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Identification the local thermophilic strains of *Bacillus licheniformis* by VITEK-2 system and *Gyrase B* gene.

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Abstract- One hundred and eight of soil samples have been collected from 17 area loci in Maysan governorate during the period from September to the October 2012 ,and cultivated on lauria agar media for isolation and identification of *B. licheniformis*. Primary identification was depended on colonial morphology, microscopic examination and biochemical tests then the isolates was identified by VITEK-2 compact system Version 05.01 using BCL identification cards (BCL-ID). The results revealed that 53(94.64%) of isolates identified as *Bacillus licheniformis* which could be classified in classes where the very good identification class with percent of identification reached to 43.39%, followed by good , acceptable, and low discrimination , which were 30.18% ,24.52% and 1.88% respectively and 3(5.35%) as unidentified organism. The isolates were identified using the PCR technique for detection of *gyrase B* gene clarify that 56(100%) isolates of *B. licheniformis* with *gyrase B* gene that encodes the subunit B protein of DNA gyrase.

Key word- *Bacillus licheniformis*, VITEK by BCL[**], *gyrase B* gene.

[**]**BCL-** Bacillus Card.

Introduction

B. licheniformis named by Weigmann 1898,is rod shaped, facultative anaerobic, gram positive, endospore-forming bacterium widely distributed as a saprophytic in the environment where they survive harsh conditions in the form of highly resistant endospores and belongs to the *B. subtilis* group [1,2,3]. Its ability to grow in thermal environment such as a hot spring it's facultative thermophilic bacteria or thermotolerant bacteria [4,5,6].Having generation time of 22 min. at 55℃ compared to 41 min. at 37℃[7].Produced substant effective as antifungl such as proteases, Mannase and Xylenase [8].*B. licheniformis* have ability the breakdown aflatoxins B1 about 74% [9]. Members of this genus has great fermentative capacity combined with low toxicity have been described as producers of biotechnologically because produce important compounds such as amylase , antibiotics, keratinase, penicillinase, pentosanase,lipase , surfactant and restriction enzymes [10]. It is widely exploited in industrial processes , in particular, the excellent protein secretion capacities of *B. licheniformis* have made it an attractive host for the large-scale production of commercially employed enzymes [11]. The VITEK 2 system is a new system that automatically carry out rapid identification on a manually prepared inoculums , this represents a major advance in the identification of aerobic endospore-formers[12].The system was equipped with an extended identification database for all routine identification tests that provide an improved efficiency in microbial diagnosis which reduces the need to perform any additional tests, so that safety for both test and user, will be improved. BCL is intended for and approved for Industry use only. Use in a clinical setting is not authorized [13, 14]. While the genomic characterization is a powerful tool for the classification of new isolated strains [15].A comparative analysis of the 16S ribosomal RNA sequence is the most commonly used genotypic method for bacterial identification. Strains that generally show 97% sequence similarity in the 16S rRNA are considered to be the same species [16]. Unfortunately, the high degrees of similarity (reaching 98.1-99.8%) have been observed for the 16S rRNA gene sequences among the *B. subtilis* group strains. *B. licheniformis* is closely related to the *B. subtitles'* group, and could not be clearly identified using phenotypic and genotypic (16S rDNA sequence analysis) techniques alone. Therefore, another genotypic technology with rapidity, accuracy and lower cost needs to be developed. Species

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specific primers are effective when used for PCR based identification involving a small number of species or a particular species. This method is based on specific primers that target highly variable regions of the universal genes. The *gyrB* gene has been used as the target for detection and identification of several species of bacteria based on the PCR assay [17]. The present study attempt aim to Isolate the Thermophilic *B. licheniformis* and Diagnosis the bacteria under study by VITEK-2 system and *Gyrase B* gene.

