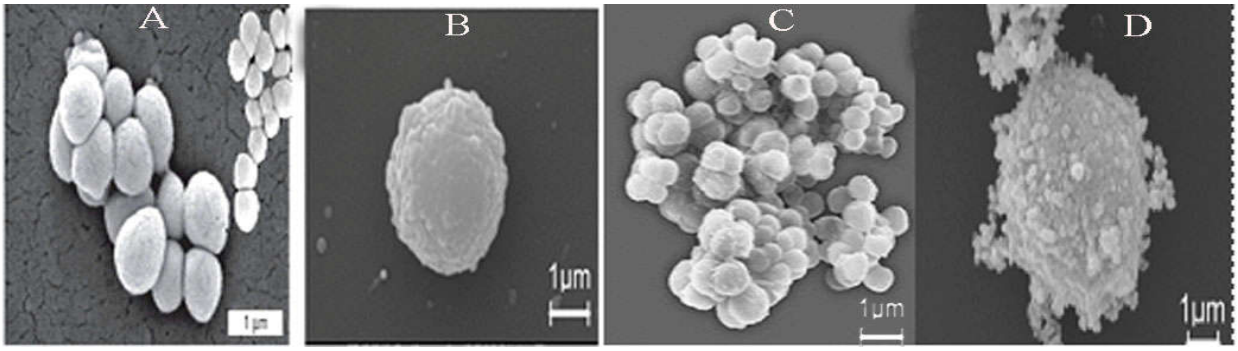


Figure (2): (A, and B) Scan electron microscopeWRS3 cell under normal condition 15% NaCl and pH10 (1um) .(C and D) Cells under highly extreme condition 28%NaCl and pH12 and Cell after 2 h in distilled water secreted salt around cell (1um)

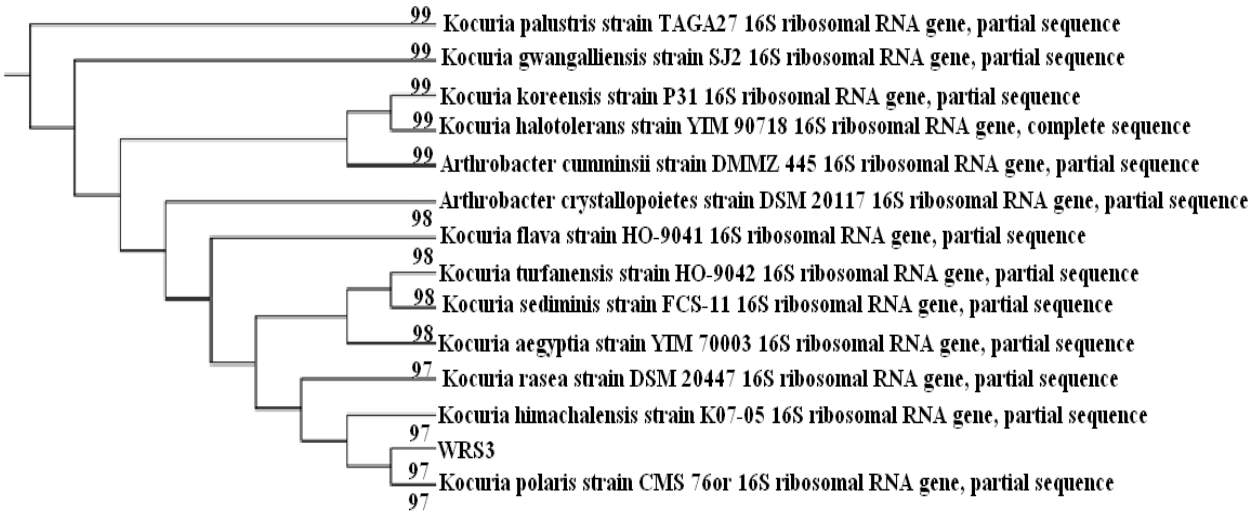


Figure 3. Dendogramofthe unknownisolateNo.WRS3 and reference strains based on the similarity matrix of phenotypic data

Determination of the Production of the alkaline lipase

The activities of lipolytic enzymes produced by isolate WRS3 was held in their concentrated supernatant and determined according to the method of Dae and Joon (1986). 0.2 ml of each concentrated supernatant was mixed with 1ml alkaline - saline soluble olive oil emulsion buffer [0.5 % olive oil emulsion buffer contain 50m M tris-HCl pH 9.5 and 2.5 molNaCl .The mixture was incubated at 50°C for 5hours. 0.2 ml reaction mixture was added to copper reagent prepared according to Lowery and Tinsley (1976). The optical densities were measured using spectrophotometer (Jenway 6105 UV/Vis) at 700 nm after 30 minutes.

The accumulation of lipolytic enzymes and maximum enzyme production may be related to percent of olive oil hydrolyzed. The best environmental growth cultural conditions, to yield the highest enzyme productivity under different factors were considered in this study. One unit of active saline-alkaline lipase was defined as the amount of enzymes, which hydrolyse one microgram of olive oil per milliliter per minute at pH9,12% NaCl and at 40°C as reported by Dae and Joon (1986).

Optimization of alkaline -saline lipase production in different conditions

Alkaline -saline lipase production in different conditions were measured as described for olive oil assay Dae and Joon (1986).

Effect of different pH on enzyme productivity

The emulsify olive oil culture media with different pHs of 7, 8, 9,10 and 11 were prepared. One milliliter of WRS3 isolate broth medium, with the turbidity of 0.5 McFarland standards (equal to 1.5×10^8/ml of bacteria) was transferred to each oilbroth culture medium and incubated at 37^{o}C, aeration speed of 160 RPM for 72 hours. Then 1 ml of each broth culture was collected and the alkaline lipase productivity was measured at 40^{o}C and 10% NaCl.

Effect of different incubation temperatures on enzyme productivity

The effect of different incubation temperatures ranging from10-60^{o}C at pH 8.0 and 10% NaCl using Tris-HCl buffer (50 mM). on lipase productivity were carried out.

Effect of different NaCl concentrations on enzyme productivity

Enzyme productivity detected at different NaCl concentration ranging from 5% to25%. Lipase productivity was measured at pH 9 and 40^{o}C.

Effect of different oils as substrates

Two percent (2% w/v) of fats and oils (olive oil, peanut oil, sun flower oil, corn oil and cotton oil) were individually added to the reaction mixture containing 4 ml of 50 mMTris-HCl buffer, pH 8.0, 1 ml of 110 mM $CaCl_2$ and 1 ml of enzyme (5 mg/ml). Lipase productivity detected at pH 9, 40^{o}C and 10% NaCl.

Effect of different concentrations of olive oil on lipase productivity

Effect of different concentrations of olive oil from 0.1 to 2.5 on lipase activity was carried out at pH 9 and 40^{o}C and 10% NaCl.

Effect of surfactants and commercial detergents on enzyme productivity

Effects of surfactants (Tween 80, Triton X-100, Sodium Dodecyl Sulphate (SDS) and commercial detergents, Piral, Presil and Arial) on enzyme productivity were determinedat pH 9 and 40^{o}C and 10% NaCl according to Shih *et al*. (2003).

Synthesis of magnetite nanoparticles (Shih *et al*., 2003)

The nanoparticles were prepared by dissolving a total of 4.05 g of $FeCl_3$•$6H_2O$ and 1.98 g of $FeCl_2$•$4H_2O$ in 100 ml of distilled water; the solution was purged with nitrogen to agitate the mixture and prevent the oxidation of Fe^{2+} ions. After 30 min of purging, 143 ml of 0.7 mol/lNH_4OH was added drop wise into the solution and the new basified solution was purged for an additional 10 min. During the addition of the solution the color will be changed from the original brown to dark brown and then to black.

