ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Isolation and identification two thermophilic and lipolytic bacterial strains from Saudi Arabia environment

Magdy M. Youssef[1, 2], Mohammed A. Al-Omair[2] and Hassan E. Abd-Elsalam[3,4*]

[1] Chemistry Department, Faculty of Science, Mansoura University, Egypt
[2] Chemistry Department, College of Science, King Faisal University, Saudi Arabia
[3] Soil and water Technology. Dep., Arid Land Cultivation Research Institute (ALCDI), City for Scientific Research and Technology Applications (CSAT), Borg El-Arab, Alex, Egypt,
[4] Biotechnology Dep. Faculty of science, Taif Univ., Saudi Arabia.

Manuscript Info

Manuscript History:

Received: 12 July 2014
Final Accepted: 25 August 2014
Published Online: September 2014

Key words:

Thermosphilic, lipase, 16S rRNA, Burkholderia pseudomallei, Staphylococcus Pasteuri

***Corresponding Author**

Hassan E. Abd-Elsalam

Abstract

In a screening program for isolation of thermophilic lipase-producing bacteria, a number of thermophilic bacteria were isolated from Al- Hassa region, Saudi Arabia. Among 93 isolates, potent bacterial candidates were characterized and identified by biochemical and PCR techniques, based on 16S rRNA sequencing. Phylogenetic analysis revealed its closeness to the thermophilic *Burkholderia pseudomallei (B. ps)* and *Staphylococcus Pasteuri (S. pa)* with optimal growth at 50°C for both strains and pH 8.0 and 7.5, respectively. An inducible nature of lipolytic enzyme synthesis using oils was demonstrated. Salt stress studies reveled that *S. Pa.* and *B. ps*. have the ability to tolerate NaCl salt up to 2% and 2.5% respectively. Both *S. Pa.* and *B. ps.* are the highest thermophilic bacteria generating lipase.

Introduction

Constitute of Lipids is a large part of the earth's biomass and lipolytic enzymes play an important role. Lipases have been recognized as very useful biocatalyst. They are the major industrial enzymes extensively used in food, pharmaceuticals, medical, textiles, and chemical industries (Akanbi *et al.*, 2010 and Mohan et al., 2008).

Microorganisms produce different classes of lipolytic enzymes, including lipases (EC 3.1.1.3), which display maximal activity towards water-insoluble long chain triglycerides, carboxylesterases (EC 3.1.1.1), which hydrolyze small ester-containing molecules at least partly soluble in water, and various types of phospholipase (Bornscheuer, 2002, Bisht and Panda, 2011 and Tirawongsaroj *et al.*, 2008).

Lipases are glycerol ester hydrolase that hydrolyse tri-, di-, and monoglycerides at an oil-water interface. They are currently attracting enormous attention because they constitute the most important group of biocatalysts for biotechnological applications (Jaeger *et al.*, 1997, Benjamin and Pandey, 1998, Jaeger and Rettz, 1998, Hasan *et al.*, 2006, and Tirawongsaroj *et al.*, 2008). Most of the lipases used in industry are microbial enzymes, of both fungal

and bacterial origin. Lipases show different substrate and positional specificities, and as a consequence, they find uses in various industries like food, chemical, pharmaceutical, cosmetic, leather processing and in the detergent industry (Sztajer and Maliszewska, 1988). Lipases, as a class of lipolytic enzymes, are stable and extremely valuable catalysts for many practical and industrial applications as the manufacture of liquid and dry detergents to dissolve fatty substances (Bjorkling *et al*., 1991).

Lipolytic enzymes, as triacylglycerol acylhydrolase and carboxylesterases have been detected in many organisms. The triacylglycerol acylhydrolases, catalyze the hydrolysis of ester bonds of triacylglycerols at the interface between an insoluble substrate and water (Verger, 1997). The hydrolytic activity of lipases and carboxylesterases are significantly enhanced by the presence of a lipid- water interface. The triacylglycerol acylhydrolase lipase enzyme can be defined as carboxylesterases that catalyze the hydrolysis and synthesis of relatively long-chain acylglycerols with acyl chain lengths of more than ten carbon atoms, with trioleoyl-glycerol as the standard substrate (**Jaeger** *et al*., 1994).

Bacterial lipases vary widely in enzymatic properties and substrate specificities. Consequently, they are currently receiving much attention because of their potential applications in various industrial processes and biotechnological applications as in fat, food ingredients, detergents, surfactants, textile industries and oil processing (Aunstrup, 1990). Microbial lipases have wide application in the processing of leather, domestic, industrial wastes and pharmaceutical industries (Ghosh *et al.* 1996, Saxena *et al*. 1999). The industrial demand for highly active preparations of lipolytic enzymes with appropriate specificity and stability to pH, temperature, ionic strength and organic solvents continues to stimulate the search for new enzyme sources (De Simone *et al*., 2001 and Madan and Mishra, 2009). Thermophilic bacteria are an important source of thermostable enzymes with properties that are often associated with stability in solvents and detergents, giving these enzymes considerable potential for many biotechnological and industrial applications (Coolbear *et al*., 1992). One of these enzymes is a thermostable lipase enzyme that has been applied to the synthesis of biopolymers and biodiesel and used for the production of agrochemicals, cosmetics, and flavors (Jäeger and Eggert, 2002).

