RESULTS

Part I

1. Isolation and purification of bacterial isolates :

The bacteria were isolated from animal blood, which obtained from slaughter house at Benha, Qualubia governorate, Egypt. The animal blood was homogenized using demineralized water and the homogenate was diluted and plated onto MGY agar media followed by incubated for overnight at 28ºC. Different morphological colonies were revealed on plates. Each one isolated colony was inoculated on MGY agar slant medium and nutrient broth medium containing 20% glycerol, by freeze-drying in 10% skimmed milk for its long-term preservation.

2. Qualitative and quantitative screening for selection of potent lipase and protease producers :

Twenty five bacterial isolates were isolated from animal blood waste samples on MGY agar medium. These isolates were purified and subjected to a screening program in order to evaluate their lipolytic and proteolytic activities by measuring clearing zone technique (CZ) (Table 3).

2.1. Lipase producer bacteria :

These isolates were subjected to a screening program in order to evaluate their lipolytic activities by measuring TCZ around the bacterial colonies. Out of 25 there was found that 4 bacterial isolates gave lipolytic activities while only one of them (No. 3) considered to gave a high lipolytic activities.

The determination of the most potent lipase producer bacterial isolates on the basis of mean diameters of clearing zones (mm) by testing

the potency of the best bacterial isolates, which selected from the previous test to attack animal blood waste. It was found that bacterial isolates No. 3, 12, 13 and 21 gave highest lipolytic activities where it reached up to 23.5, 21.4, 21.6 and 22.5 mm respectively compared with other isolates (Table 3).

2.2 Protease producer bacteria :

Twenty five bacterial isolates were subjected to a screening program in order to evaluate their proteolytic activities by measuring (GCZ) around the bacterial colonies. Three out of then isolates gave a high proteolytic activities.

The testing potency of the best proteolytic bacterial isolates base on the mean diameters of clearing zones (mm). It was found that, bacterial isolates No. 2, 6 and 8 gave the highest proteolytic activities where they reached up to 24.5, 27.3 and 30.3 mm respectively compared with other isolates (Table 3).

From the previous results concerning the qualitative screening of lipase and protease production under study. Bacterial isolates *Bacillus* spp. and *Pseudomonas* spp. were selected for their ability of produce the enzymes in high enzyme production. These two isolates were subjected to identification tests as follows.

3. Identification of the most potent bacterial isolates :

The two bacterial isolates *Pseudomonas* and *Bacillus* spp. were subjected to an identification program to the genera level was performed as shown in Table (4).

Table (3): Enzyme producer bacterial isolates growing on animal blood wastes using TCZ and GCZ techniques.

Bacterial isolates No.	Animal blood	
	Protease production (mm)*	Lipase production (mm)**
1	14.5	13.5
2	24.5	15.7
3	15.3	23.5
4	18.1	19.3
5	18.5	17.1
6	27.3	15.2
7	18.2	16.4
8	30.3	18.6
9	20.7	17.8
10	24.0	18.1
11	21.2	20.8
12	19.9	21.4
13	18.8	21.6
14	17.7	19.5
15	15.5	17.0
16	14.1	18.5
17	18.8	12.6
18	15.6	17.7
19	21.7	19.9
20	22.5	20.2
21	23.4	22.5
22	24.0	19.5
23	20.3	18.1
24	19.1	14.3
25	17.2	16.5

Table (4): Morphological, physiological an biochemical characteristics of bacterial isolates.

Characteristic	*Bacillus* sp.		*Pseudomonas* sp.	
	B. cereus	B. isolate	*Ps. Fluorescens*	Ps. isolate
Cell shape	Bacilli	Bacilli	Bacilli	Bacilli
Gram reaction	+	+	-	-
Cell diameter (µm)	3 x 1.2	3 x 1.1	0.9 x 1.2	0.8 x 1.2
Pigments	-	-	+	V
Oxidase	+	+	+	+
Amylolytic activity	+	+	-	-
Pectolytic activity	+	+	+	+
Cellulytic activity	+	+	-	-
Biolytic activity	-	V^{+}	+	+
Protolytic activity	+	-	-	V
Urease	-	V	-	-
Utilization of:				
Benzoate	-	V	+	V
Mannitol	+	+	+	+
Cellubiose	-	-	-	-
Sorbitol	-	+	-	V
Sucrose	-	+	-	-
D-arabinase	-	+	-	V
L-rhamnose	-	-	-	V
D-aspartate	-	-	-	-
Xylose	+	+	+	+
Glucose	+	+	+	+
Lactose	-	-	-	-
Maltose	-	-	-	-
Ribose	+	+	+	+
Glycerol	-	+	+	+
Fructose	+	V	-	-
Raffinose	-	-	-	-
Galactose	+	+	+	+
Ethanol	-	+	-	-
Butyrate	-	-	+	+
Arginine test	-	+	-	-
Malonate test	-	-	+	+
VP test	+	+	+	+
O/F test	+	+	+	+
Anaerobic growth	-	-	-	+
Inole	-	-	-	-
Growth at 55ºC	-	-	-	-
41ºC	-	-	-	-
37ºC	-	-	+	+
4ºC	+	+	+	+

* CF: Cairo Microbiological Resources Center (MIRCEN)

O/F : Oxidation/fermentation
VP : Voges-Proskauer
V : Variable

\+ Positive result
\- Negative result
$+^{*}$ 75% or more isolate positive
\- 80% or more isolate negative