The precipitate was magnetically separated using a permanent magnet and then washed with distilled water several times and allowed to dry in air. The resulting product was defined as synthesis of magnetite silica-coated (MSI) nanoparticles (Shih *et al*., 2003). The above-mentioned experiment was repeated until the step in which the solution was purged with nitrogen to agitate the mixture. After this step, the precursor, (TEOS) (3 ml) was carefully dropped into the reaction mixture of iron using a syringe, with mechanical stirring. The homogenization was performed for 15 min. After sonication for 15 min, 143 ml of 0.7mol/l NH_4OH was added drop wise into the mixture with continuous mechanical stirring for 30 min. The coated particles were finally separated from the liquid using a permanent magnet, washed with distilled water several times, and allowed to dry in air (Nanotech Lab.-,6 October city).

Characterization of immobilized enzyme

The medium (100 ml) was inoculated with 1ml cells supporting growth of bacterial isolate after four days adjustment metabolic product at pH9 then concentrated metabolite at 40^{o}C suspension, using microcentricon. Concentrated crude free enzyme was determined according to the methods described in the A.O.A.C. (1990).Then concentrated crude free enzyme immobilized with magnetite Fe_3O_4 nanoparticles according to (Shih *et al*., 2003).The size and shape of the nanoparticles were examined using a transmission electron microscope (TEM) (nanotech lab.).The IR spectra were recorded using a Fourier transform-infrared spectrophotometer (FT-IR)(National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt. lab.).

RESULTS AND DISCUSSION

Over the last few decades several reports have focused on extreme haloalkalotolerant microorganisms ,which they have high pH value (10-13) with varying amounts of other salts often including NaCl (up to 200 g/l) in addition to Na_2CO_3 as a major component. These organisms are obligately alkalophilic with extremely halotolerance. Extracellular alkali- and salt-tolerant enzymes that are produced by these haloalkalophilic microorganisms are applicable for industrial purpose (WIPO, 1993 and Oren, 2002). Lipase is an enzyme that capable of hydrolyzing the ester bond of triglyceride in order to produce glycerol and fatty acids. Although lipases have many different applications in detergents, cleaners, food industry, pharmaceutical industry, pulp and paper production and leather industry, the extremophile lipases have more range applications. These enzymes are resistant to the high salty, temperature and alkaline conditions (Jaeger and Reetz, 1998).
Strain WRS3 isolated from salty soda soil sample was collected from Wadi-El-Rayan, Egypt. The climatic conditions and Egyptian soil texture could yield many species of bacteria, which adapted to grow and thrive under the extreme environmental conditions, in addition to other species, which tolerate these conditions. Many reports were published for haloalkalophiles bacteria isolated from Egyptian soda soil. WadiEl-Natrun has crystalline deposit reflecting total dissolved solids of up to 40% (w/v) (Tindall *et al*., 1984; Lowe *et al*., 1993).

Phenotypic and taxonomical analyses

The bacterial cells cultivation and phenotypic characterization were tested.

Table 1. Phenotypic characteristics and comparison between isolate No. WRS3 and *Kocuriapolaris*

]	*Kocuri apolaris*	Isolate No. WRS3
Morphology		
Morphology of colony	Smooth,, opaque and circular non pigmented	Smooth,, opaque and circular with entire margins: Colony color change under sever condition to pale colony color
Colony pigment	non pigmented	Deep Dark or Light rose or pink colour (0.2-0.7ml)
At early stage		pale rose colour
At stationary phase		Deep rose or pink colour
Habitat		Sandy loem soil
Growth		Chemotrophic complex media
Cell morphology (shape)		Cocci
At stationary phase and end growth	Coccoi	Cococci or irregular cocci
Cell dimension (µm)	0.5-0.6 µm x 1-3 µm	0.5-0.55 µm or 1.5-1.55um
Gram classification	G +ve	G +ve
Gram stain	G +ve	G +ve
KOH test	ND	G+ve
Aminopeptidase activity test	ND	G+ve
Motility	Motile	Motile
Pleomorphism	ND	-
O_2requirment	Aerobic	Aerobic
- NaCl range at 35ºC	0-10%	3-30%
- At 45ºC	-	+
- Optimum	-	8-15%
pH range		
- At 35ºC	6.5-8.5	6- 12.5
- At 50ºC	-	-
- pH range in broth	6.5-8.5	7-12.5
- Optimum	7.5	7 -9.5
Temperature range	15-37 ºC	15-50 ºC
-Optimum	-	25-32 ºC
Temperature tolerance at 65ºC	-	Not survive
Growth period (at pH 10 and 20% NaCl	ND	+
At 4 ºC	-	-
At 35 ºC	72h.	72h- 120h
Effect of replaced of NaCl by some minerals:		
a) K^+ b) Mg^{+2} c) Ca^{2+}	ND	+ve and colony color pale with 3-5 g Mg^{2+} and100g K^+ while negative with Ca^{2+}
Oxidase	+	-
Nitrate reductions	+	+
Catalase	+	++
Indole	-	-
H_2S production	-	-
Urease	-	+
Gelatin liquefaction	-	-
Starch hydrolysis	+	-
Casein hydrolysis	ND	-
Hydrolysis of tributyrine	+	+
Hydrolysis of Tween 40	-	+
Hydrolysis of Tween 80	-	+
Methyl red	+	+
Voges-Proskauer	+	+
Minimal medium		
Growth with NH_4Cl		+
Growth without glucose	+	+
Growth without peptone	-	-
Growth without Mg ions	-	-
Growth without yeast extract	ND	-
Enzymes production		
-Neutral amylase - Alkaline amylase	+	+
-Neutral lipase -Alkaline lipase	+	-
-Neutral phosphatase -Alkaline phosphatase	ND	++
-Neutral protease -Alkaline protease	-	-
-Neutral cellulase -Alkaline cellulase	-	+
	ND	-
	Growth stimulations and survival	
-Growth at pH6- pH 7.5 and 3% NaCl (on agar)	+	+
-Growth at pH6- pH 7.5 and 3% NaCl (in broth)	ND	+
-Growth at pH6- pH 7.5 (in broth or agar) on 15% NaCl	ND	+
-Growth on 5-20 %KC1	ND	+ve (with change pH to 9.5)
-Growth in Na_2CO_3 1%		
-Growth in Na_2CO_3 5%	ND	+
--Growth with Casoamino acid 1%	ND	+
Growth with tryptone	+	+
Mineral salt solu. requirement	ND	++
Growth with different amino acid	ND	-
Growth with different amino acid	ND	++
		+

Continues……..

General characteristics	*Kocuria polaris*	Isolate No. WRS3
	Growth with vitamins solution	
Yeast Extract	+	+
Biotin		
Folic acid	+	+/-or week
Pyridoxine	+w	+++
Riboflavin	+w	Very week
Cyano-cobalamin	+	+week
Cell lysis in H_2O	+week	+week
- SDS	+ w	w+
	Carbohydrate utilization	-ve
Xylose		-ve
Arabinose	non oxidized carbohydrate	Approximately non oxidized carbohydrate except glucose and fructose
Rhamnose	-	-
Ribose	-	-
Glucose	-	
Fructose	-	+
Mannose	+	+
Lactose	+	-
Sucrose	-	-
Maltose	-	-
Raffinose	-	+
Trehalose	-	-
Starch	-	-
Cellulose	-	-
Salicin	-	+
Glycerol	+	-
Mannitol	+	-
Cholesterol	+	-
	Antibiotic sensitivity	-
Rifampicin	-	-
Vibramycin	ND	+
Puromycin	ND	+++
Tobramycin	ND	-
Sulfamethin	ND	++
Triple sulfa	ND	-
Sulphonamides	ND	+
Nitrofurantion	ND	+
Bactracin	ND	-
Chloramphincol	ND	-
Erythromycin	ND	-
Novobiocin	ND	++
Pencillin	ND	++
Cephalosporin	ND	+
	Antifungal effect	
Furamazone	ND	+W
Nizarol		
Lamyzol	ND	-
	Nitrogen sources requirement	
Alanine	ND	-
Arginine	+	+
Asparagine	+	+w
Cysteine	ND	++
Cystine	ND	++
Glutamic acid	+	+
Glutamine	ND	+++
Histidine	+	+
Isoleucine	ND	+w
Lysine	+	+
Methionine	ND	++
Phenyl – alanine	+	+
serine	ND	+
Threonine	ND	++
Tryptophan	ND	+
Tyrosine	ND	+
Glycine	ND	+w
Peptone	ND	++
Urea	+	+
$NaNO_3$	+	++
$(NH4)_2SO_4$	ND	+