Materials and methods

Specimen Collection and the Processing

A total of 180 soil samples , *Bacillus licheniformis* were collected from seventeenth different loci (Al-ptera ,AlMushrah ,Saeed Minehil refinery ,Noor Field ,Almemona , Alsinaf, Alkahlaa,Almajbas,Alteeb, Alduereej, Alzeoot,Algasaiba, Alsheeb, Alchuka, Almujar, Outskirts of Amarah and The edge of the Tigris River) from Maysaan governate during the period from September 2012 to the October 2012.The bacteria isolated from soil according [18]: Two gram of soil sample have been add to 10ml distilled water and mixed well, after while transfer 0.25ml from soil suspension to 25 ml Lauria broth and incubated at 55℃ for 18 hours. Prepared series of dilutions ($10^{-1,}$ 10^{-2}, 10^{-3}), each one cultured on Lauria agar and incubated at 55℃ for 18 hours. One colony picked from fresh overnight agar plates then cultured on Lauria agar and incubated at 65℃ for confirm as thermophilic ,then picked one colony form plates that contain growth and cultured on Lauria agar and incubated at 55℃ for 18 hours for diagnosis.

Identification of the *B. licheniformis*

Microscopic, Cultural and Biochemical Tests- Gram's stain was used to examine the isolated bacteria for studying the microscopic properties. Morphological colonies characteristics (size, color, edge shape) were recorded on Lauria media. *B. licheniformis* utilize citrate and grow in anaerobic conditions also diagnosis according to [3].

Identification of *B. licheniformis* by Vitek-2 Compact System.

The BCL cards were stored at 4°C prior to use. The identified isolates were confirmed with the automated VITEK-2 compact system according to Manufacturer's instructions [14] as following: Bacterial suspensions were prepared in 3 ml of 0.45 % NaCl and adjusted to a McFarland standard of 1.80 – 2.20 using the VITEK2 DensiChek (bioMérieux) .The prepared suspension were placed at the positions in cassette, BCL cards were filled automatically in the VITEK vacuum chamber, then the ID-BCL card bar code labels were scanned and the cards were placed in the card positions next to the inoculum tubes. The software then analyzed the data and reported the results after 14hr. Data were analyzed automatically using the VITEK2 database version 05.01,there are 46 biochemical tests measuring carbon source utilization, enzymatic activities, inhibition, and resistance. Organism identifications are made by converting results of the biochemical tests (an16-digit bionumber) into positive or negative test probabilities for each isolate present in the data base of the Vitek Systems.

Molecular Identification

Total DNA of All isolates was extracted from colonies grown on agar plates according to Geneaid protocol of Presto™ Mini gDNA Bacteria Kit, Taiwan. Concentration of DNA was determined spectrophotometrically (NanoDrop) by measuring its optical density at 260 nm the purity of DNA solution is indicated by ratio of OD260/OD280 which is in the range of 1.8± 0.2 for pure DNA [19]. Gel electrophoresis was used for detection of DNA by UV transilluminator[20].The PCR assay was performed to detect the *gyrase B* gene for confirmation the identification of *B.licheniformis* as shown in [Table-1], were primers synthesized by BioNEER, Korea [21].

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Table 1- Sequence and Concentration of Forward and Reverse Primer

Primer type	Primer sequence	Concentration Pmol/Ul	Product size
F- *Gyrase B*	5′-AK*ACGGAAGTCACGGGAAC-3′	202.2	550 bp
R-*Gayrase B*	5′-AGAAACTTTTCR**AGCGCTT-3′	220.4	

*K means G or T according IUPAC codes.

**R means A or G according IUPAC codes.

The Chromosomal DNA extracted from all isolates were subjected to primer by monoplex PCR. The protocol used depending on Bioneer manufacturer's instructions. All PCR components were assembled in PCR tube and mixed by refrigerated microcentrifuge at 50 rcf (850 rpm) for 10 second.

Protocol:

1. Template DNA and primers were thawed before use.

2. Template DNA and primers were added into the AccuPower® *Taq* PCR Premix tubes as in [Table-2].

Table 2-The mixture of PCR for 20 µl/ reaction

Mixture solution	
De-ionized water	6µl
Master mix	5µl
Forward primer	2µl
Reverse primer	2µl
DNA Template	5µl
Final volume 20µl	

3.The lyophilized blue pellet was completely dissolved and spun down either by using Centrifuge 15 second, vortex or by pipette up and down several times.

4. The Eppendorf PCR tubes were placed in the thermocycler and the right PCR cycling program parameter condition were conducted as in [Table-3], according to [17] with a little modification.

Table 3- PCR program that apply in the thermocyler [17].

