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ISOLATION OF PSEUDOMONAS AERUGINOSA FROM INDUSTRIAL WASTE WATER USING LIPA GENE

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ABSTRACT

Extracellular lipase producing by Pseudomonas aeruginosa have special properties not common among the lipases produce by other organisms, e.g., their thermorestance and activity at alkaline pH. Which make this enzyme have considerable value for use in numerous applications in many industries and in degrade oils in industrial wastewater. This study aimed to use PCR technique for isolation P. aeruginosa from industerial wastewater by lipA gene and to find the suitable strain which have the highest lipolytic activity by subjected the PCR product to BLAST (Basic Local Alignment Research tool) analysis, to use this suitable strain in oil rich industrial wastewater treatment and in many industrial applications by using lipase that produced from it.

KEYWORDS: Pseudomonas aeruginosa; industrial waste water; PCR.

INTRODUCTION

Pseudomonas aeruginosa is Gram-negative, facultative anaerobic rods, non-fermentative, non- sporulation, motile by polar flagellum and widely distributed in soil and water.[1,2] This bacteria is widely distributed in nature because the diversity of metabolism process that makes it ubiquitous in many environments. It is made possible by a large number of enzymes that allow P. aeruginosa to use a diversity of substances as nutrients.[3,4]

The lipA gene encoding an extracellular lipase.[5,6] LipA gene in P.aeruginosa, nucleotide sequence analysis revealed a gene of 936 bp. codes for 311 amino acids.[7]

Many Enzymes produced by P. aeruginosa have been reported and used in many commercial scales. Lipase of P. aeruginosa is widely used in industrial applications such as additives for foods (flavor modification), fine chemicals (synthesis of esters), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins), medicine (blood triglyceride assay) and production of bio diesel.[8,9]

Lipase is very important in degrading and dissolving lipids in the biological treatment of oil rich wastewaters, accelerating the process and improving time efficiency. The treatment of effluents from fats is a new and favorable application for lipases, because their ability to hydrolysis the triglycerides to free fatty acids and glycerol.[10,11] For this purpose, the main aim of the study is to through some light on the LipA gene which encoding extracellular lipase which produced by P. aeruginosa.

MATERIALS AND METHODS

Sampling

Fifty samples were collected from oil rich industrial wastewater from the factories of the general company of vegetable oils, from different departments of AL Rasheed and ALameen factory.

Labrotary identification of isolates

The samples were collected from both the general company of vegetable oils factories were offered for routine tests, include the morphological, bacteriological and biochemical tests which include grow on Cetrimide agar as a selective medium, MacConkey agar , growing at 42°C ,gram staining, oxidase, catalase test. TSI test (glucose, lactose or Sucrose fermentation and H2S production), urease test and IMViC tests (indol, methyl red (MR), voges-Proskauer (VP) and Simmon's citrate agar).[12,13,14,15]

Detection of bacterial lipolytic activity

Tow methodes were used for detection of bacterial lipolytic activity:

1-Screening of bacterial lipolytic activity

For isolation the lipolytic microbes, bacterial suspension comparison with Mcfarland standard 0.5 to containing approximately 1.5×10^{8} CFU /ml.[16] Bacterial suspension was inoculated in wells made on tributyrin agar. The formation of clear zone around the colony on

the plate was considered as positive for lipolytic microorganisms. The diameter of the zone hydrolysis was measured after incubated for 5 days at 35°C.[17]

Gas chromatography method

Separation of fatty acids

Bacterial suspension was inoculated in 100 ml of tributyrin broth and incubated at 35°C for 5 days. Then, separation the cells and particles from culture broth by centrifuge.

The suspended was transferred to separating flask with added 50 ml of Petrolium ether as organic solvent. Fatty acids solution was taken to rotary evaporator at 50°C to getrid of organic solvent and gets the fatty acids solution.[18]

Gas chromatography analysis

Analysis was carried out on GC-938A gas chromatograph with FID detector. The operation conditions were as follows: carrier gas was He (30 ml/min constant flow), the oven temperature for first 2 min was 100°C and then increased at a rate of 10°C /min until 300°C hold for 10°C/min ,injection temperature was set at 300°C and detector temperature was 325°C.

