American Journal of Microbiology and Biotechnology
2018; 5(3): 50-60
http://www.aascit.org/journal/ajmb
ISSN: 2375-3005

AASCIT American Association for Science and Technology

Identification and Characterization of Two Lipolytic and Thermophilic Bacterial Strains Isolated from Saudi Arabia Environment

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Citation
Mohammed Abdullah Al-Omair. Identification and Characterization of Two Lipolytic and Thermophilic Bacterial Strains Isolated from Saudi Arabia Environment. *American Journal of Microbiology and Biotechnology.* Vol. 5, No. 3, 2018, pp. 50-60.

Received: September 27, 2018; **Accepted**: October 26, 2018; **Published**: November 5, 2018

Abstract: Lipases are one of the most valuable classes of enzymes of high economic importance. 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. Microbial lipases have wide application in the processing of leather, domestic, industrial wastes and pharmaceutical industries. The need for thermostable lipase enzymes is steadily rising and the isolation of lipases from thermostable microorganisms is highly requisite. 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 identified based on biochemical characteristics, RAPD-PCR, and 16S rRNA gene sequencing. Phylogenetic analysis revealed their closeness to the thermophilic *Burkholderia pseudomalllei (B. pseudomalllei)* and *Staphylococcus pasteuri (S. pasteuri)* 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 revealed that *S. pasteuri* and *B. pseudomalllei* have the ability to tolerate NaCl salt up to 2% and 2.5%, respectively. Both *S. pasteuri* and *B. pseudomalllei* are the highest thermophilic bacteria generating lipase.

Keywords: Thermophilic, Lipase, Rapid PCR, 16S rRNA, *Burkholderia pseudomalllei*, *Staphylococcus pasteuri*

1. Introduction

Lipids constitute a large part of the earth's biomass. Lipolytic enzymes play an important role inlipid hydrolysis. Lipases (E.C.3.1.1.3, triacylglycerol hydrolases) show different substrate and positional specificities. They have been recognized as very useful biocatalysts and they are the major industrial enzymes extensively used in food, pharmaceuticals, medical, textiles, and chemical industries [1]. They are currently attracting enormous attention because they constitute the most important group of biocatalysts for biotechnological applications [2-7]. Most of the lipases used in industry are microbial enzymes, of both fungal and bacterial origin [8, 9]. Microorganisms produce different classes of lipolytic enzymes [10-12].

Recent studies with thermophilic microorganisms revealed enough evidence supporting reclassification of thermophilic bacteria based on the data of 16S rRNA sequence analysis. Currently, the scientists have great interest to study thermophilic bacteria due to their biotechnological importance as a source of thermostable enzymes and industrial products [13-17]. Advances in molecular biology techniques such as rapid PCR and 16S rRNA sequencing have provided the excellent opportunity for identification and characterization purposes of microorganisms at species and subspecies levels [18-22]. Among microbial lipases, 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 [23-28]. The industrial demand for highly active preparations of lipolytic enzymes with appropriate specificity and stability to pH, temperature and ionic strength continues to stimulate the search for new enzyme sources [29- 34].

Thermophilic microorganisms grow best at temperatures above 45°C and therefore, it is possible to isolate them from

different environments [35]. For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological potential in addition to scientific curiosity. In particular, phenotypic and genotypic characterization of thermophilic bacteria were done for many geothermal areas in different regions in the World [36 39]. 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 [40- 44]. 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 [1, 3, 11, 45- 47]. Thermophilic lipases show higher thermostability, higher activity at elevated temperatures 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 enzymes continues to stimulate the microorganism's production of thermostable enzymes. A small number of thermophilic lipase producing bacteria have been described in the last decades [21, 48-52]. Haki and Rakshit [53] 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 aim of this research was to isolate and purify lipolytic and thermophilic bacterial strains from Saudi Arabian environment. The isolated bacterial strains were identified by different molecular biology tools. The optimum growth conditions of the most lipolytic bacterial strains *B. pseudomalllei* and *S. pasteuri* were studied.

2. Materials and Methods

2.1. Samples Collection

Soil samples were collected from different locations in the eastern region of Saudi Arabia. These samples were contaminant with oil and kept in sterile containers to be used for isolation of lipase producing organisms.

2.1.1. Screening of Microorganism

Soil samples (10 g) were suspended in 100 ml of sterile distilled water and agitated for an hour on a shaking incubator at 50°C. The suspensions were filtrated and 10 ml of the filtrated were transferred 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 /1 lit of distilled water), supplemented with 2% olive oil as a sole carbon source in sterile flasks. The flasks were 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. One ml of the prepared dilutions was transferred into sterilized Petri dish containing Rhodamine B- olive oil nutrient agar medium and incubated at 50 ^{o}C for 24h, 48h and 72h.