ND = Not detected

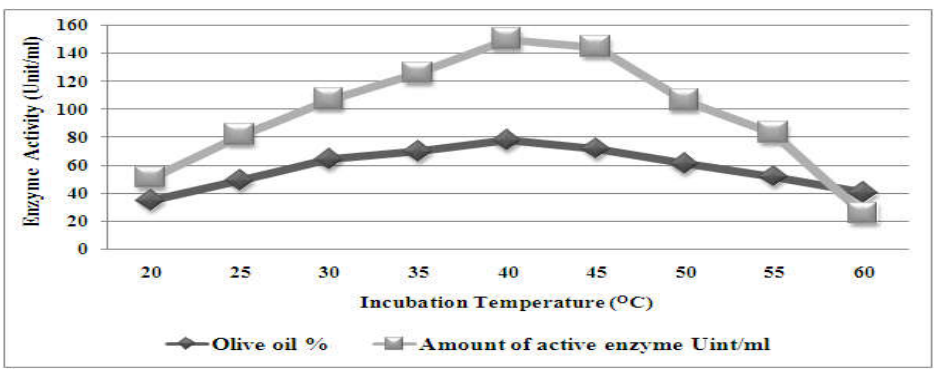


Figure 4. Effect of different incubation temperatures on productivityof active lipolyticenzymes using its concentrated cell free filtrates

The physiological conditions were growing the bacteria at different pH ranges, temperatures range, different salt concentrations, susceptibility to lysis, nitrogen and carbon sources utilization, and different enzymes detections. Gram staining was carried out by using the standard Gram reaction and was confirmed by using the KOH lysis test (Cerny, 1978). Phenotypic methods and also biochemical test include antibiogram. The grown cells showed at early stage cocci shape cell red colony color (Figure 1). While,(Figure 2 A ,B ,C& D) showed cells under normal condition (10% NaCl and pH9) ,also clear aggregation under extreme condition at end growth (28% NaCl and pH 12), the cells dimension was from 0.4 to 2µm and cell when in distilled water survive by swollen water and secreted salts arouned cell. Carbohydrateweak fermented most of them none oxidized, alsoWRS3 isolate was sensitive to some eubacterial erythromycin, novobiocin and pencillin.Phenotypic comparsion and taxonomical analyses Table (1) was clear highly similarity between isolate (WRS3) and *Kocuria Polaris*

ND = Not detected

Isolateno.WRS3 shared strain *Kocuria polars* strain in most of the taxnomical characteristics and it was considered as different variety under genus Kocuria. Euclidean distance was 97% between isolate WRS3 variety under *Kocuria polaris* overall phenetic relationship of isolate WRS3 is considered as a variety of haloalkalophile thermoduric of *Kocuria*, domain Eubacteria, Gram positive Phylum Proteobacteria, order Micrococcales, family Micrococcaceae and genus Kocuria named *Kocuria polaris*-WRS3, according to Bergey's manual of systematic bacteriology (Sneath,1986; Holt *et al*., 1994;Vos*et al*., 2009). The genus Kocuria was established by Stackebrandt *et al.* (1995) by taxonomic dissection of the genus Micrococcus, and was clearly separated from Micrococcus and Micrococcus related taxa on the basis of phylogenetic analyses using 16S rRNA gene sequences.

Members of Kocuria are Gram positive, aerobic, non-encapsulated, non-halophilic, non endospore-forming cocci characterized by the presence of menaquinones MK-7(H2) and MK-8(H2). There are eight Kocuria species with validly published names: *Kocuria kristinae, K. palustris*, *K. polaris*, *K. rhizophila*, *K. rosea*, *K. varians*, *K. marina and K. carniphila* (Reddy *et al.,* 2003; Kim *et al.,* 2004; Tvrzova*et al.,* 2005). This resultis inaccordance with Wen-Jun *et al*. (2006) who isolate dacoccoid, non-motile bacterium, designated strain YIM 70003T, was isolated from a saline, alkaline, desert-soil sample from Egypt. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the organism formed a distinct phyletic line within the genus Kocuria and was most closely related to *Kocuria polaris* DSM 14382T (98.6% sequence similarity) and *Kocuria rosea* DSM 2047T (98.2 %). The name *Kocuriaa egyptia* sp. nov.is proposed, with strain YIM 70003T. Also, Ying*et al.*(2010) investigated that, a Gram-stain-positive, halotolerant, neutrophilic, rod-shaped bacterium, strain MF38T, was isolated from a saline–alkaline soil in China and subjected to a polyphasic taxonomic characterization. The isolate grew in the presence of 0–15% (w/v) NaCl and at pH 6.5–8.5;optimum growth was observed with 3.0% (w/v) NaCl and at pH 7.0. The genomic DNA G+C content was 35.3 %. 16S rRNA gene sequence similarities of strain MF38T with type strains of described Gracilibacillus species ranged from 95.3 to 97.7 %. Strain MF38T exhibited the closest phylogenetic affinity to the type strain of *Gracilibacillus dipsosauri*, with 97.7% 16S rRNA gene sequence similarity. While, Jayakumar*et al*.(2012) reported that, extremophiles are microorganisms that have evolved to live in a variety of unusual habitats, the so-called extreme environments. They fall into a number of different classes including halophiles, alkalophiles, thermophiles, psychrophiles, and others. The groups of bacteria that can grow under alkaline conditions in the presence of NaCl are referred to as halotolerant alkaliphiles and haloalkaliphiles. The dual extremity of these extremophiles, high pH, and salt concentration make them attractive strains for exploration of novel alkaline proteases for biotechnological potential.

Lipase production

The productivity of enzymes under extremophilic conditions have become an important topic for applied research, especially those purified enzymes from extreme halophilic and haloalkalophilic organisms. Such enzymes have scientific interest because of their role in several industrial applications. Therefore the study focused on the detection of certain lipase enzymes from extreme haloalkalophils isolate.

Assay of crude lipolytic enzymes produced by haloalkalophilic isolate

Lipolytic productivity of *Kocuria polaris*-WRS3 at pH10 obtained primary by applying on the modified SCZ technique, and then tested on standard liquid media according to Hiol *et al*. (2002). Relation between substrate concentration and enzyme productivity was done as stander curve assay the productivity of lipase from microbial lipase by olive oil. A standard curve revealed a linear detection response from 0 to 200 uml. A dilution series of olive oil were prepared in the substrate solution containing 20 mml emulsion olive oil. The absorbance was converted to product concentration on the basis of a standard curve of olive oil degradation.

Parametric optimization for lipase production

Optimization of culture conditions for production of lipase

Factors affecting the productivity of lipolytic enzymes from extreme haloalkalophilic isolate *Kocuria polaris*-WRS3 strain, which was selected as the most highly lipase producer. Several parameters affecting active lipase production on saline - alkaline medium were examined following the methods recommended by Hiol *et al*. (2002).