Gayrase B gene	**Temperature̊c/time**					**Cycle number**
	Initial denaturation	**denaturation**	**annealing**	**extension**	**Final extension**	
gyrB	94˚C/4minutes	94˚C/56 sec.	56˚C/55 sec.	72˚C/1minutes	72˚C/7minutes	35

5.Seven microliters of amplified PCR product were loaded to the agarose gel wells with DNA marker to one of the wells and running by electrophoresis on 1% agarose gel [20].

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Results and Discussion

Isolation and Identification of *B. licheniformis* bacteria By conventional Methods

A total 180 samples were collected from soils from seventeenth different loci .The results has been show 133 sample with growth at 55℃. One colony picked from fresh overnight agar plates then cultured on lauria agar at 65℃ for 18 hr. The results was only 56 sample grown at this degree, the percentage for distribution of thermophilic isolate in local areas as shown in [Fig-2].Whereas various isolation methods including positive isolates number are illustrated in [Fig-1] .

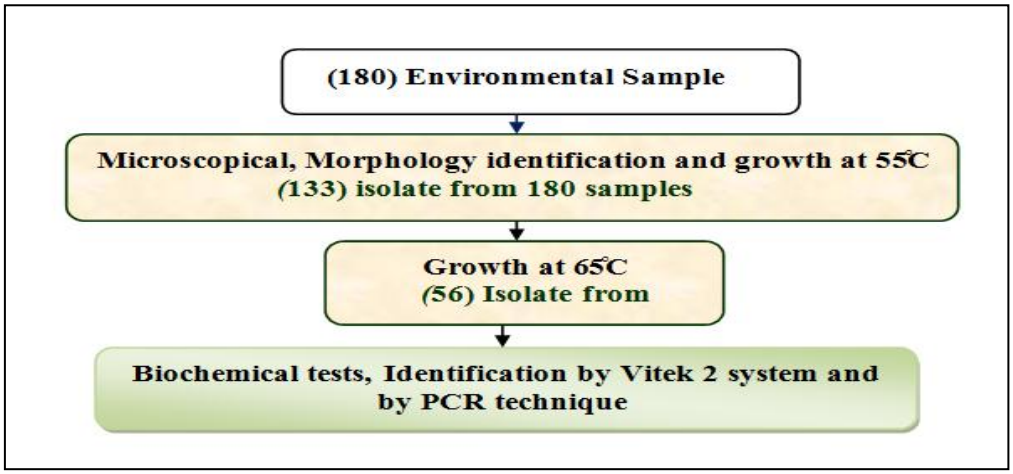

Fig. 1- Isolation and identification methods of *B. licheniformis* bacteria used in this Study .