2.1.2. Luria Bertani Medium

Luria Bertani Medium (LB medium) was made by dissolving 10 g bacto tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml de-ionized water. The pH was adjusted to 7.0 with 5 N NaOH and the volume of the solution completed to 1 litre with demonized water and sterilised by autoclaving. LB agar plates were prepared by adding 20 g agar to one liter of LB medium and sterilized by autoclaving.

2.1.3. Rhodamine B agar Media

Rhodamine B agar media were prepared as described by Kouker and Jaeger [54].

2.2. Chromosomal DNA Isolation

The total DNA was extracted from one bacterial isolates according to the method described by Sambrook *et al*., [55].

2.2.1. DNA Fingerprinting for the Isolated Bacteria

To examine the relatedness between these isolates, RAPD-PCR was carried out using three primers; the first one was BOXA1R primer (5`-CTACGGCAAGGCGACGCTGACG-`3), the second primer used in this study is the ERIC-1R primer (5'-ATGTAAGCTCCTGGGGATTCAC-3') and finally the third primer was ERIC-2 primer (5'-AAGTAAGTGACTGGGGTGAGCG-3') PCR mixtures were prepared with 1µl of genomic DNA, 5 µl of Taq buffer 10x, 200 µmol of each deoxynucleoside triphosphate, 20 pmol each primer, and 2.5 U Taq polymerase (Promega, Germany) and sterile filtered mille water to a final volume of 50 µl. The PCR program was as follows: Primary denaturation at 95°C for 4min, 38 cycles was applies as follow: 94°C for one min., 45°C for one min. and 72°C for 2min in Final extension step at 72°C for 10 min and PCR reaction kept on 4°C until removing the tubes. PCR products were separated on agarose gel electrophoresis using 1.5% w/v agarose in 0.5X Trisborate EDTA buffers. Size of each band was estimated by using DNA molecular weight marker. Finally, gel was photographed by using gel documentation system. The phylogeny tree of bacterial isolates was made according to statistical program analysis (Statistica Version 5). The highly producing lipase isolates (the data are not showed) and relatively distance isolates according to the results in the dendrogram were selected and subjected to sequence analysis of 16S rDNA genes.

2.2.2. Amplification of the 16S rRNA genes

The 16S rRNA genes were selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserved positions in the 3′ and 5′ regions of bacterial 16S rRNA genes. According to Edwards *et al*. [56], the 16S rRNA gene was amplified by using the forward 5'AGAGTTTGATCCTGGCTCAG3' and the reverse 5'GGTTACCTTG- TTACGACTT3' oligonucleotide primers. 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 revise 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 95°C for 5 min followed by repeated 35cycles as following: 1 minute at 95°C for denaturation, 1 min at 60°C for annealing, 2 min at 72°C for elongation. Reaction mixture was then Incubated at 72°C for 10 min for final extension then mixtures was hold at 4°C [55].

2.2.3. Sequencing of 16S rRNA Gene

The PCR products were routinely analyzed by electrophoresis in 1.0% agarose gels in 1x Tris-Borate-EDTA buffer [55]. The target band was purified using gel extraction kit (Qiagene, Germany). The purified PCR products were sequenced by using the forward the reverse primers. Sequencing was 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.

2.3. Effect of pH on the Bacterial Growth Rate

The influence of pH on the growth of the 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 20µl of fresh bacterial culture individually, and then incubated under shaking (120 rpm) at 50±0.1°C for 6h. The optical density (O. D.) of the growth culture was measured at 600nm.

2.3.1. Effect of Temperature on the Bacterial 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 20µl of fresh bacterial culture individually and incubated at the teased temperature for 6 hrs under shaking (120 rpm).

2.3.2. Effect of Agitation Speed (rpm) on the Bacterial Growth Rate

The effect of different agitation rates on the growth of the 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 20µl of fresh bacterial culture and incubated at 50±0.1°C for 6h.

2.3.3. Effect of Salt Concentration on the Bacterial Growth Rate

Different concentration of Sodium chloride salt 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%) to determine the best concentration 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 20µl of fresh bacterial culture incubated at 50°C for 6hrs.