3.1. General characteristics :

All morphological characteristics and stain reaction led to the fact the two bacterial isolates under identification are suggestive of being belonging to genera *Pseudomonas* gran-negative aerobic to facultative anaerobic, non-spore formers, oxidase, pectinase, lipase positive while amylase, cellubiose negative and protease variable. Acid produced from mannitol, sucrose, L-arabinose, aspartate, and galactose. The cell were able to grow 32±2ºC and grow in the presence of 0-3% of NaCl at pH 7.0. The genus of *Bacillus*, non-producing pigments, Gram-positive aerobic, spore formers- Amylase, cellulase, protease, lipase, and calatase positive while urease and caseinase negative. Acid produced from xylose, glucose mannose, ribose, sucrose, glycerol, fructose and galactose. The cells were able to grow between 28-35ºC temperature interval. The cells were able to grow in the presence of (0.2%) of NaCl at pH 7.0 (Table 4).

3.2. Specific characteristics :

Pseudomonas isolate appears fluorescence pigment on nutrient agar medium, short rod shaped 0.9 x 1.2 µ. Voges-Proskauer (VP) test and tyrosine positive. Since *Pseudomonas* EG isolate able grow anaerobically, it is suggested to belong to the species *Fluorescens* according to criteria described by consulting Bergey's Manual of Systematic Bacteriology **(Sneath, 1986).** It could be given the tentative name *Pseudomonas fluorescens* EG isolate (Table 4).

Bacillus EG isolate reveals waxy in colour on nutrient agar medium, rod shaped 3 x 0.8 µm, Voges-Proskauer (VP) test and tyrosine negative. Since *Bacillus* isolate was able to grow aerobically, it is suggested to belong to the species *Cereus* according to criteria described by consulting Bergey's Manual of Systematic Bacteriology **(Sneath,**

1986) It could be given the tentative name *Bacillus cereus* EG isolate (Table 4).

3.3. Molecular identification :

3.3.1. PCR amplification of 16S rRNA gene :

Genomic DNAs were isolated from bacterial isolates (*Ps. fluorescens* EG and *Ps. fluorescen* ATCC 1003) using a lysozyme dodecyl sulfate lysis procedure with high quality and substantially free RNA contamination. The DNA was then used as a template for PCR to amplify the 16 sRNA gene via the QLAGEN PCR system by use of an oligo (dt) Fdi and rP2 primer sets, nearly full length 16S rRNA gene could be synthesized. The amplified 165 rRNA gene was used as a template using the internal primer combination (Fdi and rP2) in PCR for conformation its specificity to the *Ps. fluorescens* 16S rRNA gene as a PCR product with a size of about 1451 bp DNA was amplified (Fig. 3A).

3.3.2. Partial sequence of 16S rRNA gene :

Cloning into pGEM-T easy vector :

The PCR product of 16S rRNA gene was extracted from the agarose gel using the Agarose Gel DNA Extraction Kit and cloned into the pGEM-T easy vector as mentioned in the materials and methods. The plasmid was then introduced into *E. coli* strain DH5 and the recombinant colonies were detected by blue/white colony screening. White and ampicillin resistant colonies were selected for further analysis. The white colonies were subcultured on LB ampicillin containing broth. The plasmid was then isolated and purified using the Wizard ® Plasmid Minipreparation System.

Fig. (3): A. Agarose gel, 1% stained with ethidium bromide showing PCR amplification of 16S rRNA gene using primers.
Lane M; λ DNA marker, Lane 1. *P. fluorescens* (ATCC 1003) and Lane 2, *Ps. Fluorescents* EG isolate expected band 1451 bp., Lane 3, *B. cereus* isolate expected ban ~ 115 bp.
B. Dot blot hybridization of DNA prepared from (1) *Ps. Fluorescens* EG. (2) *Ps. Fluorescens* (ATCC) and 3.4.5 and 6 *Ps. Fluorescens* isolated from animal blood with general eubacterial probe.

Fig. (4): Agarose gel 1.2% stained with ethidium bromide showing plasmid minipreparation of white clones containing the 16S rRNA gene fragment. (A) and Lipase gene fragment (B).
Lane M, DNA marker.

PCR confirmation :

To confirm the presence of the insert (1451 bp), the purified plasmid was used as a template for PCR detection of the recombinant colonies and a nested-PCR product with a size of about 1451 bp (Fig. 4A) was amplified.

Digestion analysis :

As an addition confirmatory test, the new plasmid was digested with *Eco* R1 restriction enzyme and a band with a size of about 1475 bp was released to prove the successful cloning the 16 rRNA gene under investigation.

DNA sequence :

The created plasmid was sequenced as mentioned before. Results in Fig. (5) showed that the nucleotide sequences of the PCR fragment which appeared to be containing 1451 nucleotides.

Sequencing analysis :

On the level of DNA sequencing, the similarity between the present sequence (16S rRNA) and that of the overseas strains belonging to different groups could be summarized as follows: In the first group, in case of AJ30301-chloro, Z7660-corona-s, AJ492828-congs AY574913-syrin and PSEATCCOq-cari strains, the similarity was formed 100%. On the level of nucletide sequence. Regarding the second group, that contains AY951875-Flose; EF694708-Flo-s and FJ470030-Flo-s strains, the identity was 100% on the level of nucleotide sequence. Considering the third group, that contains the first group and second group, the homology was 99% on the level of nucleitde sequence. The phytogenetic analysis of the present nucleotide sequence (Fig. 6) showed that the 16S rRNA

Fig. (5): Nucleotide seqauence of the 1451 bp fragment containing the *Ps. Fluorescens* EG 16S rRNA structural gene.