Effect of different incubation temperatures on enzyme productivity

The productivity of active lipase was measured at pH9 and10% NaCl for 30 min from concentrated filtrates and incubated during growth at various temperatures (Figs 4 and 5). Maximum production active enzymes were 150.2 U./ ml and 144.67U./ml at 40°C and 45°Crespectively.Also, the enzyme production at 35°C was 126.17 U. /ml. These results are parallel with those of immobilized enzyme,which gave the highest enzyme production of 174.67U/ml at 40°Ccompared to the growth at various temperatures on free enzyme and immobilized lipase.

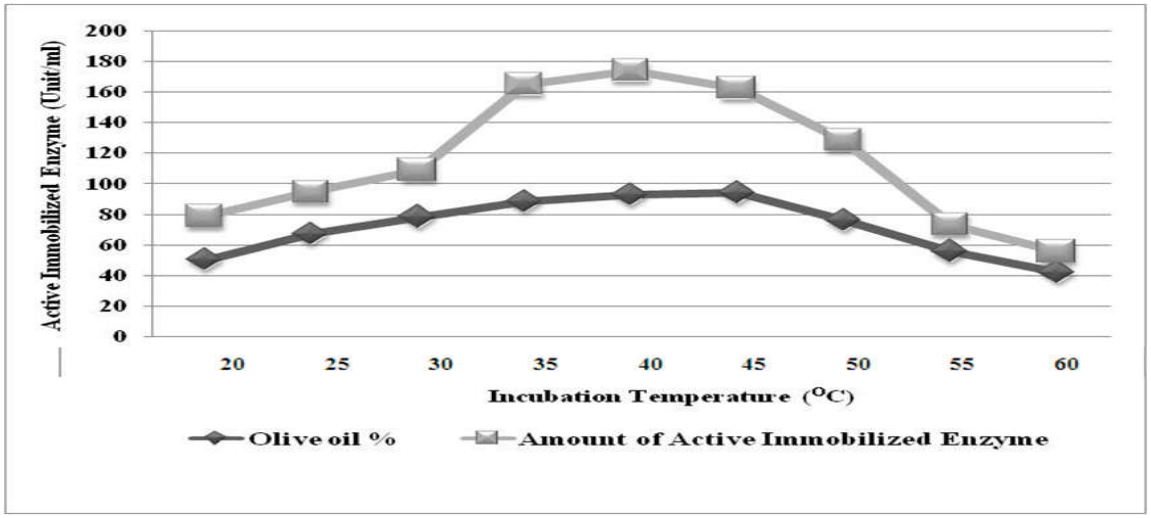


Figure 5. Effect of different incubation temperatures on productivity of lipolytice enzymes using its concentrated immobilized lipase

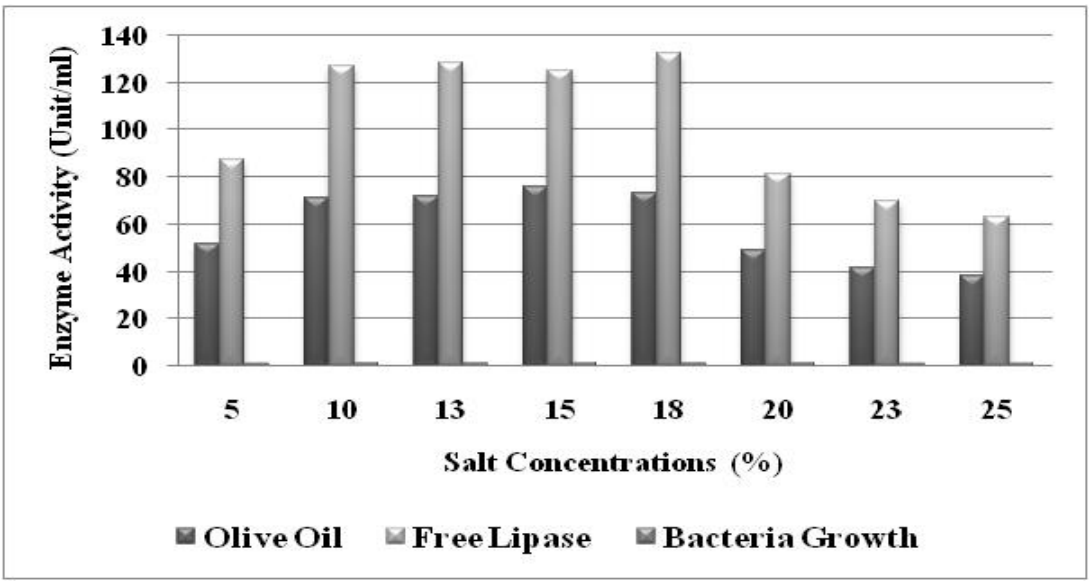


Figure 6. Effect of NaCl concentrations on productivety of lipolytic activity of free active enzyme and immobilized lipase

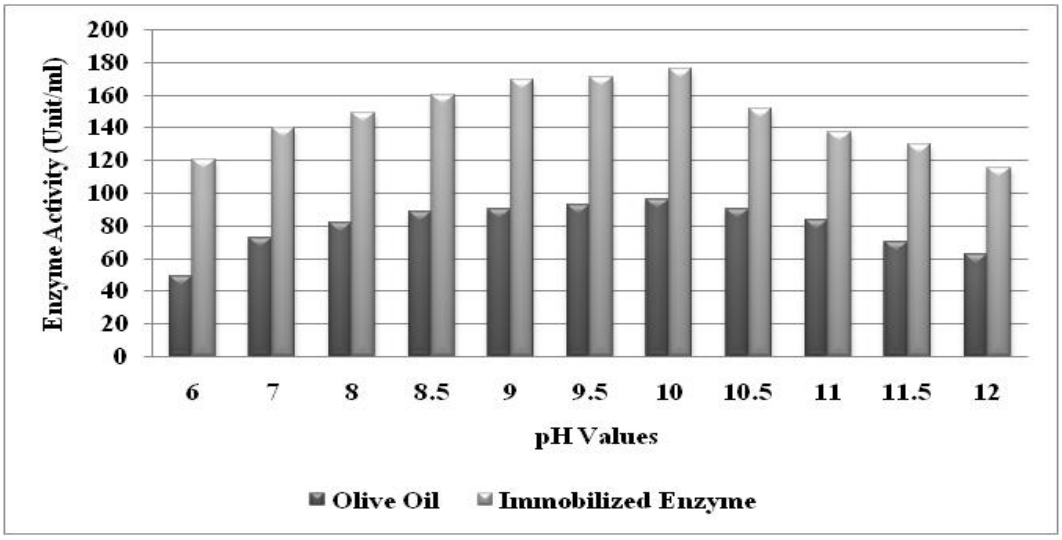


Figure 7. Effect of different pH values (by a carbonate buffer) on activity of immobilized lipase