3. Results and Discussion

3.1. Screening of Lipase Producing Bacteria

A total 43 putative strains of thermophilc bacteria were isolated from oil contaminant soil samples in Saudi Arabia. These isolates were identified by conventional and molecular techniques by comparing with reference strains. Each of the thermophilic bacterial isolates was streaked out and purified on Rhodamine B– Olive oil nutrient agar media (Figure 1 A & B). The Rhodamine B- olive oil Petri dishes were visualized on UV- transilluminator at wave length 350 nm [52]. Pink colonies mean non-lypolytic bacteria while the orange colonies mean lypolytic bacteria. The fluorescence observed was due to the hydrolyzed substrate reacting with Rhodamine B. Hou and Johnston [56] suggested that the development of fluorescent was due to Rhodamine B dimers complexed with mono- or diglycerides and fatty acid. Two of the isolated bacterial strains exhibited the highest lypolytic activity (Figure 1 C). 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 lipolytic colony (data not shown). These two strains were identified as *Burkholderia pseudomalllei* (*B. pseudomalllei*) and *Staphylococcus pasteuri* (*S. pasteuri*).

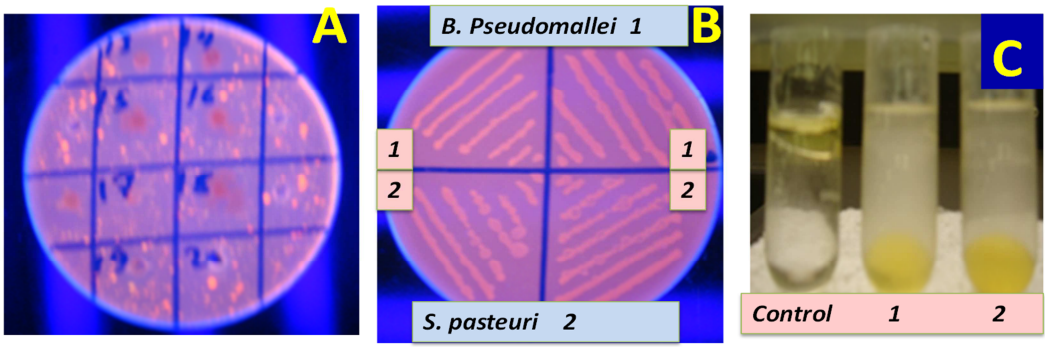

Figure 1. *(A) lypolytic and non-lypolytic bacteria isolated from Saudi Arabia environment on Rhodamine B- olive oil nutrient agar plate visualized by UV light (B) Purified lipolytic B. pseudomallei and S. pasteuri on Rhodamine B- olive oil nutrient agar plate and (c) Effect of the thermophilic B. pseudomallei (1) and S. pasteuri (2) on 10% olive oil compared to the control.*

Each bacterial strain was stained with Malachite green and safranin stains to identify the spore formation of isolated strains. The investigation of the isolated bacterial strains in terms of morphologic, and physiological properties showed that all the strains were Gram positive, thermophilic and endospore forming.

3.2. RAPD Fingerprinting of Bacterial Isolates DNA

All the isolated bacteria were subjected to RAPD-PCR using three primers: BOXA1R, ERIC-1R and ERIC-2 primers (Figure 2). The RAPD band patterns resulting from the three primers were analyzed using UPGMA method to construct a similarity matrix and to generate a dendrogram indicating the relationship between the eleven tested isolates of thermophilic highly lipolytic bacterial isolates. The presence or absence of any particular DNA bands was the only parameter considered in the computer analysis. The dendrogram generated to show linkage distance (Figure 3) indicated that the isolates were classified into two main groups branched (I and II) from one ancestor at 50% linkage distance, branch (I) was strain *Burkholderia pseudomallei* and the other branch (II) was divided at linkage distance 41% into two clusters (i and ii), cluster (i) was strain *Staphylococcus pasteuri.* At linkage distance 38%, all isolates at cluster (ii) were divided into two sub-cluster, while were divided into five groups at 23% linkage distance and the five group were divided into eight 7 classes at distance linkage percent 13%. Whenever, at distance linkage 7%, no similarity was observed.