Molecular Methods for Identification of Pseudomonas aeruginosa by using lipA gene

DNA extraction

A sterile inoculating loop touched four or five isolated colonies of the bacterial culture inculated nutrient broth and incubated overnight at 37°C, one ml of it transferred to 1.5 ml microcentrifuge tube, centrifuged for 1 minute at 14-16000x g ,then discarded the supernatant .The bacterial genomic DNA etxtract according to manufacture protocol was done using reagent genomic DNA Kit (Geneaid – Thailand).

Amplification of lipase gene in Pseudomonas aeruginosa by lipA 948

The DNA amplified by PCR using lipA 948 primer (F- 5'GGA TCC ATG AAG AAG AAG TCT CTG CT 3') and (R- 5' AAG CTT CTA CAG GCT GGC GTT CTT 3').[7]

The PCR was performed in 25 µl reaction maxture containing 12.5 µl of Green Master Mix (1X) (promega), 3 µl of genomic DNA of bacteria, 1µl of each forward and reverse primers and 7.5 µl of nucleases free water.

The PCR program comprised initial denaturation step at 94°C for 5 min. ,a denaturation step of 94°C for 1 min.,annealing at 60°C for 45 sec. ,extention at 72°C for 1 min. and final extention step of 72°C for 10 min. 30 serial cycles of reactin was performed.

RESULTS AND DISSCUSSION

The fifty samples collected from different places of both factories ,only 34 (68%) isolates belong to Pseudomonas aeruginosa, which gave positive results for the tests used to ensure the presence of P. aeruginosa.

Detection of bacterial lipolytic activity

1-Screening and selection

The screening of bacterial lipolytic activity was based on the obtained values of clear zones diameter in tributyrin agar.[19] After 5 days at 35°C.[17]

According to Yazaji and Alhaj.[17] Prasad.[20] and Sulochana et al.[21] they considered that the optimum temperature for the production of lipase in P. aeruginosa is 35°C . Three isolates (P-1, P-2 and P-16) characterized the good production of lipase. The diameters of these isolates ranging between (2.1_2.7) cm. The isolate (P-16) shows the highest lipolytic activity with a clear zone (2.7 cm) in diameter. According to Al-Safar.[22,23] the local isolate could be selected on the basis of diameter of clear zone around colony when using serratia in her study. In this study the local isolate was P.aeruginosa (P-16). Figure (1) showed the clear zone around colonies.

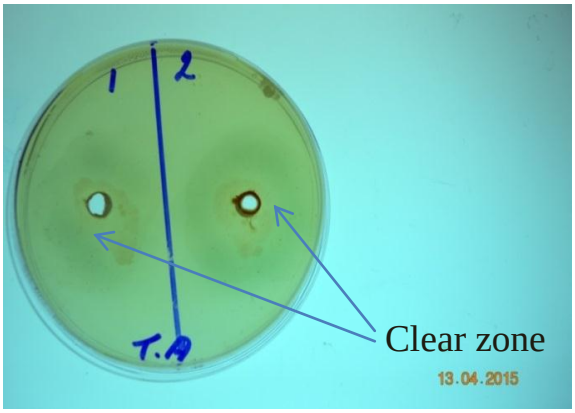

Figure (1): Clear zone of lipolytic activity around P. aeruginosa colonies.

Gas chromatography-Flame ionization detector

According to Ali et al.[24] Dahiya et al.[25] the obtained values of separating fatty acids by Gas chromatograph analysis (Figure2 ,3 and 4) based on comparing it with fatty acid standard (in this study were Butyric, Palmatic, Linoleic, Stearic and Oleic acid).

Table (1) shows the lipolytic activity values of isolates by Gas chromatography method. It has been found that the local isolate P. aeruginosa (P-16) was the best. This result identified with the screening of bacterial lipolytic activity by tributyrin.

Table 1: The fatty acids value (%) after degredation by P. aeruginosa.