Thermophilic lipases show higher thermostability, higher activity at elevated temperature and often show more resistance to chemical denaturation. This makes them ideal tools in industrial and chemical processes where relatively high reaction temperatures and/ or organic solvents are used. The industrial demand search for the thermostable continues to stimulate the microorganism's produces of thermostable enzymes. A small number of thermophilic lipase producing bacteria have been described in the last decades (Kambovrova *et al*., 2003 and Dharmusthii and Luchai,1999). Haki and Rakshit (2003) reported that a few thermostable lipases have been isolated from thermophiles and hyperthermophiles. The knowledge of thermostable lipolytic enzymes in industrial applications is increasing at a rapid and exciting rate. The aims of this research were isolate, purify thermophilic bacteria and identify through studies the Molecular and biochemical characterization of the thermophilic bacterial isolates from Saudi Arabian environment.

Material and Methods:

Sample collection

The soil samples were collected from different locations in the eastern region of Kingdom of Saudi Arabia contaminant with oil in a sterile container for the isolation of lipase producing organisms under laboratory condition.

Screening of microorganism

Soil samples were brought to the laboratory and 10 g of sample was suspended in 100 ml of sterile distilled water, agitated for half an hour on a shaker at 50 ºC. transfer 10 ml of this homogenize solution to 100 ml of minimal media (KH_2PO_4 3.0 g, Na_2HPO_4 6.0 g, NaCl 5.0 g, NH_4Cl 2.0 g, $MgSO_4$ 0.1 g in 1 lit of distilled water), supplemented with 2% olive oil as a sole carbon source in sterile flasks and incubated at 50°C in a shaking incubator at 180 rpm for two weeks. The viable bacterial count was performed by serial dilution technique using 10^{-2} - 10^{-3} inoculate (Daley, 1979). One ml of the prepared dilutions was transferred into sterilized Petri dish containing LB

media or Rhodamine B- olive oil nutrient agar medium and incubated at 50 ^{o}C for 24h, 48h and 72h. The Rhodamine B- olive oil Petri dishes were visualized on UV- Transilluminator at wave length 350 nm. Pink colonies mean non lypolytic bacteria while the orange colonies mean lypolytic bacteria (Sandoval and Marty; 2007).

Ninety three lipolytic thermophilic bacterial were isolated, each of the thermophilic bacterial isolates was streaked out and purified on LB and Rhodamine B – Olive oil nutrient agar media.

Each bacterial isolate was stained with Malachite green and safranin stains to identify the spore formation of isolated thermophilic bacterial strains. All bacterial isolate was stained with Gram stains to identify Gram +ve and Gram –ve bacterial strains. The dominant organisms were isolated and individually streaked on tributyrin (Hi media 071) agar plates and the formation of halo zone around the colony on tributyrin agar was considered as the positive colony

One ml of the prepared dilutions was transferred into sterilized Petri dish containing LB media or Rhodamine B- olive oil nutrient agar medium and incubated at 50 ^{o}C for 24h, 48h and 72h. The Rhodamine B- olive oil petri dishes were visualized at 350 nm UV box as illustrated in Figure 1. Pink colonies mean non lypolytic bacteria while the orange colonies mean lipolytic bacteria (Sandoval and Marty; 2007).

Molecular Identification of the selected bacterial isolates:

The bacterial isolate showing the highest lipolytic activities (43 isolates) were identified using 16 S rDNA sequencing

DNA Isolation and PCR Amplification of 16S rDNA:

The DNA was extracted from one bacterial isolate that considered the best selected isolates, according to the method described by Sambrook *et al*., (1989).

Amplification of the 16S r RNA genes:

According to Edwards *et al*.(1997) and Wilson *et al*. (1997), the 16S r-RNA gene was amplified by using two universal primers and the sequences of these primers were illustrated in table (1)

Table (1) Sequence of the two primers used in amplification of 16S r RNA gene.

Primer	Nucleotide sequence
F (27)	5'AGAGTTTGATCCT GGCTCAG3'
R (1492)	5'GGTTACCTTGTTACGACTT3'

The PCR amplification were carried out in a total volume 50µl containing 5 µl 10x buffer, 4 µl 25 mM $MgCl_2$, 4µl 2.5mM dNTPs, 2 µl 10 pmol of each forward and Reviser primers, 2µl of 50 ng bacterial genomic DNA and 0.4 µl (5 U μl^{-1}) Taq DNA polymerase (Promega, Germany). Deionized water was added to complete the volume to 50µl. PCR amplification was performed in a 9700 thermal cycler PCR (Eppendorf, Germany) programmed for one cycle at 95ºC for 5 min followed by repeated 35cycles as following: 1 minute at 95ºC for denaturation, 1 min at 60ºC for annealing, 2 min at72ºC for elongation. Reaction mixture was then Incubated at 72ºC for 10 min for final extension then mixtures was hold at 4ºC (Sambrook *et al*., 1989).