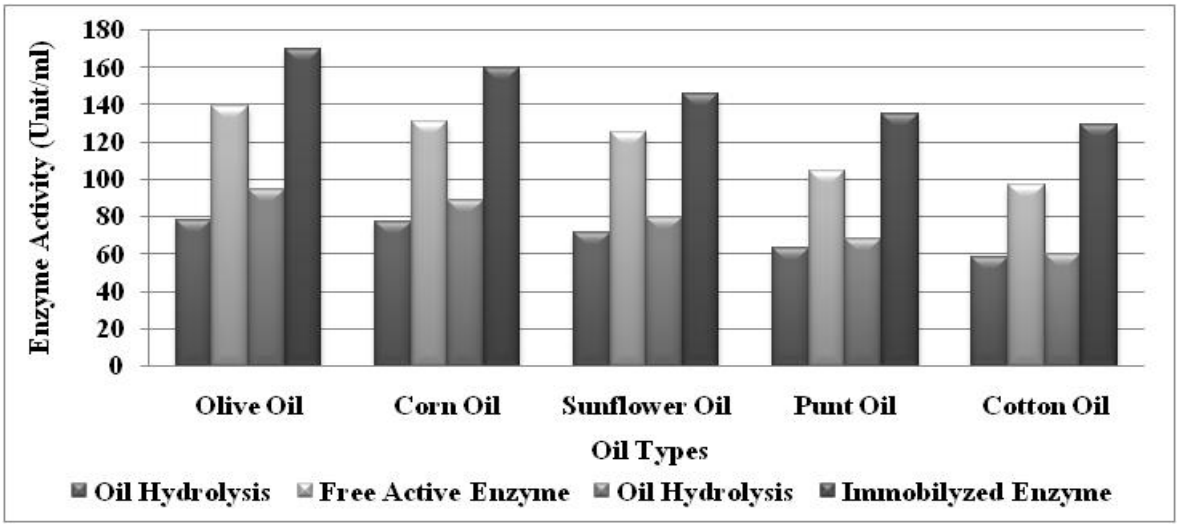


Figure 8. Effect of different kinds of oils on lipase produced by *Kocuriapolaris*-WRS3isolate using immobilized enzyme and free active enzyme

Table 2. Effect of different concentrations of olive oil on lipase produced in media, by haloalkalotolerant isolate No. RWS3 using its concentrated cell free filtrate

Olive oil concentration % inbroth media	hydrolysis % in assay exp	Amount of active Enzyme production (U/ml)	Growthas O.D (at 650 nm)
0.1	52.05	86.75	1.045
0.2	63.6	105	1.062
0.3	69.16	112.6	1.252
0.5	76.4	127.33	1.336
0.8	79. 50	130	1.447
1	83.1	135.17	1.450
1.5	80.3	133.5	1.482
2	78.5	134.83	1.49
2.5	78.5	131.483	1.451

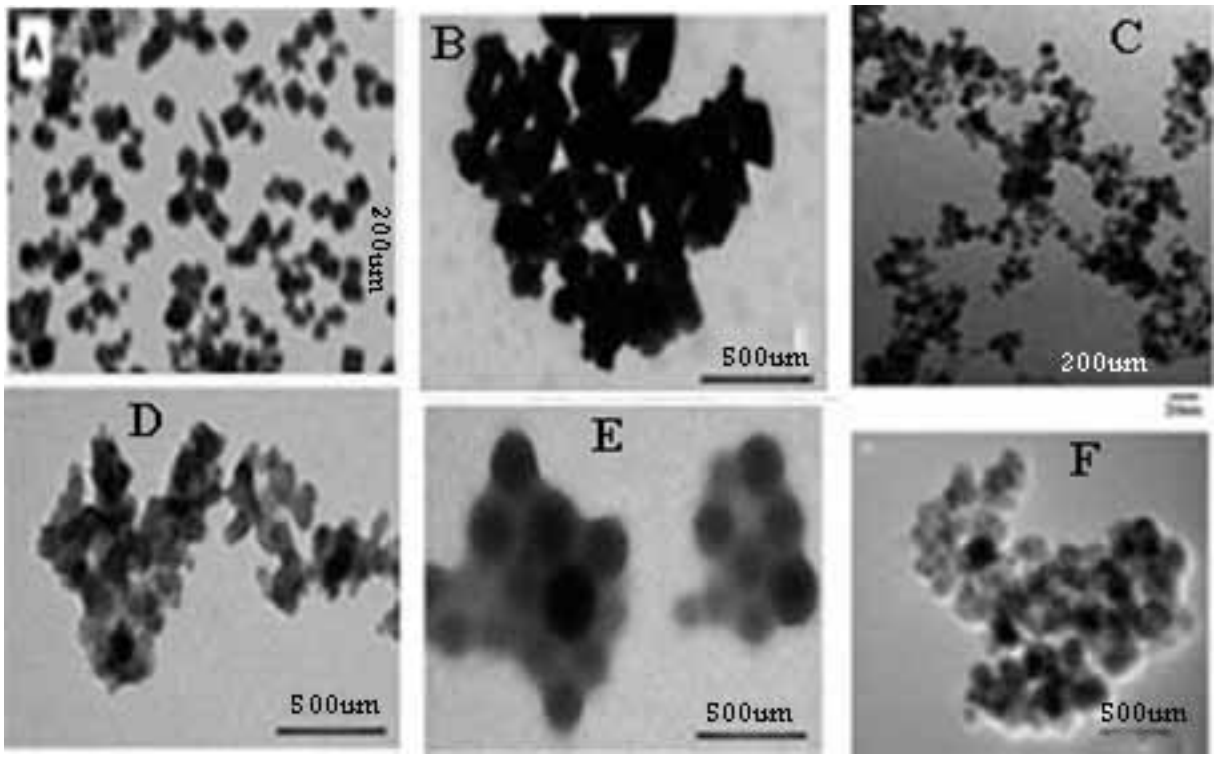


Figure 9.Transmission electron microscope images ofstructure and shape of the support fornanoparticles. The particle size and morphology of Fe_3O_4, Fe_3O_4/SiO_2, and Fe_3O_4/SiO_2/enzyme were evaluated from the TEM micrographs(A,B and C) Fe_3O_4 nanoparticles, size distribution is 200-500 nm with and without silica (D, E and F) Fe_3O_4 size distribution is 500 nm cover with immobilized lipase silica aggregated together

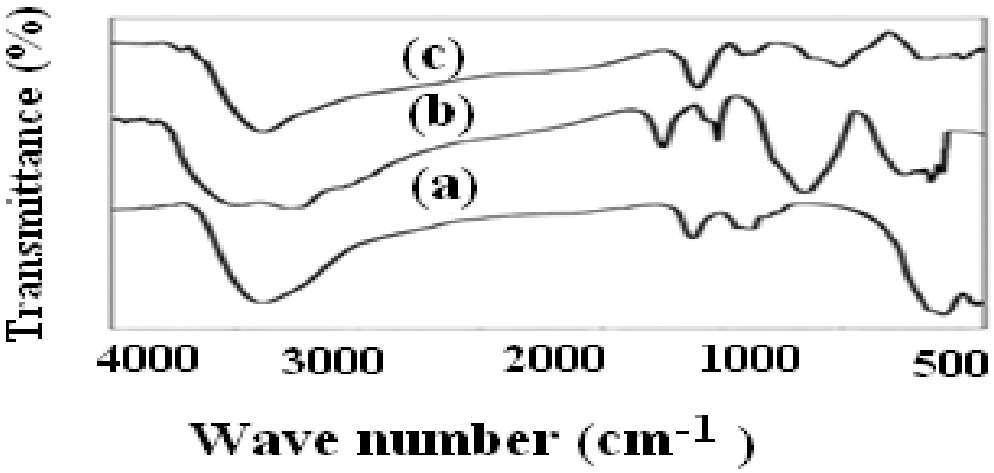


Figure 10. (a) Fourier transform-infrared spectrophotometer spectra of Fe_3O_4, Wave number (b) MSI without immobilized lipase, and (c) MSI with immobilized lipase