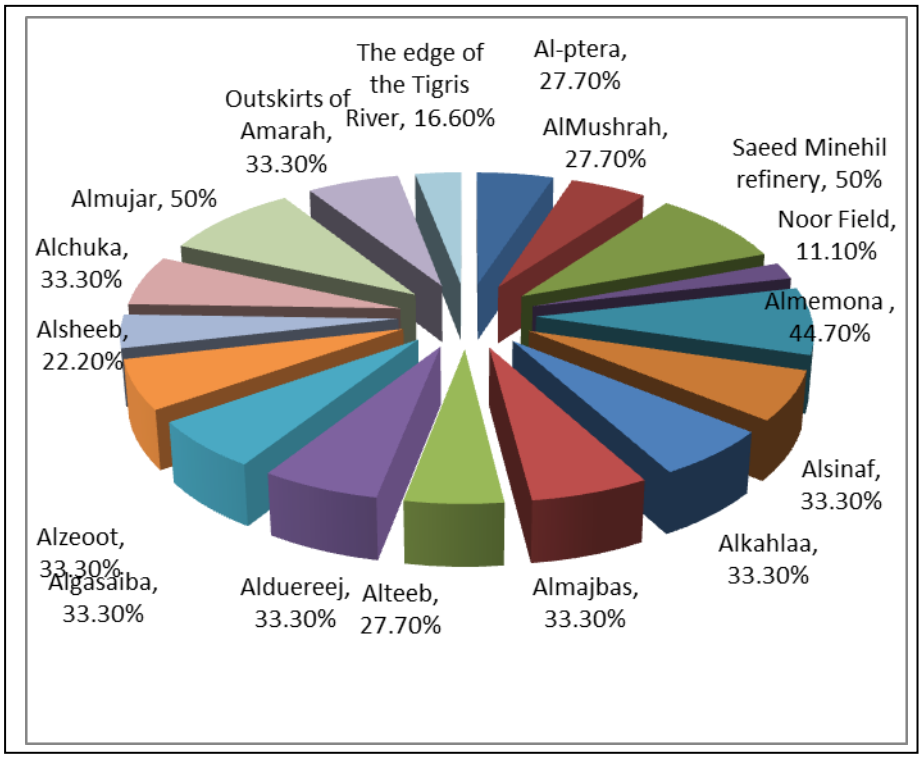

Fig. 2- The percentage of the presence of thermophilic *B. licheniformis* in the areas under study

Identification of *B. licheniformis* is depended on the colonial morphology, microscopically and biochemical tests as initial identification. The colonies of *B. licheniformis* are grown on culture media once revealed the typical characteristics being described by referential studies, that the colonies are

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irregular form, undulate margin, opaque, creamy color, glistening , mucoid and rhizoid. *B. licheniformis* β-hemolytic colonies colonies [3,22] . The results of biochemical tests are adopted in the[Table-4] and agree with [3] for diagnosis of *B. licheniformis*, where the results indicated that (56) isolates belong to thermophilic *B. licheniformis* which grow at 65℃; it's facultative thermophilic bacteria [23].In general, the genus *B. licheniformis* are facultative anaerobic, Gram positive , rod shaped and motile whose natural habitat is in the environment as a saprophytic [24].

Table 4-Biochemical tests of *B. licheniformis* isolates

Primary tests	Results	Primary tests	Results
Oxidase test	**+ ve**	Anaerobic conditions	**+ve**
Catalase test	**+ ve**	Urease	**+ ve**
Motility test	**+ ve**	Citrate test	**+ ve**
Indol test	**-ve**	MR test	**+ ve**
Hydrolysis of gelatin	**+ ve**	Hydrolysis of starch	**+ ve**
VP	**+ ve**	Growth with lysozyme	**-ve**
Growth at pH		**Growth in NaCl**	
5.7	**±**	2%	**+ ve**
6.8	**+ ve**	5%	**+ ve**
Growth at		7%	**+ ve**
10℃	**- ve**	10%	**+ ve**
30℃	**+ ve**		
40℃	**+ ve**		
55℃	**+ ve**		
65℃	**+ ve**		

+ ve: positive result , -ve :negative result , ± : variable result,VP : Vogues-Proskauer test ,MR: methyl red test

Identification of *B. licheniformis* by Vitek 2 System

Only 56 of isolates had been identified depending on cultural and morphological characteristics reinforced by conventional biochemical test and confirmed by Vitek 2 system Version 05.01 with the BCL cards Show the results revealed that 53(94.64%) of isolates identified as *Bacillus licheniformis* and 3(5.35%) as Unidentified organism. The [Table-5] presented the results of Vitek 2 system, which could be classified in classes where the very good identification class with percent of identification reached to 43.39%, followed by good , acceptable, and low discrimination , which were 30.18% ,24.52% and 1.88% respectively.

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Table 5-Distribution of Vitek 2 results in different classes of probability.

Classes of probability	No (%) of identification
Very good	23(43.39)
good	16(30.18)
acceptable	13(24.52)
Low discrimination	1(1.88)

Probability of very good identification= 93-95%

Probability of good identification= 89-92%, Probability of acceptable identification =85-88%, Probability of low discrimination with extra tests= 50%