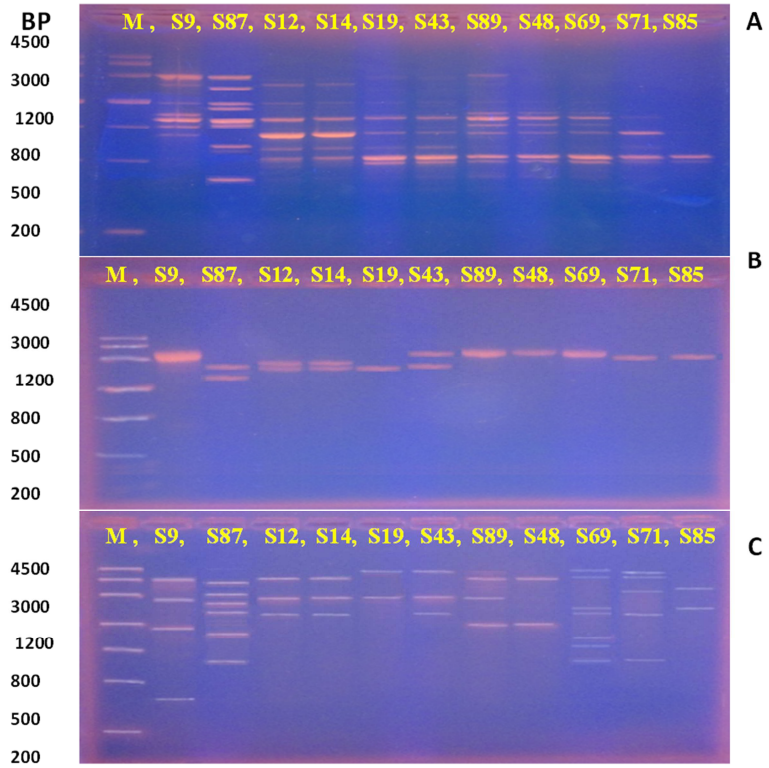

Figure 2. *RAPD-PCR for 11 bacteria isolates; A: BOX1AR primer; B: ERIC-1R primer, C: ERIC-2 primer. M: QIAGEN GelPilot DNA Molecular Weight (Marker Wide Range Ladder), S9: Staphylococcus Pasteuri and S87: Burkholderia pseudomallei.*

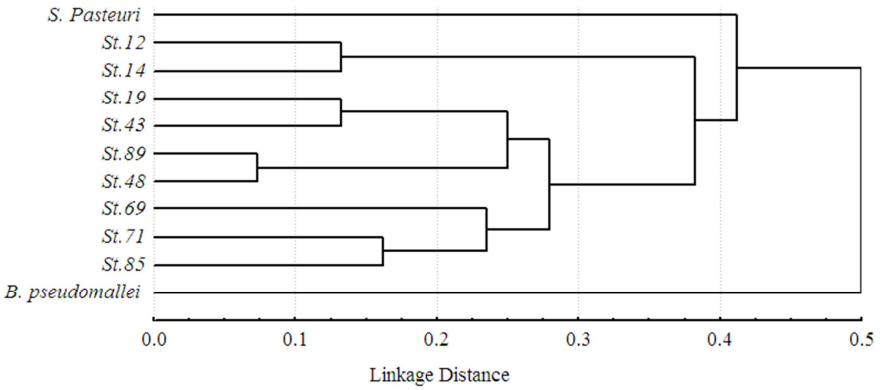

Figure 3. *Dendrogram obtained by clustering (UPGMA method) based on the band pattern obtained by the RAPD-PCR analysis of the selected thermophilc lipolytic isolates. S9: Staphylococcus Pasteuri and S87: Burkholderia pseudomall.*

3.3. 16S rRNA Sequence Analysis

Genomic DNA of the isolated bacterial strains was purified and 16S rRNA genes were amplified as illustrated in Figure 4. Approximately 1000 bp of the 16S rRNA genes were sequenced for all the isolated bacterial strains. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rRNA sequences. Sequence analysis of 16S rRNA showed that there was a strong similarity (≥ 98%) between the isolated bacterial strains and representative strains in the gene bank of *Bacillus licheniformis, Bacillus saphuricus, Pseudoalteromonas sp, Staphylococcus Pasteuri, Bacillus sp, Bacillus subtilis, Bacillus cereus and Burkholderia pseudomalle.* The sequence analysis of 16S rRNA may indicate that the data is helpful for identification of bacteria at genus level, but not at species. Comparing the sequence of the 16S rRNA 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 pseudomalllei* MSHR346 (CP001408), *Burkholderia pseudomalllei* 668 (CP000570), *Burkholderia pseudomalllei* 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 shown in Table 1. The highly two lipolytic activity thermophilic lipase-producing bacterial strains were *Burkholderia pseudomallei* and *Staphylococcus pasteuri*.

3.4. Alignments and Phylogenetic Analysis

The phylogenetic analysis of the isolated bacteria based on 16S rRNA genes were carried out by pair-wise and multiple DNA sequence alignment comparison using clustalW version 4 [57]. Bootstrap neighbor-joining tree [58] was generated using mega version 4.0 [59]. The phylogeny of bacterial strains *B. pseudomaleil, S. pasteuri* and closely related species were analyzed using the multi sequences alignment program and the results are presented in a phylogenetic (Figure 5).