Sequencing of 16S rRNA Gene:

The PCR products were routinely analyzed by electrophoresis in 1.0% agarose gels in 1x Tris-Borate-EDTA buffer (Sambrook *et al*., 1989). The target band was purified using gel extraction kit (Qiagene, Germany).

The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers as described (Table 1). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) at Macrogen lab in south Korea. Approximately 1000 bp was sequenced and phylogenetic analysis of the thermophilic lipase-producing bacteria isolates based on 16S r-RNA genes were carried out by pair-wise and multiple DNA sequence alignment comparison using clustalW version 4 (Thompson *et al.*, 1994). Bootstrap neighbor-joining tree (Saitou and Nei, 1987) was generated using mega version 4.0 (Tamura et al., 2007).

Growth optimization for promising isolates

The best two thermophilic lipase-producing bacteria isolates were selected to various characterizations. The optimization factors including pH value, incubation temperature, agitation and salinity are demonstrated to obtain the best optimum conditions for highly growth and lipolytic activity.

Effect of pH on the growth rate

The influence of pH on the growth of the two selected thermophilic lipase-producing bacterial isolates was assessed using LB medium. The pH of the medium was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 using 1.0 M HCl or 1.0 M NaOH. The prepared medium was autoclaved and inoculated with both the two selected isolates individually, then incubated under shaking (120 rpm) at 50±0.1ºC for 6h. In 100 ml conical flask, 20 ml of LB media with different pH was used. Approximately 20µl of fresh bacterial culture was inoculated. The optical density (O.D.) of the growth culture was measured at 600nm.

Effect of temperature on the growth rate.

Different incubation temperatures were used at 25, 30, 35, 40, 45, 50, 55, 60, and 65°C. LB medium was prepared and the optimum pH was adjusted as previously mentioned, the medium was autoclaved and inoculated with the selected isolates and incubated at previously temperature for 6 hrs under shaking (120 rpm). The prepared medium was autoclaved and inoculated with both of the two selected bacterial isolates. In 100 ml conical flask, 20 ml of LB media, approximately 20µl of fresh bacterial culture was inoculated and incubated at the different selected temperatures. The OD of the growth culture was measured at 600nm.

Effect of agitation speed (rpm) on the growth rate:

The influence of different agitation rates on the growth of the two selected thermophilic lipase-producing bacterial isolates were assessed using LB medium. The agitation rate was adjusted at 0, 50, 100, 150, 200, 250 and 300 rpm by using water bath incubator shaker. The medium was prepared, autoclaved and inoculated with both isolates individually at 50±0.1ºC for 6h. In 100 ml conical flask, 20 ml of LB media at pH 7.0 was used. Approximately 20µl of fresh bacterial culture was inoculated. The optical density (O.D.) of the growth culture was measured at 600nm.

Effect of salt concentration on the growth rate

Different concentration of salt (Sodium chloride) were used in LB medium (0.5, 1.0,1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 %) were used to determine the best one for high growth rates of the selected isolates, the optimum pH and temperature were adjusted as previously mentioned. The medium was autoclaved and inoculated with the selected isolates and incubated for 6 hrs. In 100 ml conical flask, 20 ml of LB media, 20µl of fresh bacterial culture incubated at 50 ºC. The OD of the growth culture was measured at 600nm.

Results and Discussion:

From the 16S rRNA sequencing analysis (data not shown) of the remainder bacterial isolates, we identified the thermophilc lipase-producing bacterial isolates as *Bacillus licheniformis, Bacillus saphuricus, Pseudoalteromonas sp, Staphylococcus Pasteuri, Bacillus sp, Bacillus subtilis, Bacillus cereus and Burkholderia pseudomall.*

The highly two lipolytic activity thermophilic lipase-producing bacterial strains were *Burkholderia pseudomall* and *Staphylococcus pasteuri*.

Alignments and phylogenetic analysis

The selected isolates were identified by partial sequencing of the PCR amplified 16S rDNA gene. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rRNA sequences. Comparing the sequence of the 16S rDNA gene of the isolates with the sequences in GenBank revealed that one of the highly two lipolytic activity thermophilic lipase-producing bacterial isolates is similar to *Burkholderia pseudomallei* MSHR346 (CP001408), *Burkholderia pseudomallei* 668 (CP000570), *Burkholderia pseudomallei* 1710b (CP000124), and *Burkholderia mallei* ATCC 23344 (CP000010) with 99% similarity while, *Burkholderia thailandensis* E264 (CP000086) with 97% similarity and the other isolate is similar to Uncultured bacterium (FM874204), *Staphylococcus sp*. dv8 (FJ773995), *Staphylococcus pasteuri*. BQN3T-02d (FJ380992), *Staphylococcus pasteuri*. BQN3C-01d (FJ380983), and *Staphylococcus pasteuri*. NJ-1(FJ435675) with 84% similarity as showed in Table (2).