Fig. (6): Phytogenetic tree based on the 16S rRNA gene sequences of *Ps. Fluorescens* EG strain and *Ps. Fluorescens* spp. The branching pattern was generated by the neighbor-joining method, the numbers at the nods indicate the levels of boot strap support based on a neighbor-joining analysis of 100 resample data sets.

Ps. fluroscens Egypt strain, was classified in a separated cluster whereas the homology was 88% with the first and second groups.

3.3.4. Southern blot hybridization :

By using Southern blot technique (DNA-DNA hybridization) with the universal oligonucleotides probe against denatured DNA immobilized on Hyband N nylon membrane (Fig. 3-B), performed to analyze the genetic relatedness of *Ps. fluorescens* EG isolate with other *Pseudomonas* spp. The EG-isolate showed 74% relative binding ratio (RBR) with *Ps. fluorescens* (KACC 1003). Avery low hybridized signal was observed with other *Psudomonas* spp,. Based on this evidence, along with the biochemical and physiological anslysis and the 16S rRNA, it can be inferred that the EG isolate is *Ps. fluorescens*.

3.3.2.1. PCR amplification of *lip A* gene :

The total DNAs were isolated from *Ps. fluorescens* EG isolate using a lysozyme dodecyl sulfate lysis procedure with high quality and substantially free RNA contamination. The DNAs were used as a template for PCR to amplify the lipase gene (*Lip A* gene) via the QIAGEN PCR system by use of an oligo (dt) GF1 and GR2 primer sets, nearly fall length *Lip A* gene could be synthesized. The amplified *Lip A* gene was used as a template using the internal primer combination (GF1 and GR2) in PCR for conformation its specificity to the *Ps. fluorescens Lip A* gene, as a PCR product with a size of about 1315 bp (Fig. 3B).

3.3.2.2. Cloning and nucleotide sequence of *Lip A* gene :

The PCR product of *Lip A* gene was extracted from the agarose gel using the Agarose Gel DNA Extraction Kit and cloned into the plasmid pTTY2 vector as mentioned in the materials and methods. The plasmid was then introduced into *E. coli* strain DHS and the recombinant colonies

were detected by blue/white colony screening. White and ampicillin resistant colonies were selected for further analysis. The white colonies were subcultural on LB ampicillin containing broth. The plasmid was then isolated and purified using the Wizard ® Plasmid Minipreparation System.

PCR conformation:

To conform the presence of the insert (1320 bp) the purified plasmid was used as a template for PCR detection of the recombinant colonies and a nested-PCR product with a size of about 1320 bp (Fig. 4B) was amplified.

Digestion analysis :

As an addition confirmatory test, the new plasmid was digested with ECO R1 restriction enzyme and a band with a size of about 1320 bp was released to prove the successful cloning the *Lip A* gene under investigation.

***Lip A* gene sequence :**

The created plasmid was sequenced as montioned before. The result illustrate in Fig. (7-A) show the nucleotide sequence of PCR fragment which appeared to be containing 1350 bp. The nucleotide sequence of the *Lip A* gene was recorded in Genebank with Accession number FJ639839 seq.

Sequencing analysis :

The similarity between the present sequence *Lip A* gene and that of the overseas strains belonging to different groups could be summarized as follows: In case of, AB109034 seq.; AB109040. seq., AB109039 seq (first group) strains the similarity was formed 93% on the level of nucleotide sequence. Regarding, the second group, that include

AB025596. seq; DQ906143 seq and AY721617 seq strains the identity was 88%. On the level of nucleotide sequence. Considering the third group, that include AF202538 seq strain with similarity 41% of the level of nucleotide sequence (Fig. 7-B).

The phylogenetic analysis of the present nucleotide sequence (Fig. 7-B) showed that, the *Lip A* gene from *Ps. fluorescens* EG strain was classified in a separated cluster where as the homology was 41% with the first, second and third groups.

2.6. Deduced amino acids sequence of *Lip A* gene :

The predicated numbers of amino acids were produced from translation of *Lip A* gene nucleotide sequence of *Ps. fluorescens* were 438 amino acids starting with methionine and ended with arginine (Fig. 8). Amino acids composition of *Lip A* gene sequence was showed that the type of aminoacids consists of 20 amino acids. Allanine has a high frequency with percentage 11.87%, valine, lle, serine, leu Glycine, Aspartic ghrinine and Asn has moderate frequency with percentage 4.57, 5.48; 8.90; 8.90, 7.99; 6.85; 7.76 and 7.08% respectively, while proline, Glu Arginine, Gln, treptophan tyrosine; phenylalanine; it is crystine, methionine and Lys has a low frequency and percentage 4.34, 2.28, 4.12, 2.05, 2.28; 3.65, 3.65, 2.28, 1.37, 1.37; 8.475% respectively (Table 5), as well as stop codon.

2.7. Nucleotide sequence accession numbers :

The *Lip* gene and 16S rDNA gene of *Ps. fluorescens* EG. Strain intergenic spacer region sequences determined in this study have been deposited in the EMBL, Gene Bank, and DDB1 nucleotide databases under the following accession numbers: FJ639838 respectively.

Fig. (7): Nucleotide sequence of *Lip A* gene of *Ps. Fluorescens* EG strain (A) and Dendrogram showing clusters of *Ps. Fluorescens* strain based on their nucleotide sequences (B).