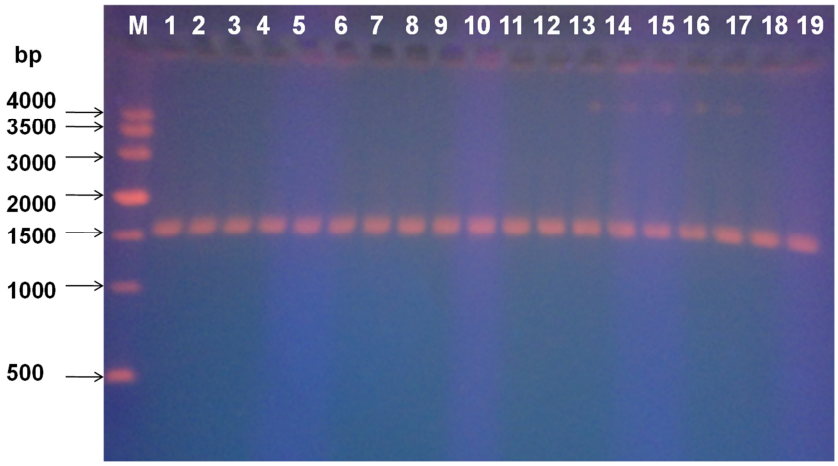

Figure 4. *1% Agarose gel electrophoresis illustrating PCR –amplification of 16S rRNA gene for bacterial isolates. M is Gelpiolt ladder DNA marker and lanes 1 to 19 represent the isolated 16S rRNA gene of thermophilic bacterial strains that collected from Saudi Arabia.*

Table 1. *Similarity percentage of 16S rDNA sequences for the selected isolates compared to those obtained from database.*

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

The phylogenetic tree in figure 5 demonstrated the linkage of *B. pseudomalllei* and *S. pasteuri*; this was constructed using 30 different isolates from Gene Data Bank (http://www.ncbi.nlm.nih.gov). Similar data can be generated using the partial 16S rRNA sequence data set for *B. pseudomalllei* and *S. pasteuri*. The phylogenetic tree generated from *MEGA* version 4 and ClustalW for isolates *B. pseudomalllei* and *S. pasteuri* indicated that there are two clusters; one of them contains *B. pseudomalllei.*