No.	Fatty acid standard	Time	P- 1	P- 2	P- 16
1	Palmatic acid	13.8	1.42	_	1.64
2	Linoleic acid	14.4	_	0. 85	_
3	Stearic acid	15.8	0. 63	0. 64	1.42
4	Oleic acid	16.06	1.08	0. 59	1.69
5	Butyric acid	20.41	0. 6	_	13.8

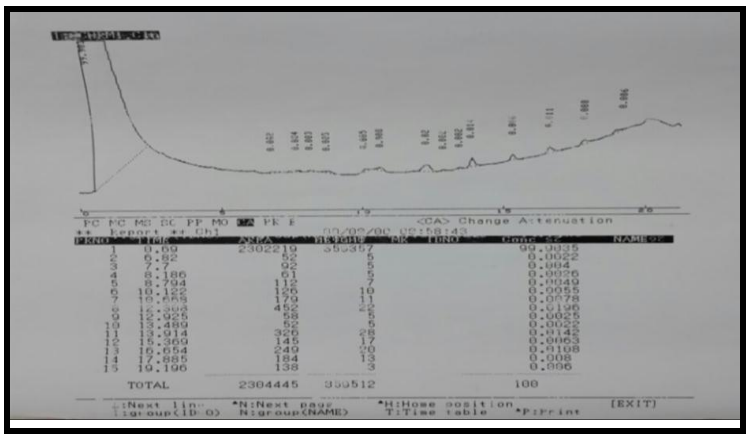

Figure (2): The analysis of P-1 fatty acids by GC -Flame ionization.

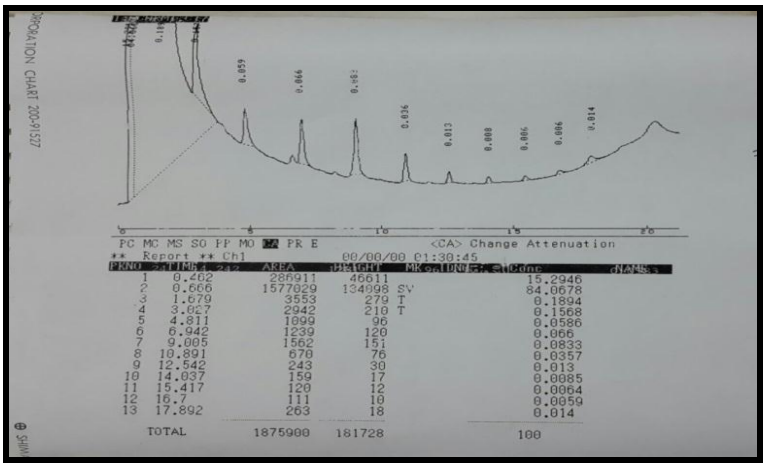

Figure (3): The analysis of P-2 fatty acids by GC -Flame ionization.

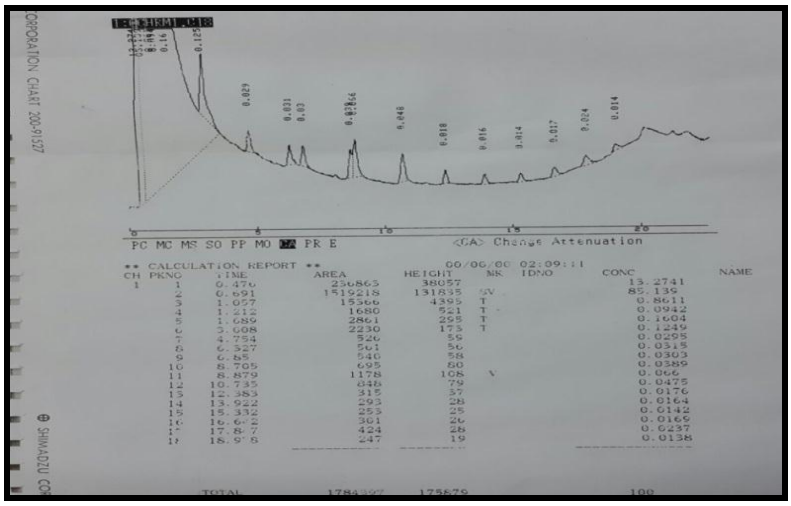

Figure (4): The analysis of P-16 fatty acids by GC -Flame ionization.

MOLECULAR METHODS

The first step was DNA extracted from all isolates of P. aeruginosa by DNA extraction kit (Geneaid – Thailand). Then, detected by gel electrophoresis .As shown in Figure (5).