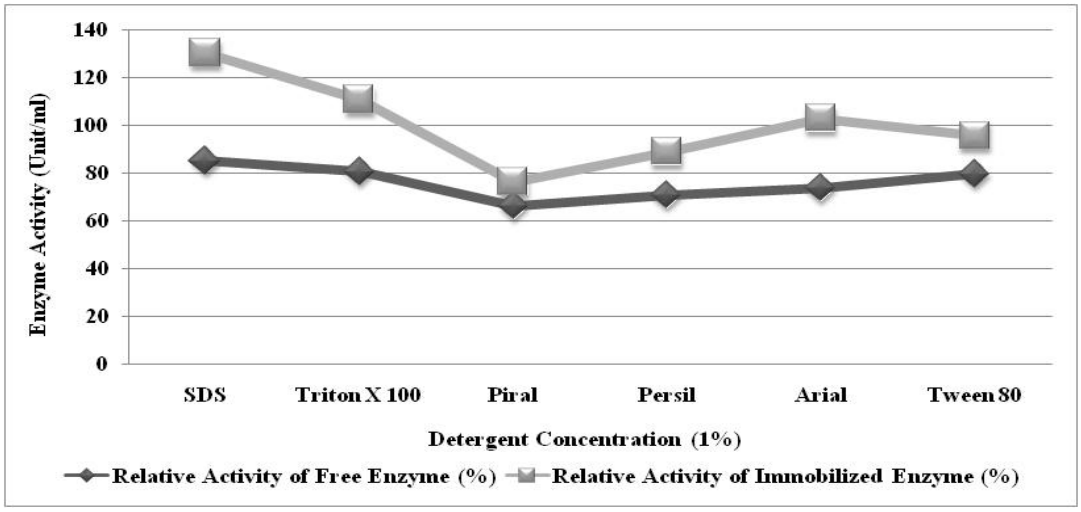


Figure 11. Effects of various detergents on lipase productivity on free and immobilized enzyme

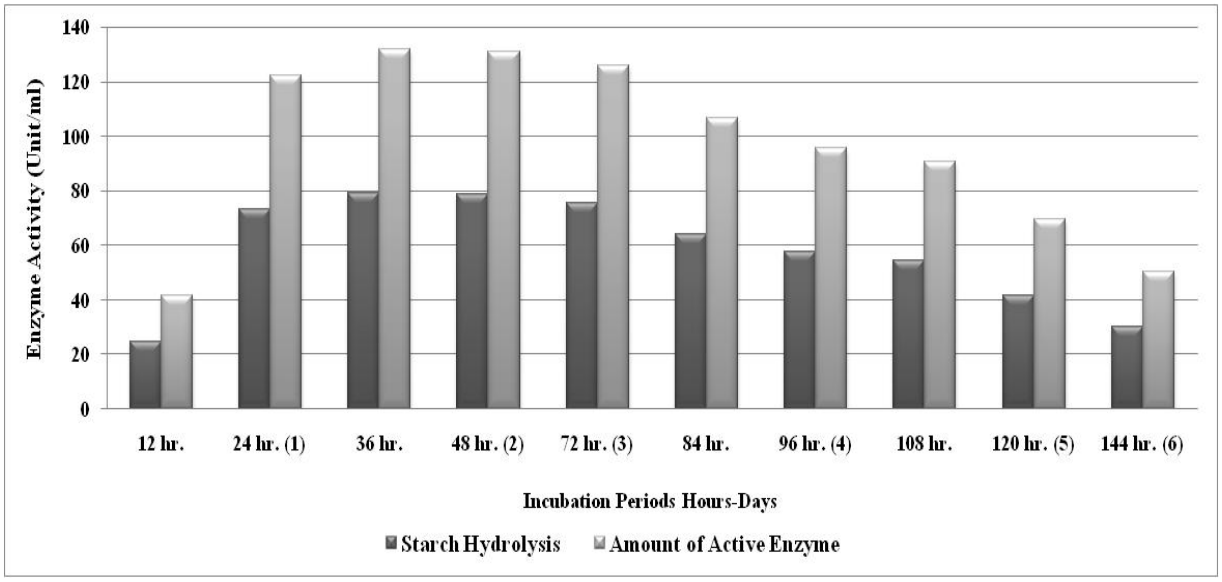


Figure 12. Effect of different incubation periods, on productivity of lipolytic enzymes, using its concentrated cell free filtrate

Maximum production of active enzymes was 160.2 and 174.67 U. /ml at 40°C, respectively. While production of active enzymes was 165.17 U./ ml and 128.67U. /ml at 35°C and 50°C , respectively, due to the accumulation of lipolytic enzymes and maximum enzyme (Fig.5). This result is inaccordance with Lailaja and Chandrase karan (2013) who found that, *Bacillus smithii* BTMS 11, isolated from marine sediment, produced alkaline and thermo stable lipase.The enzyme was found to have 50°C and pH8 as optimum conditions for maximal activity. However, the enzyme was active over wide range of temperatures (30-80°C) and pH (7.0-10.0).Halo-alkaliphilic lipolytic bacteria have been recognized for many years, and enzyme lipase have emerged as key product of rapidly growing biotechnology industries, such as food,detergent, chemical and have biomedical applications1. Lipase catalyzes lipids and liberates fatty acids and glycerol additionally; they also catalyze the synthesis of glyceride Gupta et al (2004).Alkaline or saline conditions over the last two decades were highly active between 40 and 60°C (Hiol *et al*., 2002).The lipase showed a novel property of marked activation on prolonged incubation at alkaline pH wash performance analysis of the commercial detergent for removal of fatty stains improved upon addition of this lipase Almost all lipases enzymes studied under extreme

The productivity of active free lipase and immoblized enzyme was measured at pH10 and different concentration of NaCl (Fig 6). The results cleared that lipase yield was increased by increasing salt concentration from10% to 18% salt, with shortened incubation periods. This is in accordance with the present results where high productivities of lipase were obtained with 18% NaCl than with 5 and 25 %. Preliminary tests for high production of lipase from concentrated broth filtrate adjusted at pH from 7 to 12 in presence of 15% NaCl.

High enzymes productivity was detected (90.33-130.17 -U/ ml) at pH of 9 to 10.5, with vigorous growth, while the productivity was the highest due to the immobilized enzyme.The results in (Fig 7) cleared that lipase yield was increased by increasing pH's value from pH7 till pH10 So far, the production of lipase by halophilic microorganisms of *Salini vibrio* and *Natronococus*has been reported (Amoozegar *et al*., 2008). Halophiles use different methods to resist the NaCl bactericidal effect. There are a lot of similar fundamental methods to send sodium ions into the outside of the cell. The usual method includes the accumulation of ion K+ and Cl- inside the cell to keep osmotic balance. Different types of oil (Fig. 8) were tested forthe selection of the best substrate for free lipase production.Also different oils were tested on immobilized enzyme.

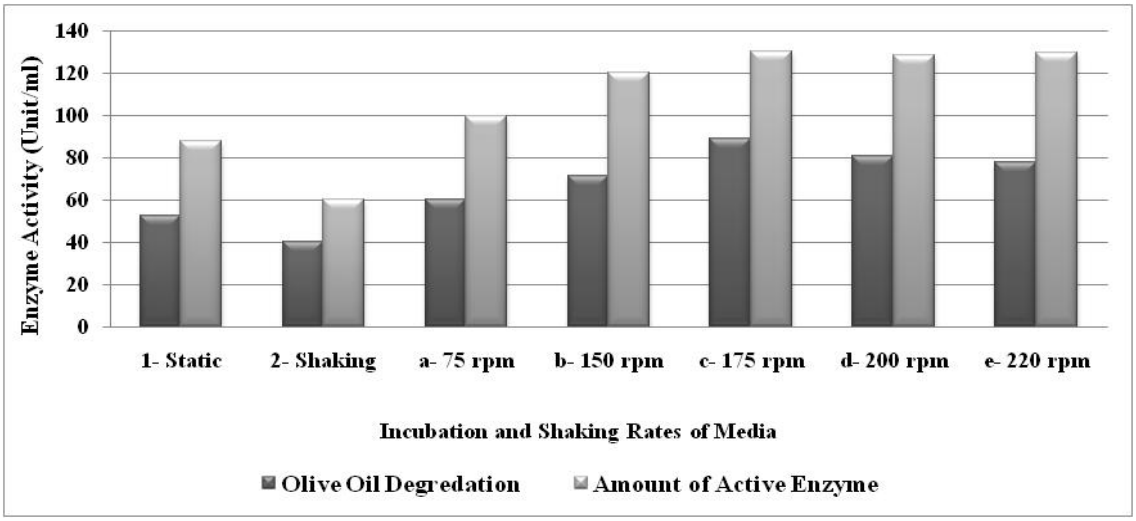


Figure 13. Effect of aeration productivity of lipolytic enzymes using its concentrated cell free filtrate