Table 2. Similarity percentage of 16S rDNA sequences for the selected isolates compared to those obtained from database.

Isolate	Identity	% Identity	Accession No.
Burkholderia pseudomallei	*Burkholderia pseudomallei* MSHR346	99%	CP001408
	Burkholderia pseudomallei 668	99%	CP000570
	Burkholderia pseudomallei 1710b	99%	CP000124
	Burkholderia mallei ATCC 23344	99%	CP000010
	Burkholderia thailandensis E264	97%	CP000086
Staphylococcus Pasteuri	Uncultured bacterium	84%	FM874204
	Staphylococcus sp. dv8	84%	FJ773995
	Staphylococcus pasteuri. BQN3T-02d	84%	FJ380992
	Staphylococcus pasteuri. BQN3C-01d	84%	FJ380983
	Staphylococcus pasteuri. NJ-1	84%	FJ435675

The phylogeny of the strains *Burkholderia pseudomaleil, Staphylococcus Pasteuri* and closely related species were analyzed using the multisequences alignment program and the results are presented in a phylogenetic tree is represented in Figure (1).

The phylogenetic tree in figure (1) demonstrated the linkage of *Burkholderia pseudomallei* and *Staphylococcus pasteuri*; this was constructed using 30 different isolates from GenData Bank (http://www.ncbi.nlm.nih.gov). Similar data can be generated using the partial 16S rRNA sequence data set for *Burkholderia pseudomallei* and *Staphylococcus Pasteuri*. The phylogenetic tree generated from *MEGA* version 4 and ClustalW for isolates *Burkholderia pseudomallei* and *Staphylococcus Pasteuri* indicated that there are two clusters, one of them contains *Burkholderia pseudomallei.*

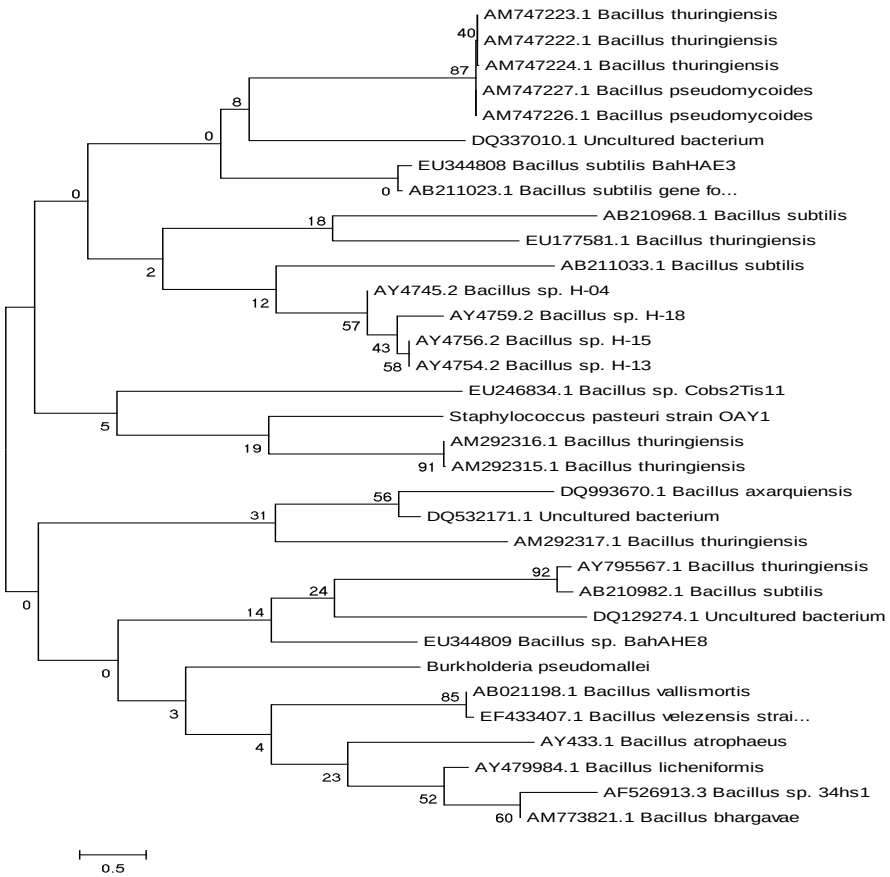

Figure 1: Phylogenetic tree of the *Burkholderia pseudomall* and *Staphylococcus Pasteuri* isolates and their related genera has been linked based on partial 16S rRNA sequence comparisons. Their names and respective accession numbers are given in the tree.