Fig. (8): Amino acid sequence of *Lip A* gene of *Ps. Fluorescens* EG strain (A) Dendrogram showing clusters of *Pseudomonas* strains based on their amino acids sequences (B).

Table (5): Predicted amino acids composition of *Lip A* gene sequence of *Ps. fluorescens*.

Type of A.A.*	Name	No.	Frequency (%)
P	Proline	19	4.34
V	Valine	20	4.57
E	Glutamic	10	2.28
R	Arginine	18	4.12
I	Ilenine	24	5.48
S	Serine	39	8.90
Q	Gln	9	2.05
L	Leucine	39	8.90
W	Trpitophan	10	2.28
G	Glycine	35	7.99
Y	Tyrosine	16	3.65
F	Phenylalanine	16	3.65
D	Aspergine	30	6.85
H	Histidine	10	2.28
C	Cystine	6	1.37
M	Methionine	6	1.37
T	Thrionine	34	7.76
X	**	*	*
K	Lysine	15	8.42
A	Alanine	52	11.87
N	Aspartic	31	7.08
Total no. of A.A.		438	100%

* A.A. amino acid.
** Stop codon.

Part II. Enzymes production :

1. Protein content :

Protein content were determined in bacteria isolates related to BSA as stander protein. Table (6) revealed that protein content and enzyme activity in *B. cereus* and *Ps. fluorescens*.

Table (6): Protein content and enzymes activities of *B. cereus* and *Ps. aeruginosa*.

Bacterial isolates	Protein content mg/g cells	Lipase		Protease	
		U/g cells	Specific activity	U/g cells	Specific activity
B. cereus	2.134	35.56	25.10	233.04	205.5
Ps. fluorescens	1.595	311.34	195.20	72.97	45.75

- Specific activity (unit/mg protein)
- U/g unit/fram cell

B. cereus produced high protein content (2.134 mg/g cells) while *Ps. fluorescens* was produced low (1.595 mg/g cell) protein content. On the other hand, *B. cereus* produced high amount of protease (233.04 U/g cells) with high activity (205.5). While it produced low amount of lipase (35.56 U/g cell) with low activity (25.10). On the contrary, *Ps. fluorescens* produced high amount of lipase (311.34 U/g cell) with high activity (195.20) while low amount of protease with low activity (45.75).

2. Qualitative of proteins :

Qualitative of bacterial proteins were determined on the basis of the number, intensity, molecular weight and reproducibility of SDS-PAGE bands (Fig. 9). The bands with the same mobility were treated as identical fragments, weak bands with negligible intensity and smear bands were both excluded from final analysis. Fig. (9) demonstrated the SDS-PAGE profile obtained with *B. cereus* and *Ps. fluorescens*. The number and density of band varied between them. In SDS-PAGE of the

total protein of *B. cereus* and *Ps. fluorescens* where tributyrin and gelatin induced cells the over production of lipase and protease were detected (Fig. 9).

When tributyrin and gelatin were emulsified with buffer agarose and poured onto washed SDS-polyacrylamide gel, lipolytkic and proteolytic activities were shown at the new band position by clearing the opaque triglyceride and polypeptide. This staining results implied that this protein band corresponded to the lipase and protease produced in *B. cereus* and *Ps. Fluorescens* (Fig. 9).

Figure (12) shows a comparison of the total cellular protein from the cells with the without induction.

Fig. (9): SDS-PAGE analysis of protease and lipase expressed in *B. cereus* and *Ps. Fluorescents,* Lane is located with the following molecular mass standards, Lane 1,2,3,4 total cellular proteins from *B. cereus* (ATCC), *B. cereus* EG, *Ps. Fluorescens* (ATCC) and *Ps. Fluorescens* EG. Lanes 5 and 6 purified protease and lipase enzymes respectively.

3. Factors controlling of enzyme production :

3.a. Incubation temperature: Effect of different incubation temperatures on lipase and protease production were recorded in Table (7). It could be concluded from results that, the maximum lipase (s) and protease(s) productivity were attained at 35ºC in the presence of animal blood (slaughter houses wastes viz, 525,25, 2.75 µ/ml (Lipase) and 5.75 and 475.25 µ/ml (protease) for both *Ps. fluorescens* EG and *B. cereus* EG isolates, respectively. Below or above this particular degree temperature gave values gradually decreased as compared to optimal temperature values recorded for both bacterial strains.

Table (7): Relationship between incubation temperatures to lipase and protease(s) productivities and bacterial strains.

Incubation temp. (ºC)	Lipase(s) (µ/ml)		Protease(s) (µ/ml)	
	Ps. fluorescens	*B. cereus*	*Ps. fluorescens*	*B. cereus*
5	0.0	0.00	0.0	10.17
10	59.12	0.00	5.75	60.28
15	75.35	0.00	7.25	75.39
20	95.45	0.75	10.34	95.41
25	150.75	0.89	15.25	175.52
30	375.25	1.45	25.16	225.63
35	525.15	2.75	35.75	475.25
40	72.25	1.12	5.51	12.12
45	1.50	0.80	2.21	10.25
50	0.00	0.00	0.00	7.21

2.2. Incubation period :

Data in Table (8) showed the relation between lipase and protease(s) productivities and time of incubation. For a general view of constitutive level of lipase and protease(s) activities and the inducing effect of animal blood substrate. The enzymes productivities were detected at time intervals of 10 to 100 intervals 10 hr. incubation for the

two bacterial isolates on the production medium. The level of lipase and protease(s) increased gradually with increasing the incubation period up to 70 hr for *Ps. fluorescens* and (725.25 and 952.25) and *B. cereus* isolates (275.95 and 1725.75 µl/ml) respectively. Then decreased value in the enzymes productivities could be observed.