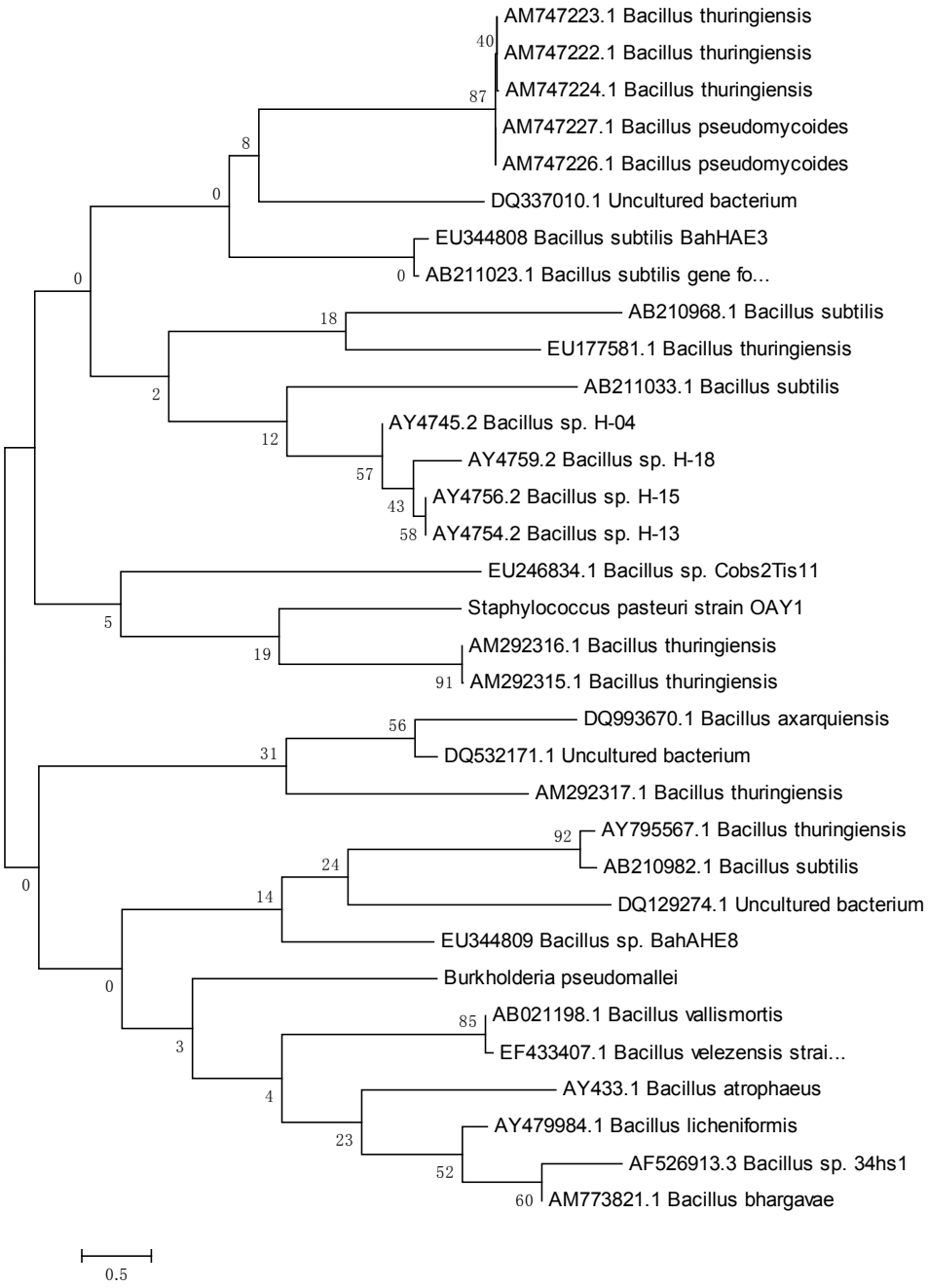

Figure 5. *Phylogenetic tree of the B. pseudomallei and S. pasteuri isolates and their related genera has been linked based on partial 16S rRNA sequence comparisons. Their names and respective accession numbers are given in the tree.*

3.5. Growth Optimization Selected Strains

In the present work, bacterial isolates *B. pseudomallei* and *S. pasteuri* exhibited the best lipolytic activity. Therefore, the growth optimization factors of both strains were characterized. 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.

3.5.1. Effect of pH on the Growth Rate

The influence of pH on the growth of *B. pseudomalllei* and *S. pasteuri* was assessed using LB medium. The pH of the 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 represented in Figure 6. For *S. pasteuri*, the growth rate was increased with decreasing the acidity (increasing pH) of the LB medium up to pH 7.5 for, where the maximum growth at this degree of acidity was 0.798 (O.D. reading) and the minimum (O.D. 0.007) was observed at pH 8 up to pH 10. The optimum pH for *B. pseudomalllei* was 8.0, where the maximum growth 0.700 (O.D. reading) as illustrated in Figure 4, while the growth of *B. pseudomalllei* decreased

dramatically to be 0.021 (O.D.) at pH 10. Therefore, from the obtained results, we can conclude that the optimum pH for growth of both *B. pseudomalllei* and *S. Pasteuri* were 8.0 and 7.5. respectively.

3.5.2. Effect of Incubation Temperature on the Growth Rate

Different incubation temperatures (25, 30, 35, 40, 45, 50, 55, 60, and 65°C) were used to study the optimum temperature for growth rate of *B. pseudomalllei* and *S. pasteuri*. The cultures were incubated at the optimum pH in incubator water bath shaker for 6 hrs at 120 rpm. The obtained results are represented in Figure 7 for bacterial strains *S. pasteuri* and *B. pseudomalllei*. The growth rate of *S. pasteuri* was increased with increasing the incubation temperature, where the highest growth rate (O.D: 1.088) was observed at 50°C. On the other hand, the growth rate was dramatically decreased when the temperature increased over 50°C to be 0.156 and 0.067 at 60°C and 65°C, respectively for *B. pseudomalllei*, the highest growth rate was recorded at 50°C (O.D: 1.167) and the growth rate was declined when the incubation temperature increased up to 60°C (O.D: 0.111) and at 65°C (O.D. 0.044).

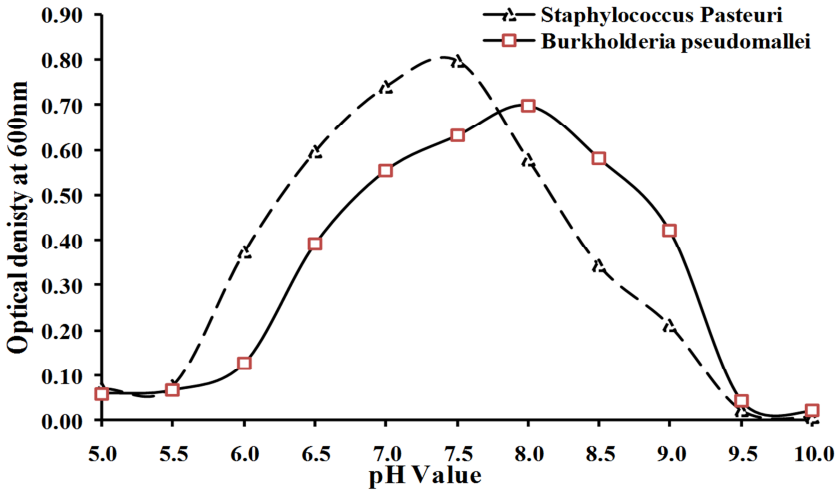

Figure 6. *The relationship between pH and growth rate of B. pseudomalllei and S. Pasteuri.*