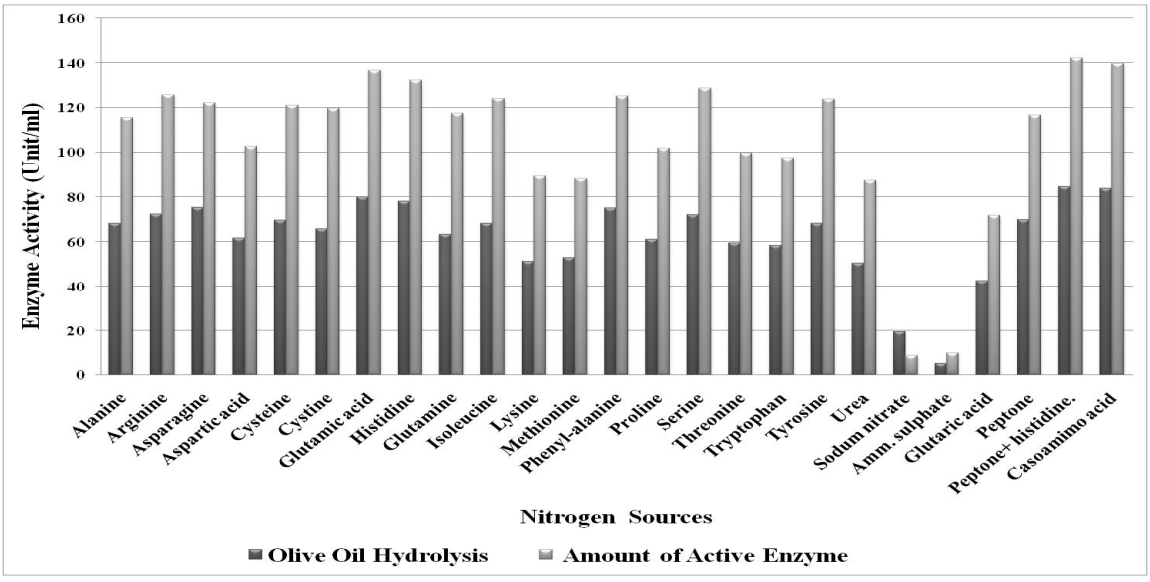


Figure (14): Effect of different nitrogen sources on productivity of lipolytic enzymes - using its concentrated cell free filtrate

It is clear that olive oil, corn oil and sun flower oil gave higher production of enzyme productivity. Also Effect of different concentrations of olive oil on lipase produced in media table (2) was examined and higher production of enzyme productivity between 0.8 to 1.5 ml. In a recent study Elham *et al.* (2016) reported that, the extremophile lipases have more range applications. These enzymes are resistant to the high salty, temperature and alkaline conditions. The best environmental conditions and their interactions for lipase production were obtained .They studies the production of lipase by a novel halotolerant and psychrotolerant bacterium, *Planomicrobium okeanokoites* ABN-IAUF-2.The most enzyme production was measured after 72 hours incubation at 20°C in the presence of hazelnut oil as carbon source and yeast extract as nitrogen source and pH 7. The analysis of Taguchi test showed that the most effective factors in enzyme production were carbon source with 54.65% and nitrogen source with 19% of effectiveness. The particle size and morphology of Fe3O4, Fe3O4/SiO2, and Fe_3O_4/SiO_2/enzyme were evaluated from the TEM micrographs (Fig.9). It was noteworthy that the size distribution was 200-500 nm, the nanoparticles are irregular spherical in shape and their aggregation can be discerned clearly. In (Fig. 10) FTIR clear (a) Magnetic silica nanoparticles (MSI) without immobilized lipase, and (c) MSI with immobilized lipase (b) and (c), the coated silica layer can be observed as a typical core–shell structure of the Fe_3O_4/SiO_2nanoparticles.Thedispersity of the Fe_3O_4/SiO_2 nanoparticles was also improved, and the average size increased.

Wenlei and Ning (2009) reported that, magnetic Fe_3O_4 nanoparticles treated with (3-aminopropyl) triethoxysilane were used as immobilization material. Lipase was covalently bound to the amino-functionalized magnetic nanoparticles by using glutaraldehyde as a coupling reagent with the activity recovery up to 70% and the enzyme binding efficiency of 84%. Moreover, the immobilized lipase was found to be able to catalyze the trans esterification of soybean oil with methanol to produce fatty acid methyl esters (better known as biodiesel). Further study showed that the immobilized lipase could be used four times without significant decrease of activity. conditions as the surfactants showed different rates of lipase exhibited better resistance strongly to Sodium Dodecyl Sulphate (SDS), Triton X-100 Arial, Tween 80 and Persil, respectively, (Fig. 11). After lipase adsorption, the degree of particle aggregation increase was investigated the effects of various detergent on activity of lipase, however, a change in the lipase activity on free enzyme and immobilized enzyme demonstrated thecommercial detergents at the same conditions as the surfactants showed different rates of lipase exhibited better resistance stronglyto Sodium Dodecyl Sulphate (SDS),Triton X-100, Arial,tween 80 and Persil, respectively, (Fig.11).Highly lipase from *Kocuria polaris*-WRS3 exhibited better resistance to commercial detergents There was a slight decrease in free enzyme thanproductivity of the immobilized lipase. Lei *et al*. (2011) investigated that,Silica and its derivatives when coated onto the surface of magnetic nanoparticles may help to change their surface properties. With the appropriate coating, the magnetic dipolar attraction between the magnetic nanoparticles may be screened, thus minimizing or even preventing aggregation. The coating film could also provide a chemically inert layer against the nanoparticles, which is particularly useful in biological systems.

The larger specific surface area and surface reactive groups that are introduced by further modification of silica materials are favorable during the preparation of silica carriers for immobilized enzymes, and these carriers are very suitable for adsorption and immobilization of the adsorbed protein abundantly and steadily. The dispersity of the Fe_3O_4/SiO_2 nanoparticles was also improved, and the average size increased. In a recent study our results agree with Ghada *et al*. (2015) who investigated the effects of various detergents on activity of lipase. They demonstrate the effects of various detergents on enzyme activity. *Aspergillus terreus* lipase was sensitive to SDS than the other. In 1.0% SDS concentration, the enzymes showed 70% activity after 1 h storage. This level of stability was similar to or higher than those reported for other bacteria. The enzyme was relatively stable when stored with tween-80, DMSO and Triton X-100 (i.e., the activity remaining higher than 80%). It is not compatible with our results, *Schmidt- Dannert et al*. (1994) who reported a total loss of lipolytic activity in the presence of Tween 20 and Tween 80, but no effect was observed when incubated with Triton X-100. Also they found a total loss of activity in the presence of SDS but in contrast, activity was enhanced in the presence of Triton X-100, Tween 20 and Tween 80. Moreover, Rathi *et al*. (2001) studied the effect of commercial detergents as Ariel, Persil, pearl, extra, and tied, in addition to triton 100 and SDS on lipase activity. They found that the lipase from *B. cepacia* exhibited better resistance to commercial detergents (57-80% residual activity) than lipolase (40-80% residual activity) after 1h of incubation at 37ºC and pH 11.0.