Table (8): Relation of microbial isolates to incubation period on enzymes production on animal blood medium.

Microbial isolates / Incubation period	Lipase production (µ/ml)		Protease production (µ/ml)	
	Ps. fluorescens	*B. cereus*	*Ps. fluorescens*	*B. cereus*
10 hr	25.15	10.31	10.25	25.72
20 hr	70.32	21.75	70.31	100.15
30 hr	250.58	31.57	212.75	212.32
40 hr	521.70	50.81	531.31	975.85
50 hr	615.94	75.32	875.21	1221.91
60 hr	650.82	95.62	850.71	1520.72
70 hr	725.25	275.95	952.25	1725.75
80 hr	412.25	35.35	120.15	857.23
90 hr	85.12	20.75	31.17	125.31
100 hr	51.72	5.21	10.21	25.75

3.a. Initial pH values :

For this study, the animal blood medium (SHBM) was adjusted at different initial pH values covering the range of (3-10). It could be concluded from the results recorded in Table (9) represented the optimum initial pH value capable of promoting lipase(s) biosynthesis by *Ps. fluorescens* and protease(s) bioassayed by *B. cereus* were found to be at

the value of 7.0. Since the enzymes yields, reached up to 25.21; 35.70 (*Ps. fluorescens*) and 15.21; 450.13 (µ/ml), (*B. cereus*) for lipase and protease respectively. The enzymes productivity decreased gradually above and below then optimal pH values.

Table (9): Effect of initial pH values on lipase and protease productivities by bacterial isolates allowed to grow on SHBM for 48 h under submerged fermentation condition.

Initial pH value	Lipase production (µ/ml)		Protease production (µ/ml)	
	Ps. fluorescens	*B. cereus*	*Ps. fluorescens*	*B. cereus*
3	3.0	0.0	4.50	10.21
4	5.12	0.0	6.75	15.43
5	6.25	0.75	11.21	20.65
6	7.25	4.15	25.12	50.81
7	25.21	15.21	35.70	450.13
8	23.35	9.40	19.16	350.34
9	9.15	5.21	12.25	300.62
10	2.75	4.00	5.34	10.65

4.a. Substrate concentrations

It could be concluded from the results recorded in Table (10) and illustrated that, the maximum lipase and protease(s) productivities were attained in the presence of 2.5 g animal blood, Baxter to the in the case of *Ps. fluorescens* and *B. cereus* EG isolates.

Table (10) : Relation of substrate concentration application to lipase an protease production by bacterial isolates.

Substrate concentration (g/flask)*	Lipase production (µ/g)		Protease production (µ/g)	
	Ps. fluorescens	*B. cereus*	*Ps. fluorescens*	*B. cereus*
0.50	0.0	0.0	0.0	5.17
1.00	3.25	0.0	50.0	75.95
1.500	4.12	0.0	39.28	150.83
2.00	5.75	3.64	50.28	300.64
2.50	17.25	9.47	75.37	425.42
3.00	6.21	7.25	71.49	250.25
3.50	5.75	5.13	50.54	50.17
4.00	4.95	4.31	10.62	10.92
4.50	4.01	2.52	8.71	5.85
5.00	3.25	1.74	2.89	2.73

* Flask 250 ml.

5.a. Carbon sources :

The effect of certain ten different carbon sources listed in Table (11) were introduced into the applied production medium of lipase and protease productivities by *Ps. fluorescens* and *B. cereus* EG-isolates under animal blood (SHW) condition were studied. It was clear that all the different carbon sources exhibited various indicative degrees lower than control by both most potent bacterial strain.

6.a. Nitrogenous sources :

The effect of 8 various nitrogenous sources (organic and inorganic) on lipase and protease(s), productivities by *Ps. fluorescens* and *B. cereus* were applied as shown in Table (12). The maximum value of lipase and protease productivities recorded at 242.95 and 1528.75 µ/ml respectively in applied ammonium molybdate with *Ps. fluorescens* respectively. While with *B. cereus* 215.75 and 1375.12 µl/ml in applied ammonium dihydrogen phosphate respectively.

Table (11): Relation of different carbon sources application to lipase and protease productivities by bacterial, EG strains.

Carbon sources	Lipase production (µ/ml)		Protease production (µ/ml)	
	Ps. fluorescens	*B. cereus*	*Ps. fluorescens*	*B. cereus*
D (-) glucose	120.15	275.1	15.0	292.75
D (-) fructose	225.12	45.2	19.9	35.73
D (+) Mannose	18.89	50.3	12.8	45.52
D (+) Xylose	21.70	75.4	14.7	19.41
Ribose	32.61	31.5	13.6	17.15
Lactose	115.52	12.75	18.5	25.23
Sucrose	27.43	10.0	25.4	30.91
Raffinose	19.34	12.7	10.3	10.12
Starch	31.25	14.8	120.2	18.85
Dextrin	33.16	20.9	30.0	17.74

Table (12): Relation of different nitrogenous source application to lipase and protease productivities by bacterial isolates.