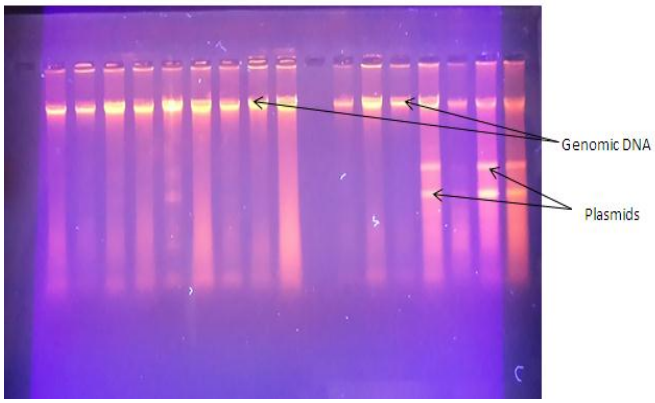

Figure (5): Extracted DNA electrophoresis on 1% agarose (70 vol/ 90 min) to check purity and integrity

The second step was lipase gene amplification with lipA 948 primer by PCR technique, which appeared that all 34 isolates give positive result (100%).According to Wu et al.[7] the designe of this primer for the lipase gene was based on the full length DNA sequences for P. aeruginosa lipase genes in NCBI GenBank. (Figure 6).

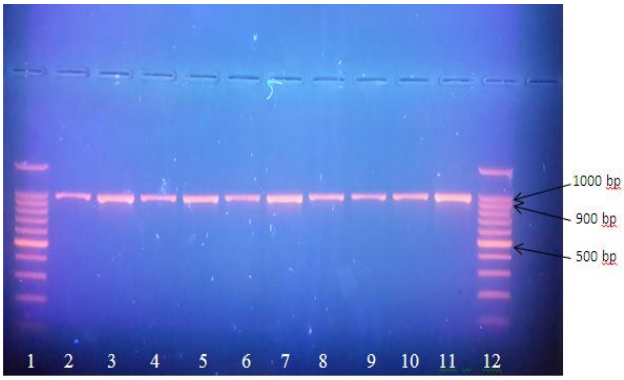

Figure 6: Agarose gel electrophoresis of PCR amplification products of P. aeruginosa lipA 948. Lanes 2-11: positive results, lanes: 1,12 ladder.

Analysis lipA sequences with National Center for Biotechnology Information

Analysis lipA sequences was done depending on comparison between studied sequences for primer lipA 948 with the records of national center for biotechnology information database specifically to BLASTn (Basic Local Alignment Research tool) for similarity search with the preexisting sequence available in NCBI/Genbank to confirm the sequences and then to detect any DNA alterations in sequences of the genes in the isolate (P-16) which shows the highest lipolytic activity. [6][26] Figure (7) show the alignment in isolate (P-16) by using BLASTn.

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Query  38   TGCCAGCCCTCTGATCCAGGCCAGCACCTACACCCAGACCAAATACCCCATCGTGCTGGC  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  98   CCACGGCATGCTCGGCTTCGACAACATCCTCGGGGTCGACTACTGGTTCGGCATTCCCAG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  147  CCACGGCATGCTCGGCTTCGACAACATCCTCGGGGTCGACTACTGGTTCGGCATTCCCAG  206

Query  158  CGCCTTGCGCCGTGACGGTGCCCAGGTCTACGTCACCGAAGTCAGCCAGTTGGACACCTC  217
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  207  CGCCTTGCGCCGTGACGGTGCCCAGGTCTACGTCACCGAAGTCAGCCAGTTGGACACCTC  266

Query  218  GGAAGTCCGCGGCGAGCAGTTGCTGCAACAGGTGGAGGAAATCGTCGCCCTCAGCGGCCA  277
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  267  GGAAGTCCGCGGCGAGCAGTTGCTGCAACAGGTGGAGGAAATCGTCGCCCTCAGCGGCCA  326

Query  278  GCCCAAGGTCAACCTGATCGGCCACAGCCACGGCGGGCCGACCATCCGCTACGTCGCCGC  337
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  GCCCAAGGTCAACCTGATCGGCCACAGCCACGGCGGGCCGACCATCCGCTACGTCGCCGC  386