In addition, Mostfa and El-Hadi (2010) also found a total loss of activity in the presence of SDS while, activity was enhanced in the presence of Triton X-100, Tween 20 and Tween 80. Matsumae & Shibatani (1994) demonstrates the effects of various detergents on enzyme activity. *P. aeruginosa* KM110 lipase was sensitive to SDS than the others. In 1.0% SDS concentration, the enzymes showed 70% activity after 1 h storage.This level of stability was similar to or higher than those reported for other bacteria .The enzyme was relatively stable when stored with Tween-80, DMSO and Triton X-100 (i.e., the activity remaining higher than 80%). Also, Lailaja and Chandrasekaran (2013) found that,Effect of a number of metal salts, solvents, surfactants, and other typical enzyme inhibitors on lipase activity was studied to determine the novel characteristics of the enzyme. More than 90 % of the enzyme activity was observed even after 3h of incubation in the presence of commercial detergents Surf, Sunlight, Ariel, Henko, Tide and Ujala which indicating the detergent compatibility with *B. smithii* lipase. Production of lipolytic enzyme was measured during different growth and incubation periods as well as under static and shaking conditions in media with 15% NaCl at pH10 and 45°C, containing 1% olive oil. Results of (Figs. 12 and 13) denoted high enzyme activity between 122.7 and 132 U / ml during first 24h until mid-stationary phase (72hrs.) and the maximum productivity of active enzymes was at 48hr. then the production of active enzymes began to decrease after 72hrs their minimum level after 6 days incubation. The effect of aeration of cultures on enzyme activities was also tested (Fig.13). High enzymes productivity was obtained at aeration speed 150- 220 rpm between 48h. to 72h Microbial lipase today occupies a place of prominence among biocatalysts owing to their ability to catalyze a wide variety of reactions inaqueous and non-they

study, *Aspergillus terreus* showed high lipase production in submerged culture (15.463 ± 0.39 U/mg).The optimum conditions for lipase production by *A. Terreus* were pH range 7-9, a temperature of 30-45ºC for a period of 6 days and ionic streng th 60 mMNaCl. Lipase production was also detected when *A. terreus* was grown on different hydrocarbons, olive oil or tween 80 but not on sugars indicating that the enzyme is inducible by hydrocarbons as well as oils. The kinetics studies showed that the Km value for *A. terreus* was 8.12 µmol, the optimum pH for lipase activity was 6, the optimum temperature was 30 ± 1ºC and lipase activity could tolerate high levels of ionic strength; however the fungal growth and lipase productivity were very sensitive to increase ionic strength Ghada *et al*. (2015).

Data revealed that high enzymes productivity was obtained (120.9 -129.33 U/ml), at 150- 220 rpm. Nitrogen sources were supplied individually to Horikoshi oil medium (Fig. 14).The results indicate that extracellular lipase produced from isolate WRS3 was stimulated and exhibited the highest activity (80 %,139 -142 U/ml) by the addition of peptone + histidine or casoamino acid + histidine to the culture medium. High productivity was also exhibited when casoamino acid was replaced in the culture medium by the following nitrogen sources :histidine, serine, phenylalanine, glutamic acid, asparagine and arginine. They gave enzymes activity of more than 122-130U/ml. However, sodium nitrate and ammonium sulphate repressed the enzyme production in media. Deyaa *et al.* (2016) investigated that, thermophilic and alkaliphilic lipases are meeting a growing global attention as their increased importance in several industrial fields. From over 23 bacterial strains, novel strain with high lipolytic activity was isolated from Southern Sinai, Egypt, and it was identified as *Geobacillus thermoleovoran*s DA2 using 16S rRNA as well as morphological and biochemical features. The lipase was produced in presence of fatty restaurant wastes as an inducing substrate. The optimized conditions for lipase production were recorded to be temperature 60^{o}C, pH 10, and incubation time for 48 hrs.

Enzymatic production increased when the organism was grown in a medium containing galactose as carbon source and ammonium phosphate as nitrogen source at concentrations of 1 and 0.5% (w/v), respectively. Moreover, the optimum conditions for lipase production such as substrate concentration, inoculums size, and agitation rate were found to be 10% (w/v), 4% (v/v), and 120rpm, respectively. The thermoalkaline lipase with Triton X-100 had the best degreasing agent by lowering the total lipid content to 2.6% as compared to kerosene (7.5%) or the sole crude enzyme (8.9%). It can be concluded that the chemical leather process can be substituted with TA lipase for boosting the quality of leather and reducing the environmental hazards. Alkaline saline lipase production from strain *Kocuria polaris*-WRS3 was crude and exhibited maximal activity under alkalotolerant and halophilic thermoduric conditions. Strain WRS3 exhibited the maximum productivity of active lipase that could be obtained in a large scale with the following modification of culture media g /L: olive oil,or corn oil 5ml, casoamino acid 5, histidine 2.5, NaCl concentration between 10 and 18% ,$CaCl_2$ 0.3,KC1 1.0, K_2HPO_4 1.0, $MgSO_4$ -7H_2O 0.2, yeast extract 3.0,Sodium citrate 3.0 , all these compound were dissolved in 900 ml , then sterilized and one ml of the stock mineral salt solution pH would be adjusted between 8.5 and 10.5 by using carbonate buffer. Inoculated media were incubated at 35^{o}C and 40^{o}C. Tambekar *et al*. (2015) reported that, Halo-alkaliphiles grow high pH and high salt concentration can be a source of novel enzymes. The enzymes produced by these bacteria have great importance in industry due to its high thermo and pH stability. The alkaline lipase producing bacteria are generally found in sea water or alkaline lakes such as Lonar Lake. A total of 17 bacterial cultures were isolated as methylotrophs from alkaline Lonar lake, 6 showed lipolytic activity and one strain DHT12 was prominent lipase producer which was studied further for its phenotypic and biochemical characters. The bacterium DHT12 was screened for production and partial characterizations of lipase, and 16SrRNA sequencings identified as *Pseudomonas aeruginosa.* The maximum lipase production was reported at pH 9, the activity was 3.7 unit/mL and optimally active at 60^{o}C. Thus the enzyme produced by this bacterium have promising applications in many industries like leather, soaps and detergents, pharmaceuticals, biofuel, oleochemical, food and textile. Also, Aliyu and Zahangir (2015) reported that, the isolation of microbial strains from extreme environments assists in producing lipases with unique characteristics. Advances in biotechnology associated with cloning, expression, and mutagenesis as well as directed evolution contribute to conferring different properties to microorganisms. Thus, the ability of lipase stobe stable in acid, alkali, salt, and organic solvents without deteriorating and detergents contributes immensely to their wideindustrial application. Generally results obtained in this study show that olive oil was the most suitable substrate for maximum lipase production by haloalkalophilic strain WRS3 which defined physiologically and phenotypic as *Kocuria Polaris* WRS3.Silica and its derivatives when coated onto the surface of magnetic nanoparticles may help to change their surface properties .The preparation of silica carriers for immobilized enzymes, were very suitable for adsorption and immobilization . Studies concentrated to enhance lipase production by this strain placing it under a different parameters of the lipase production as well as increasing enzyme expression with immobilized enzyme than free enzyme.After lipase adsorption, the degree of particle aggregation increase and the effects of various detergent on activity of lipase**,**was investigated.Lipase productivity was strongly resistance to Sodium Dodecyl Sulphate (SDS) and Triton X-100, Arial, Tween 80 and Persil, respectively. The remarkable productivity of *Kocuriapolaris*-WRS3lipase in this range has proved it to be a potential alkaline lipase similar, this lipase candidate for industrial applications such as detergent, leather and fine chemical industries. Also, magnetic nanoparticles provide an economically efficient and selective system for enzyme immobilization.

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