Growth optimization selected strains

The growth optimization of the highly two selected lipolytic activity thermophilic lipase-producing bacterial strains was studied with some parameters such as pH value, incubation temperature, agitation speed and salinity concentration demonstrated to obtain the best optimum conditions for highly growth and lipolytic activity of both Burkholderia pseudomallei and Staphylococcus Pasteuri.

A- Effect of pH on the growth rate

The influence of hydrogen ion concentration (pH) on the growth of the two selected thermophilic strains (Burkholderia pseudomallei and Staphylococcus Pasteuri) was assessed using LB medium. The pH medium was adjusted in the range from pH 5.0 up to pH 10.0 using 1.0 M HCl or 1.0 M NaOH and incubation for 6 hrs at 50°C after inoculation the bacterial strains. The optical density (O.D.) of the growth culture was measured at 600nm. As showed in figure (2). The growth rate was increased with decreasing the acidity (increasing pH) of the LB medium up to pH 7.5 for Staphylococcus Pasteuri, where the maximum growth at this degree of the acidity was 0.798 (O.D reading) while decreasing the growth from pH 8 up to pH 10 to be 0.007. While, in respect of Burkholderia pseudomallei the optimum pH was 8.0 where, the maximum growth 0.700 (O.D. reading) as illustrated in figure (2), while decreased dramatically the growth of Burkholderia pseudomallei to be 0.021 (O.D.) at pH 10.

So, from the results, we can conclude that the maximum pH condition for growth of both Burkholderia pseudomallei and Staphylococcus Pasteuri were 7.5 and 8.0 respectively, when the incubation the two strain for 6 hrs at 50°C.

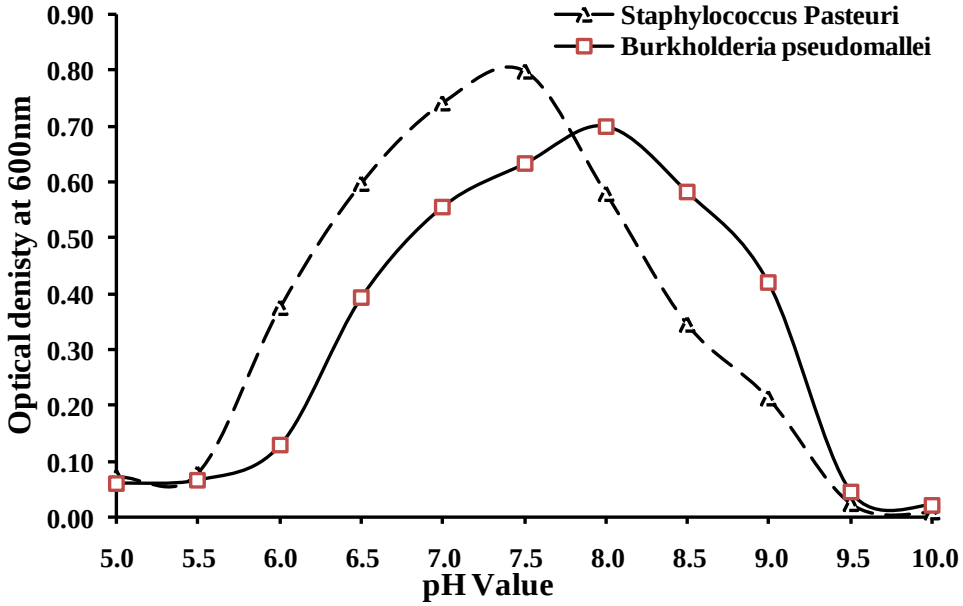

Figure 2: The relationship between pH and growth rate of Burkholderia pseudomallei and Staphylococcus Pasteuri for 6 hrs incubation at 50°C.

B- Effect of incubation temperature on the growth rate.

Different incubation temperatures (25, 30, 35, 40, 45, 50, 55, 60, and 65ºC) were used to study the optimum temperature for growth rate of each selected strains (Burkholderia pseudomallei and Staphylococcus Pasteuri) at pH 7.0 and incubated in incubator water bath shaker for 6 hrs at 120 rpm. The obtained results are showed in figures 12 and 13 for bacterial strains Staphylococcus Pasteuri and Burkholderia pseudomallei, respectively. In figure (3) the growth rate of Staphylococcus Pasteuri was increased with increasing the temperature of the strain incubation and the Staphylococcus Pasteuri recorded the highest growth rate (O.D: 1.088) at 50ºC, while the growth rate was dramatically decreased when the temperature increased over 50ºC to be 0.156 at 60ºC and 0.067 at 65ºC. Also, in respect of Burkholderia pseudomallei and from the results that showed in figure (3), was the same pattern as in the growth of Staphylococcus Pasteuri, the highest growth rate was recorded at 50ºC (O.D: 1.167) and the growth rate was fall when increased the temperature of incubation up to 60ºC (O.D: 0.111) and at 65 ºC the O.D of the growth became 0.044.