Query  338  CGTACGTCCCGACCTGATCGCTTCCGCCACCAGCGTCGGCGCCCCGCACAAGGGTTCGGA  397
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  387  CGTACGTCCCGACCTGATCGCTTCCGCCACCAGCGTCGGCGCCCCGCACAAGGGTTCGGA  446

Query  398  CACCGCCGACTTCCTGCGCCAGATCCCACCGGGTTCGGCCGGCGAGGCAGTCCTCTCCGG  457
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  447  CACCGCCGACTTCCTGCGCCAGATCCCACCGGGTTCGGCCGGCGAGGCAGTCCTCTCCGG  506

Query  458  GCTGGTCAACAGCCTCGGCGCGCTGATCAGCTTCCTTTCCAGCGGCAGCACCGGTACGCA  517
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Query  518  GAATTCACTGGGCTCGCTGGAGTCGCTGAACAGCGAGGGTGCCGCGCGCTTCAACGCCAA  577
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  567  GAATTCACTGGGCTCGCTGGAGTCGCTGAACAGCGAGGGTGCCGCGCGCTTCAACGCCAA  626

Query  578  GTACCCGCAGGGCATCCCCACCTCGGCCTGCGGCGAAGGCGCCTACAAGGTCAACGGCGT  637
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Sbjct  627  GTACCCGCAGGGCATCCCCACCTCGGCCTGCGGCGAAGGCGCCTACAAGGTCAACGGCGT  686

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Query  818  CGAGGTGAACCAGGTCTTCGGCCTCACCAGCCTGTTCGAGACCAGCCCGGTCAGCGTCTA  877
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  867  CGAGGTGAACCAGGTCTTCGGCCTCACCAGCCTGTTCGAGACCAGCCCGGTCAGCGTCTA  926

Query  878  CCGCCAGCACGCCAACGGCCTGAAGAACGCCAGC-TGT  914
            |||||||||||||||| |||||||||||||||| ||| 
Sbjct  927  CCGCCAGCACGCCAACCGCCTGAAGAACGCCAGCCTGT  964
```

Figure (7): The alignment in isolate (P-16) by using BLASTn.

Table 2: Types of mutations in lipA gene sequence in P. aeruginosa.

Sample	Wild Type	Mutant Type	Site	Amino acid	Mutution	Effect
P 16	CGC CTG	GGC -TG	943 961	Arg / Gly Deletion C	Substitution Deletion	Missense Frameshift

The results of the afore-mentioned show that the sequences of local isolate **(** P- 16) which has the highest lipolytic activity was close to the nearest strain in NCBI Pseudomonas aeruginosa strain 8380 with 99% percentage of identity. Figure (8) show the effect of mutations.

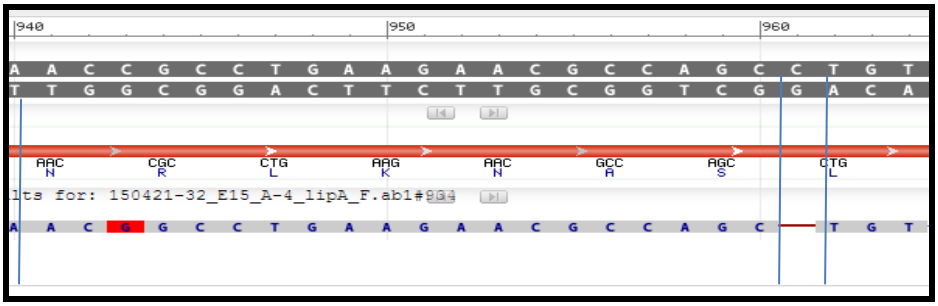

Figure (8): The effect of mutations in isolate (P- 16).

Missense mutation which may lead to change in the phenotype, because they may lead to change of amino acids and then in the protein , not all the mutation missense lead to significant changes in the protein because it can be replaced amino acid with another very similar in terms of chemical characteristic and in this case, the protein is still working normally, or it could replace amino acids in a region of the protein that does not significantly affect the secondary protein structure or function.[27]

Frame shift mutation leads to change the reading type, and that leading to quite different from the original translation, then to large change in protein translator.[28]

The increase in demand of lipases in various fields made the researchers improve the production abilities of the strains by mutation and selection.[29,30]

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