Microbial isolate / Nitrogenous sources	Lipase production		Protease production	
	Ps. fluorescens	*B. cereus*	*Ps. fluorescens*	*B. cereus*
Peptone	25.212	18.75	157.21	257.13
Ammonium acetate	8.34	5.75	10.25	15.21
Ammonium molybdate	242.95	12.04	1528.75	1250.50
Ammonium dihydrogen phosphate	90.10	215.75	572.81	1375.12
Ammonium sulphate	85.29	18.82	350.62	257.33
Ammonium nitrate	102.38	17.80	212.44	125.25
Sodium nitrate	45.47	75.44	57.22	75.14
Potassium nitrate	35.56	50.26	20.11	40.15

3. Characterization of enzymes :

Some characters of the partially purified lipase and protease(s) produced by *Ps. fluorescens* and *B. cereus* EG isolates respectively, growing on animal blood (SHW) as a substrate and incubated under optimal nutritional conditions. These characters include purities: Thermostability pH stability; substrate concentration; enzymes concentration and inhibitors or activators on hydrolytic activity.

3.a. Enzymes purification :

The potent bacterial isolates were allowed to grow on production medium under optimal semi solid fermentation conditions for production of lipase and protease(s) by *Ps. fluorescens* and *B. cereus* respectively, at the end of incubation period one liter of production media were collected separately. The enzymes were precipitated by centrifugation at 6000 rpm for 20 min under cooling. The supernatant (bacterial cell) was discarded and the precipitate was connected.

The active enzyme protein was obtained by saturated ammonium sulfate 80% were specific activity 1567.82 and 15.27 µ/ml and protein content 0.825 and 0.812 mg/ml corresponding to a specific activity of 1900.38 and 18.80 μ/mg^{-1} protein of protease and lipase respectively (Table 13). Only 50 ml was obtained at the end of the process and dialyzed against tap water, followed by dialysis against sucrose crystal until a volume 3 ml was obtained.

Results illustrated in Table (13) showed that, a sharp one peak was obtained in both enzymes purification and specific activities reached its maximal value up to 1900.38 and 18.80 μ/mg^{-1} protein for protease and lipase enzymes respectively.

Table (13) summarized the purification steps of lipase produced by *Ps. fluorescens* and protease produced by *B. cereus* EG isolates.

Table (13): Ammonium sulfate precipitation pattern of the lipase and protease produced by *Ps. fluorescens* and *B. cereus*.

$(NH_{42}SO_4$ (%)	Protease				Lipase			
	Activity µ/ml (x)	Protein content mg/ml (y)	Specific activity (x/y)	Purificat-ion could	Activity µ/ml (x)	Protein content mg/ml (y)	Specific activity (x/y)	Purificat-ion could
CFF	1.52	0.25	6.08	1.0	0.42	0.450	0.93	1.0
20	23.42	0.310	75.54	11.38	1.00	0.540	1.69	1.31
40	142.65	0.425	335.64	37.61	2.12	0.650	3.26	1.44
60	543.21	0.612	887.59	144.43	5.21	0.725	7.18	1.611
80	1567.82	0.825	1900.38	310.38	15.27	0.812	18.80	18.90
100	734.52	0.521	1409.82	230.00	0.25	0.657	0.38	1.46

3.b. Amino acids composition of purified enzymes :

Free amino acids extracted from the enzymes protein were analyzed using OV225 glass column and a flames ionization detector in GLC technique. About 18 chromatographic peaks at different retention times (min) (Rts) were observed in the chromatograms corresponding to the free amino acids in enzymes or\\protein. The retention time values for authentic amino acid derivatives frequently identified were presented in Table (14) and Fig. (10 a, b). However, two unknown peaks could be exactly identified owing to the absence of two authentic amino acid samples. They appeared in the extracted of Rt 19.1 and 25.5 min and were designated by a and b respectively.

The changes in the relative percentages of the individual free amino acid constituents and profiles in the chromatogram of lipase and protease proteins were presented in Table (14) and Fig. (10 a,b). These data illustrated that, methionine and tyrosine peaks were absent in lipase protein. Moreover, alanine, valine, were predominant amino acids each of them was more than (10%) from the total free amino acids. On the other hand, thrionine, glycine, isoleucine, leucine, lycine serine, cystine, aspartic, phenylanine, glutamic, histidine and two unknown were found with moderate amounts (3%). However, arginine was found with low concentration with less than (1%) from the total amino acids (Table 14 and Fig. 10 a,b). The amino acids composition of protease proteins 18 amino acids, 16 amino acids present and 2 unknown. However, alanine, valine and tyrosine were predominant amino acids each of them more than (15%) from the total amino acids. On the other hand thereonine, glutamic and histidine were found high concentration between (10% to 15%) of total amino acids. As well as unknown (a) glycine, isoleucine, leucine, lysine, serine, unknown (b); cystine methionine, aspartic and phenylalanine were found with moderate amount with less than (10%). However Arginine was found with low concentration with less than (1%) from the total amino acids (Table 14 and Fig. 10b).

3.c. Protein fraction of enzymes :

Qualitative of enzymes proteins, number, intensity, molecular weight and reproducibility of SDS-PAGE bands were determined. Bands with the same mobility were treated as identical fractions. Weak bands with negligible intensity and smear bands were both excluded from final anlysis.

Table (14): Retention time and relative percentage of free amino acids in the lipase and protease enzymes by a gas chromatography equipped with 3% OV225 glass column and a Hames ionization detector.