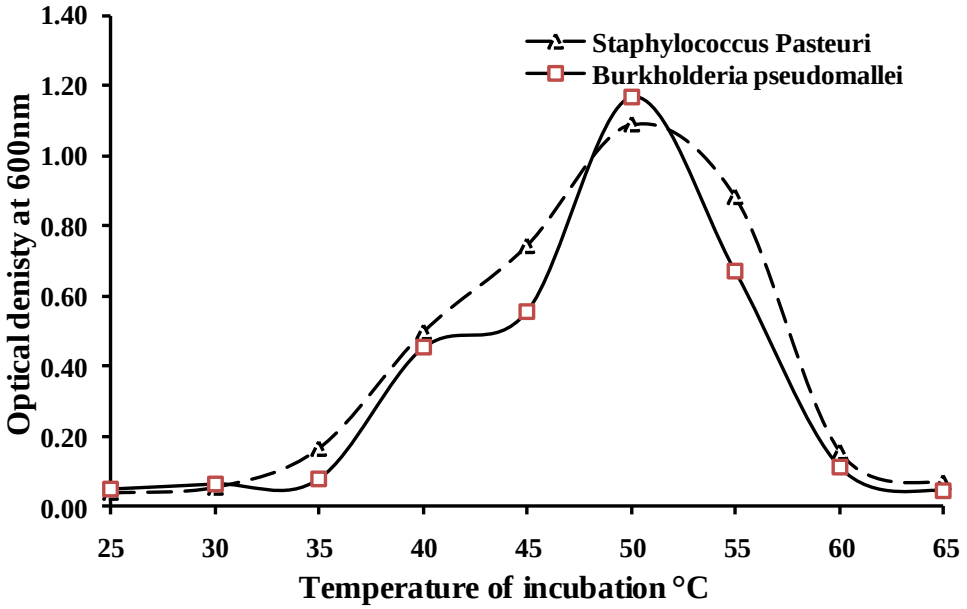

Figure 3: The relationship between temperature and growth rate of Burkholderia pseudomallei and Staphylococcus Pasteuri after incubation for 6 hrs at different temperature degrees.

C- Effect of agitation speed on the growth rate:

Effect of agitation speed on growth rate of Burkholderia pseudomallei and Staphylococcus Pasteuri strains was studied at speed of agitation from static stage (zero shaking) to 300 rpm. As showed in figure (4), growth rate of Staphylococcus was increased gradually with increasing agitation speed, the growth rate increased approximately twofold (O.D: 1.526) at speed 200 rpm compared with the growth rate (O.D: 0.887) at agitation speed 150 rpm, then slow gradually increased the growth rate to reach the maximum at agitation speed 300 rpm. In respect of Burkholderia pseudomallei as showed in figure (4), the growth rate was increased linearity to reach the maximum growth (O.D: 1.212) at agitation speed 200 rpm and decreased the growth rate with increase agitation speed up to 300 rpm.

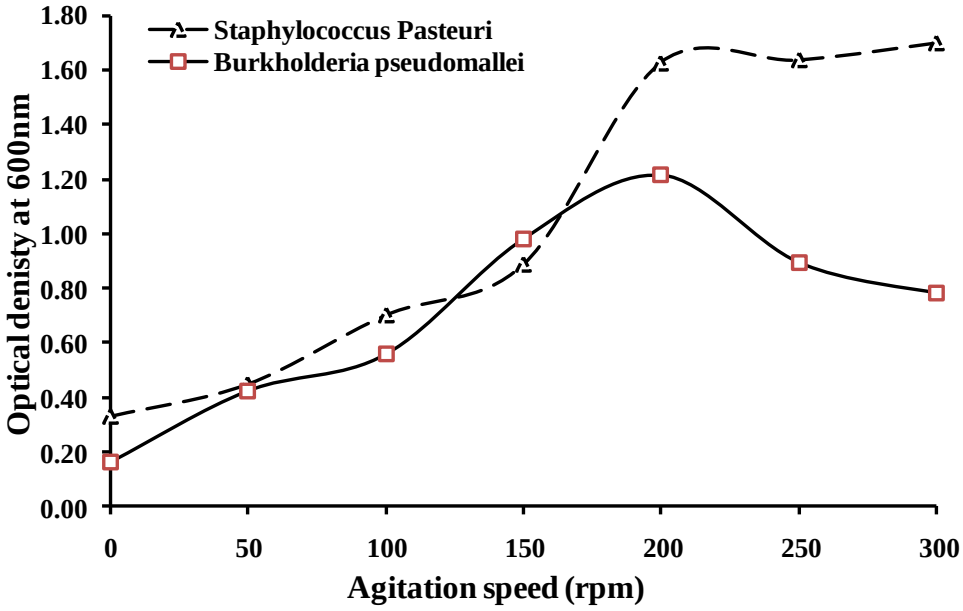

Figure 4: The relationship between agitation speed (rpm) and growth rate of Burkholderia pseudomallei and Staphylococcus Pasteuri after incubation for 6 hrs at 45°C.