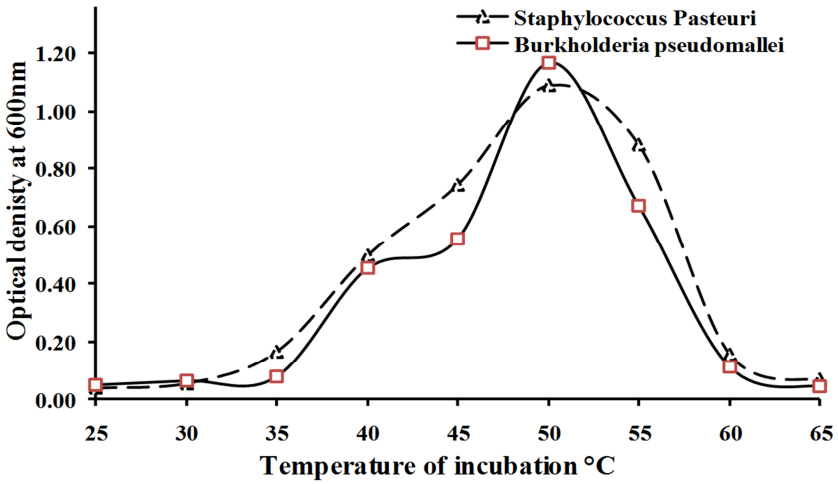

Figure 7. *The relationship between temperature of incubation and growth rate of B. pseudomalllei and S. pasteuri.*

3.5.3. Effect of Agitation Speed on the Growth Rate

Effect of agitation speed on growth rate of *B. pseudomalllei* and *S. pasteuri* strains was studied at speed of agitation from static stage (zero shaking) to 300 rpm. As represented in Figure 8, the growth rate of *S. pasteuri* was increased gradually with increasing agitation speed, the growth rate increased approximately two folds (O.D: 1.526) at speed 200 rpm compared with the growth rate (O.D: 0.887) at agitation speed 150 rpm. The growth rate of *S. pasteuri* was gradually increased to the maximum at agitation speed 300 rpm. With respect to *B. pseudomalllei,* the growth rate was increased linearity to its maximum (O.D: 1.212) at agitation speed 200 rpm as illustrated in Figure 8. Beyond 200 rpm, the growth rate of *B. pseudomalllei* was decreased with increasing the agitation speed up to 300 rpm.

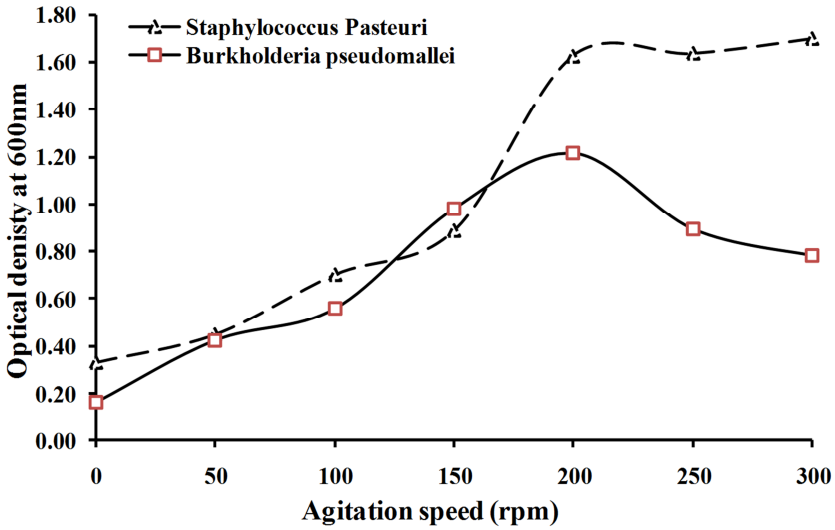

Figure 8. *The relationship between agitation speed (rpm) and growth rate of B. pseudomalllei and S. pasteuri.*

3.5.4. Effect of Salt Concentration on the Growth Rate

Effect of salt stress at different rates of salt as NaCl (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 *B. pseudomalllei* and *S. pasteuri* were used todetermine the best salt concentration required for the highest growth rates.. As represented in Figure 9, *S. pasteuri* recorded the highest growth rate (O.D: 1.258) at 2% of NaCl in the growth medium, while the growth rate was decreased gradually with increasing NaCl concentration. For *S. pasteuri*, the growth rate decreased (O.D 0.358) at 6% of NaCl. In case of strain *B. pseudomalllei*, as shown in Figure 9, the growth rate increased from O.D. = 0.712 to 1.086 with increasing concentration of NaCl 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% NaCl was 0.248).