Retention time (min)		Amino acids	Relative percentage	
			Lipase(s)	Protease(s)
1	16.5	Alanine	12.1	15.2
2	17.2	Valine	11.2	17.3
3	18.2	Threonine	01.4	10.5
4	19.1	Unknown [(a)]	01.6	09.7
5	14.8	Glycine	01.8	02.8
6	21.2	Isoleucine	01.3	04.7
7	22.0	Leucine	01.5	02.8
8	24.0	Lycine	01.7	01.6
9	24.8	Serine	02.9	03.4
10	25.5	Unknown [(b)]	02.3	04.2
11	26.8	Arginine	00.9	00.8
12	27.2	Cysteine	02.8	01.7
13	29.0	Methionine	-	05.8
14	29.8	Aspartic	02.9	03.6
15	31.5	Pheylalanine	04.6	04.4
16	32.5	Glutamic	03.7	14.2
17	33.5	Hiditine	03.53	21.1
18	38.5	Tyrosine	-	15.5

Fig. (9) demonstrates the SDS-PAGE profile obtained with lipase and protease enzymes. The number of scored one band of each lipase and protease respectively. The molecular weight of newly protein fractions ranged from about 670 kDa of lipase enzyme and 52.5 kDa of protease

enzyme, the obtained 8 newly protein patterns were considered as protein genetic markers.

3.d. Enzyme stability :

The aim of this study the stability of the concentration of purified lipase and protease enzymes in the presence of thermal, pH, chlorine, oxidizing agents and commercial detergents at higher temperature because of the fact that, an ideal detergent enzyme should be stable and active in the detergent solution and should be have adequate thermal stability to be effective in a wide range of washing temperatures.

3.d.1. Thermostability of the purified enzymes :

Data recorded in Table (15) showed that lipase and protease activities showed that, the enzymes exhibited they are optimal activities at 50-60ºC, where they reached 650.25 an 2377.0 µ/ml respectively.

Table (15): Thermostability of the purified lipase and protease produced by *Ps. fluorescens* and *B. subtilis*.

Enzymes / Temperature	Lipase activity (U/ml)	Protease activity (U/ml)
40	425.25	1250.15
50	575.45	1857.27
60	650.03	2250.42
70	75.65	1510.65
80	52.88	120.83
90	35.07	52.72
100	20.15	25.91

3.d.2. pH stability of purified enzymes :

Interestingly, results illustrated in Table (16) showed that the purified lipase and protease produced by *Ps. fluroesens* and *B. cereus* exhibited they are maximal activities at pH 7 and 8.5 where they reached up to 135.25 and 4226.98 µ/ml. The enzymes still active up to pH 10.

Table (16): pH stability of the purified lipase and protease activities.

pH values	Activity (U/ml)	
	Lipase	Protease
5	47.25	1525.27
6	50.14	1957.45
7	135.25	2100.83
8	110.75	2575.76
9	125.25	3157.54
10	115.13	2265.32

3.d.3. Concentration of enzymes :

The activity of lipase and protease was found that, there are a continuous increasing of enzyme concentration units where they reach up to 350.28 and 3758.75 µ/ml respectively as shown in Table (17) .

Table (17): Effect of purified enzymes concentration on activities produced by *Ps. fluorescens* and *B. subtilis*.

Enzyme (µ/l)	Enzyme activities (µ/ml)	
	Lipase	Protease
25	25.15	251.7
50	51.31	725.6
75	110.52	1007.5
100	150.74	1125.4
125	210.83	1575.3
150	285.91	2075.2
175	321.25	2525.1
200	350.28	3758.75

3..4. Sodium hypochlorite on enzymes activities :

The aim of this study the effect of chlorine on the enzymes compatibility. In the fact that enzyme incompatibility with chlorine is the reason for its failure to use as a part of cleaning agent in some detergent formulations which contain chlorine as bleaching agent. The results recorded in Table (18) illustrated that, the chlorine concentration up to 10 µl/ml increased the lipolytic activity and the total activity was 149.6% as compared to control. While increasing chlorine concentration up to 15 µl/ml indicated on obvious decrease in lipolytic activity up to 60.8% remaining activity. On the other hand, data recorded in Table (18) indicated that chlorine at 5 µl/ml did not exert any effect on stability of protease at all intervals time. While increasing the chlorine concentration due to decreasing the protease activity by different ratios depending on the time of enzyme exposure to chlorine.

3.d.5. Oxidizing agents and surfactants :

The results recorded in Table (19) and represented graphically in Fig. (26) showed that, the application of triton x 100 exhibited a very high enhancement in lipolytic and proteolytic activities in which the maximum stimulation were occurred with 2 to 10% (v/v) triton, where the activities increased 1025.20% to 2433.51% respectively. As well as lipase was stable in all applied concentration of SDS. On the contrary protease was unstable in all applied concentration of SDS. On the other hand H_2O_2 excreted an obvious decreased effect on lipase and protease activities at all applied concentrations. However, sodium cholate showed a remarkable increase activities at all concentration.

Table (19): Effect of oxidizing agents and surfactants on lipase protease activities.