D- Effect of salt concentration on the growth rate

Effect of salt stress at different rates of salt as Sodium chloride (0.5, 1.0,1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 %) in LB medium on the growth rates of strains Burkholderia pseudomallei and Staphylococcus Pasteuri were used to determine the best salt concentration produce high growth rates of the selected strains, at stability both of pH, agitation and temperature as previously mentioned and incubated for 6 hrs in 100 ml conical flask, and the O.D of the growth rate was measured at 600 nm against control of the same media. As showed in the figure (5) the Staphylococcus Pasteuri recorded the highest growth rate (O.D: 1.258) at 2% of sodium chloride concentration in the growth medium while the growth rate was decrease gradually with increasing sodium chloride concentration, where the growth rate became 0.358 as O.D at 6% of sodium chloride concentration. In respect of strain Burkholderia pseudomallei as showed in figure (5), the growth rate increased from 0.712 to 1.086 as O.D in the medium with increasing concentration of sodium chloride from 0.5% to 2.5%, while the strain growth rate decreased gradually with increasing concentration of the salt from 2.5 to 6% (O. D at 6% concentration was 0.248).

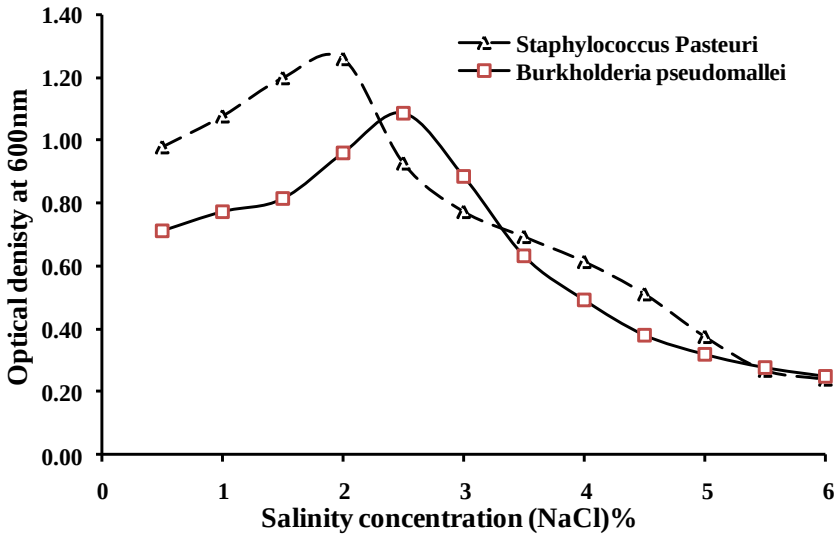

Figure 5: The relationship between salt concentration as sodium chloride (%) and growth rate of Burkholderia pseudomallei and Staphylococcus Pasteuri after incubation for 6 hrs at 50°C.

Conclusion:

In a screening thermophilic lipase-producing bacteria in kingdom of Saudi Arabia environment, forty three native bacterial strains were isolated from different contaminated soil and water sites from Al- Hassa region, potent bacterial candidates were characterized and identified by biochemical and PCR techniques, based on 16S rRNA sequencing. Two of the thermophilic lipase-producing bacterial isolates recorded highest lipolytic activity, Phylogenetic analysis revealed its closeness to the thermophilic Burkholderia pseudomallei and Staphylococcus Pasteuri.

The maximum growth rate of the highly lipolytic activity bacterial strains Burkholderia pseudomallei and Staphylococcus Pasteuri, was studied with both temperature of incubation growth, acidity of growth medium (pH), salinity concentration in the growth medium and agitation rate of incubation growth were evaluated.

Temperature influences on the growth rate of both Staphylococcus Pasteuri and Burkholderia pseudomallei, where the maximum growth was found at 50ºC. So, we can conclude that the two bacterial strains: Staphylococcus Pasteuri and Burkholderia pseudomallei are thermophilic bacteria. In respect of acidity, from the previous results the best acidity condition to growth of the two strains are grown in slightly alkalinity condition in the medium at pH 7.5 and 8.0 for both Staphylococcus Pasteuri and Burkholderia pseudomallei respectively.

The growth rate of Staphylococcus Pasteuri increased graduated with increasing agitation speed of its growth medium, while the maximum growth of Burkholderia pseudomallei was found with agitation speed 200 rpm. The two strain of used bacteria (Staphylococcus Pasteuri and Burkholderia pseudomallei) were tolerate the sodium chloride salt in the growth medium and the maximum growth rate were at 2% and 2.5%, respectively.

References:

Akanbi, TO, AL Kamaruzaman, F Abu Bakar, N. Sheikh Abdul Hamid, S. Radu, MY Abdul Manap, and N. Saari (2010) Highly thermostable extracellular lipase-producing Bacillus strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing. Internat. Food Res. J 17: 45-53.