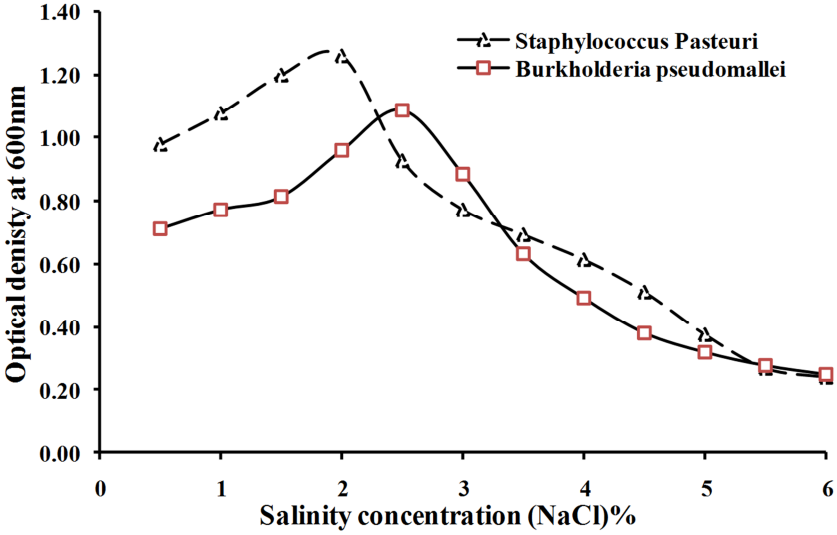

Figure 9. *The relationship between salt concentration as sodium chloride (%) and growth rate of B. pseudomalllei and S. pasteuri.*

4. Conclusion

In screening thermophilic lipase-producing bacteria in kingdom of Saudi Arabia environment, forty three native bacterial strains were isolated from different contaminated soil from Al- Hassa region; potent bacterial candidates were characterized and identified by biochemical and PCR techniques, based on 16S rRNA sequencing. In this study, the 16S rRNA gene sequence provided frequent phylogenetically useful information. Direct sequence determination of 16S rRNA gene fragments represents a highly accurate and versatile method for identification of bacteria to the species level. Therefore, the identification of lipase-producing bacteria should not only rely on phenotypic methods, but should be confirmed by the beneficial genotypic techniques such as 16S rRNA sequence analysis.

Two of the thermophilic lipase-producing bacterial isolates recorded the highest lipolytic activity, Phylogenetic analysis revealed their closeness to the thermophilic *B. pseudomalllei*

and *S. pasteuri.* The maximum growth rate of the highly lipolytic activity bacterial strains *B. pseudomalllei and S. pasteuri* was assessed at different temperatures of incubation, acidity of growth medium (pH), salinity concentrations and agitation rates.

Temperature influences the growth rate of both *S. pasteuri and B. pseudomalllei,* where the maximum growth was found at 50°C. Hence, we conclude that the two bacterial strains: *S. pasteuri and B. pseudomalllei* are thermophilic bacteria. With respect to acidity of the medium, the obtained results indicated that the maximum growth of the two bacterial strains was attained under slightly alkaline conditions, at pH 7.5 and 8.0 for both *S. pasteuri* and *B. pseudomalllei*, respectively.

The growth rate of *S. pasteuri* increased gradualy with increasing agitation speed of its growth medium, while the maximum growth of *B. pseudomalllei* was observed at agitation speed of 200 rpm. Both *S. pasteuri* and *B. pseudomalllei* bacterial strains tolerated the sodium chloride salt in the growth medium and the maximum growth rate wase at 2% and 2.5%, respectively.

Acknowledgements

The authors appreciate King Abdulaziz City for Science and Technology General Directorate of Research Grants Programs, Kingdom of Saudi Arabia for the financial support Project No. 08- Bio13-6. The author also acknowledges Prof. Magdy M. Youssef, Head of Biochemistry Division, Chemistry Department, Faculty of Science, Mansoura University, Egypt for his cooperation in the Molecular Biology techniques and editing the manuscript.

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