Enzymes / Oxidizing agents conc. (w/v)		Lipase		Protease	
		Activity µ/ml	Relative activity (%)	Activity µ/ml	Relative activity (%)
Control	0.0	50.00	100	275.00	100
Sodium dedecyl sulphate (SDS)	2	50.75	101.5	1421.15	516.78
	4	120.50	241.0	1230.24	447.36
	6	252.10	504.2	750.35	2.72
	8	450.25	400.5	000.75	0.00
	10	505.75	1011.5	000.00	0.00
H_2O_2	2	20.50	41.00	1502.15	546.23
	4	19.50	38.92	1520.14	552.77
	6	15.21	30.42	1520.41	55.2.87
	8	10.21	20.42	1150.52	418.37
	10	07.75	15.50	750.73	272.99
Sodium cholate	2	120.15	240.30	1250.93	454.88
	4	240.28	480.56	1421.80	517.01
	6	512.40	102.80	2121.78	771.55
	8	759.64	1519.28	2571.37	935.04
	10	1120.82	2241.64	3121.45	1135.07
Tween x 100	2	520.29	1025.20	1352.32	491.75
	4	720.47	1419.64	1834.56	667.11
	6	972.65	1916.55	1972.74	717.36
	8	1122.83	2212.47	2731.98	993.94
	10	1235.01	2433.51	3421.15	1244.05

Part III. Application of biological bacterial to technical and industrial :

A. Production of fibrinogen :

The rapid blood clotting in teleost animals has been well known for many years, and it is found that blood coagulation in animal is fundamentally similar to mammalian blood coagulation. The purified animal fibrinogen for the use of fibrin glue as a haemostatic agent, and characterized a mainly with respect to mechanical structure and viscoelustic properties of the resulting fibrin gels, in addition to possible immunological response.

A.1. Fibrinogen purified :

The fibrinogen was isolated from animal blood using centrifugation in combination with 100% saturated ammonium sulphate. NH_3 buffer pH 6.6. The diafiltration and concentration process was done with a cross flow of 2.5 L/min and a transmembrane pressure (TMP) of 0.6 bars. The resulting diafiltrated protein solution was concentrated 10 fold. However, the use of undesirable or possible toxic components such as Tris, aprotinin, benzamidine and Σ-aminocaproic acid in these protocols is incompatible with our goal. An alternative purification protocol for animal fibrinogen with only consumable components is presented.

The protein concentration were determined related to bovine fibrinogen as the standard protein. It was 2.37 mg/ml blood.

Unreduced SDS-PAGE analysis :

The first precipitation step of fibrinogen showed contamination of Co-precipitated proteins with molecular mass <150 kDa (Fig. 1-A, Lane 1) proteins were removed in a second precipitation step (Fig. 11, Lane 2). Further desalting and concentration (dia-ultrafilteration) of the second

precipitate tielded a highly clottable fibrinogen without any detectable effect on final protein composition (Lane 3).

Reduced SDS-PAGE analysis :

Analysis the SDS-PAGE patterns of the subunit chains of animal blood fibrinogen appears that the A ∝-chains stain poorly with coomassie and are heterogenous with a molecular weight of 90-110 kDa compared to A ∝-chains of human fibrinogen with a molecular weight of approximately, 67 kDa. The ββ and Y chains of animal blood and human fibrinogen are of equal size with molecular weight of approximately 55 and 50 kDa respectively.

Clottability :

Clottability of animal blood fibrinogen was determined using blood thrombin (4.5 NIH μ/ml) as a clotting agent. The animal blood fibrinogen was highly clottable the blood with 90%.

B. Application of enzymes in Bio-detergents :

Application of lipase and protease in detergents were carried out on clean cloth samples stained with different materials such as salad, chocolate, blood and oils in the Arial and without Arial and treated with 50ºC. The color strength values (K/S) for each cloth sample were compared to un stained cloth as control sample.

The results were tabulated in Table (20) it was cleared that, all treatments (enzymes + Arials) could be considered the best treatments for removal of salad, blood, chocolate and oils, where they gave the highest values of whiteness; reflectance and lowest values of yellowness as well as color strength value (K/S). Regarding cloth stained with blood and treated with lipase and protease with or without detergent (Arial) at 50ºC. The results in Table (20) clean cloth stained with blood, salad,

Fig. (11): A: SDS-PAGE (10%) analysis of nonreduced aliquots from the fibrinogen precipitation steps. Lane 4 protein marker; Lane 3 animal blood plasma, Lane 2. fibrinogen fraction of the first ammonium sulphate precipitation, Lane 4. stained with silver nitrate, the second ammonium sulphate.

B: Reduced animal blood and human fibrinogen precipitated from plasma at 56ºC for 3 min. Laner 7. protein marker, Lanes (1-3) purified animal fibrinogen and lanes (4-6) human fibrinogen. The protein were stained with coomassie brilliant blue G.

chocolate and oils at 50ºC gave the highest whiteness and lowest yellowness value where as, (62.1 x 3.5), (60.4 x 7.5); (61.5 x 5.3); 71.5 x 13.5) for lipase and (75.7 x 9.75); (61.6 x 5.3), (68.5 x 2.5) and (45.7 x 30.1) for protease respectively, comparing with water or Arial only (without enzymes). As well as, enzymes without detergent, gives a good result for stained removal from cloth spice. On the other hand, the K/S value and yellowness exerted lowest values at the same treatments where as (63.4 x 0.12); (60.5 x 0.11); (62.7 x 0.17) and (65.1 x 018) for lipase and Arial as well as (61.2 x 0.32q); (65.4 x 0.15); (67.7 x 0.10) and (25.7 x 1.4) for protease and Arial respectively.

In addition, it was observed, the lipase had high ability to remove the oil stain from cloth spiece at 50ºC compared with protease enzyme (Table 20).