Aunstrup, K. (1990). Novo Nordisk foresees bright future for genetically engineered industrial enzymes. Eur. Biotechnol. Lett. 94:3.

Benjamin, S. and Pandey, A. (1998). Candida rugosa lipases: Molecular biology and Versatility in Biotechnology. Yeast. 14: 1069-1087.

Bisht, S. S. and A. K. Panda (2011) Biochemical Characterization and 16S rRNA Sequencing of Few Lipase-Producing Thermophilic Bacteria from Taptapani HotWater Spring, Orissa, India. Biotechnol. Res. Internat.

Bjorkling, F., Godtfredson, S.E. and Kirk, O, (1991).The future impact of industrial lipases, Trends in Biotechnol. 9, 360-363.

Bornscheuer, U.T. (2002) Microbial carboxyl esterases: classification properties and application in biocatalysis. FEMES Microbiol.Rew.26: 73-81.

Coolbear, T., R. M. Daniel, and H. W. Morgan. (1992). The enzymes from extreme thermophiles: bacterial sources, thermostabilities and industrial relevance. Adv. Biochem. Eng. Biotechnol. 45:57-98.

De Simone, G., V. Menchise, G. Manco, L. Mandrich, N. Sorrentino, D. Lang, M. Rossi, and C. Pedone. (2001). The crystal structure of a hyper-thermophilic carboxylesterase from the archaeon Archaeoglobus fulgidus. J. Mol. Biol. 314**:**507-518.

Dharmusthii, S. and S. Luchai, (1999). Production, purification and characterization of thermophilic lipase from Bacillus sp. THL0127.FEMS Microbiol. Lett., 179: 241-246.

Edwards, U., T. Rogall, H. Blocker, M. Emde and E.C. Bottger, 1989. Isolation and direct nucleotide determination of entire genes. Characterisation of a gene encoding for 16S ribosomal RNA. Nucleic Acids Res., 17: 7843–7853.

Ghosh P K, Saxena R K, Gupta R, Yadav R P and Davidson W S, (1996).Microbial lipases: production and applications Sci. Prog 79 119-157.

Haki, G. D., and S. K. Rakshit. (2003). Developments in industrially important thermostable enzymes: a review. Bioresour. Technol. 89:17-34.

Hasan F, Shah A. A, Hameed A (2006).: Industrial applications of microbial lipases. Enzyme and Microb Technol, 39:235-251.

Jäeger K E and Eggert T. (2002). Lipase for biotechnology. Current Opinion Biotechnology, **13**, 390-397.

Jaeger K-E and Reetz MT. (1998). Microbial lipases form versatile tools for biotechnology. Trends Biotechnol. 16:396–403.

Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. Heuvel, and O. Misset. (1994). Bacterial lipases. FEMS Microbiol. Rev. 15:29-63.

Jaeger, K-E., Schneidinger, B.; Rosenau, F.; Werner, M.; Lang, D.; Dijkstra, B.W.; Schimossek, K.; Zonata, A. and Reetz, M.T. (1997). Bacterial lipases for biotechnology application. J. Mol. Catal. , 3: 3-12.

Kambovrova, M., N. Kirilova, R. Mandeva and A. Derekova, (2003). Properties of thermostable lipase from a thermophilic Bacillus stearothermophilus MC7. J. of Molecular Catalysis B. Enzymatic, 22: 307-313.

Madan, B. and Mishra, P. (2009). Overexpression, Purification and Characterization of Organic Solvent Stable Lipase from Bacillus licheniformis RSP-09. J Mol Microbiol Biotechnol:17(3):118-123.

Mohan, T. Selva; A. Palavesam and G. Immanvel (2008).Isolation and characterization of lipase-producing Bacillus strains from oil mill waste. African J. Biotech. 7: 2728-2735.

Rademaker, Jan and J. Bruijn, Frans, 1997. "Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis."InGustavo Caetano-Anolles and Peter M. Gresshoff, DNA Markers Protocals, Applications and Overviews. (pp. 151-172) New York: Wiley-Liss, Inc.

Saitou N & Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning; A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Saxena R K, Ghosh P K, Gupta R , Davidson W S, Bradoo S and Gulati R .(1999). Potential biocatalysis and future industry, *Curr. Sci*. 77: 110-115.

Sztajer, H. and Maliszewska, I. (1988) The Effect of culture condition on lipolitic productivity of microorganisms. Biotechnol. Lett. 10: 199-204.

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Thompson, J. D.; Higgins, D. G. and. Gibson, T. J (1994). ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22:4673-4680.

Tirawongsaroj, P., R. Sriprang, P. Harnpicharnchai ,T. Thongaram, V. Champreda, S. Tanapongpipat, K. Pootanakit and L. Eurwilaichitr (2008) Novel thermophilic and thermostable lipolytic enzymes from a Thailand hot spring metagenomic library. J. Biotechnol. 133: 42–49.

Verger, R. (1997). Interfacial activation of lipases: facts and artifacts. Trends Biotechnol. 15:32-38.