The results for 46 test by Vitek-2 show all identified isolates positive for L-Pyrrolidonyl-Arylamidase, Ala-Phe-Pro Arylamidase, Cyclodextrine, Methyl-A-D-Glucopyranoside acidification, Maltotriose, D-Mannitol, D-Mannose, N-Acetyl-D-Glucosamine, Palatinose, Beta-Glucosidase, D-Trehalose, D-Glucose, Esculin hydrolyse, Polymyxin_B Resistance and NaCl 6.5%. This study agree with [25] in positive test for Cyclodextrine, Maltotriose, D-Mannitol, D-Mannose, N-Acetyl-D-Glucosamine, Palatinose, Beta-Glucosidase, D-Trehalose, D-Glucose, Esculin hydrolyse, Polymyxin_B Resistance and NaCl 6.5%.Also results show all isolates negative for L-Lysine-Arylamidase, L-Aspartate Arylamidase, L-Proline Arylamidase, Methyl-D-Xyloside, Alpha-Mannosidase, D-Melezitose, Beta-Mannosidase, Inulin, Putrescine assimilation and Kanamycin Resistance, this agree with [25].While the rest of tests variable in results between positive and negative, while the unidentified isolates only three.

The number of identified strain in this study according to the 16-digit bionumber of laboratory reports 36 strain for *B. licheniformis* and three share in bionumber (123237155544627) , four in (0332370555062221), six in (0130361555062261), two in (1132371555466271), two in (1132371555266221),three in (0332371755042221),four in (1333361555442271) and the other with different bionumber, there are a large number of different bionumbers, making it possible to resolve species into subgroups, the bionumber has limited epidemiological value because of the frequent occurrence of a few major numbers, with many different strains giving the same bionumber[26], while unidentified organism each one with different bionumber as shown in [Table-5].The results as unidentified may be Technical error because were re-tested and changed to identify with PCR technique.

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Table 5-Major Vitek-2 BCL card bionumbers of isolates

Species	Major Bionumber	No. of strains
B. licheniformis	123237155544627	3
	1332371755476261	1
	1332371155446271	1
	0330371555062221	1
	1130371555466271	1
	1336371555466271	1
	1372371755476261	1
	0330360555066221	1
	1236361555452261	1
	0333370555062221	1
	0332371555062231	1
	0332360555076231	1
	0332371555062261	1
	0330351555062261	1
	0332361555066271	1
	0130371555062271	1
	0332370555062221	4
	0130361555062261	6
	1132771555076261	1
	1132371555066261	1
	1132371555476261	1
	1332371755476271	1
	1136371555466271	1
	1172361555476261	1
	1132371555466271	2
	0322371555062221	1
	1132371555266221	2
	1330361555472271	1

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	0332371755042221	3
	0330361555062261	1
	0330770555066231	1
	1330361555072271	1
	1330371757466271	1
	1332371555476261	1
	1372371555466261	1
	1333361555442271	4
Unidentified organism	0020370551062221	1
Unidentified organism	0230360555506222	1
Unidentified organism	1230370555466231	1

Molecular Identification of *B. Licheniformis* by PCR Technique

Polymerase chain reaction technique has been used to amplify genes of *gyrase B* gene from genomic DNA of all *B. licheniformis* isolates and unidentified organism. DNA is extracted from all isolates and detect about the extracted DNA by Gel Electrophoresis. The results of isolates diagnosis using the PCR technique for *gyrase B* gene detection clarify that 56(100%) isolates of *B. licheniformis* with *gyrase B* gene that encodes the subunit B protein of DNA gyrase and this agree with [17], while the three unidentified organisms also show positive results for *gyrase B* gene as shown in [Fig-3]. Results of the present study demonstrate none-significant differences between the methods used (vitek-2) and PCR in the diagnosis for *B. licheniformis* isolates. Where this method of PCR was more sensitive compared to other methods because *B. licheniformis* close related to the *B. subtillis* group and could not be clearly identified using phenotypic and genotypic(16S rDNA sequence analysis) [27], depending on the diagnostic gene *gyrB* ,that selected specific-specific primer to this gene according to [17]. All isolates of *B. licheniformis* bacteria gave a positive result to detected for *gyrB* gene as shown in [Fig-3]. Compared to 16S rDNA, the sequence of subunit B (*gyrB*) has a relatively high mutation rate because of the protein-coding nature, i.e., synonymous substitutions mainly at the third codon position [28], and for this reason, species identifications have been resolved that otherwise would have been impossible by 16S rDNA sequencing[29]. Huang and Lee, 2009 successfully used the *gyrB* sequence and DNA hybridization to discriminate species of the *B. subtilis* group. However, the sequencing or DNA hybridization assay is complicated, time intensive, expensive and difficult to use routinely in research.

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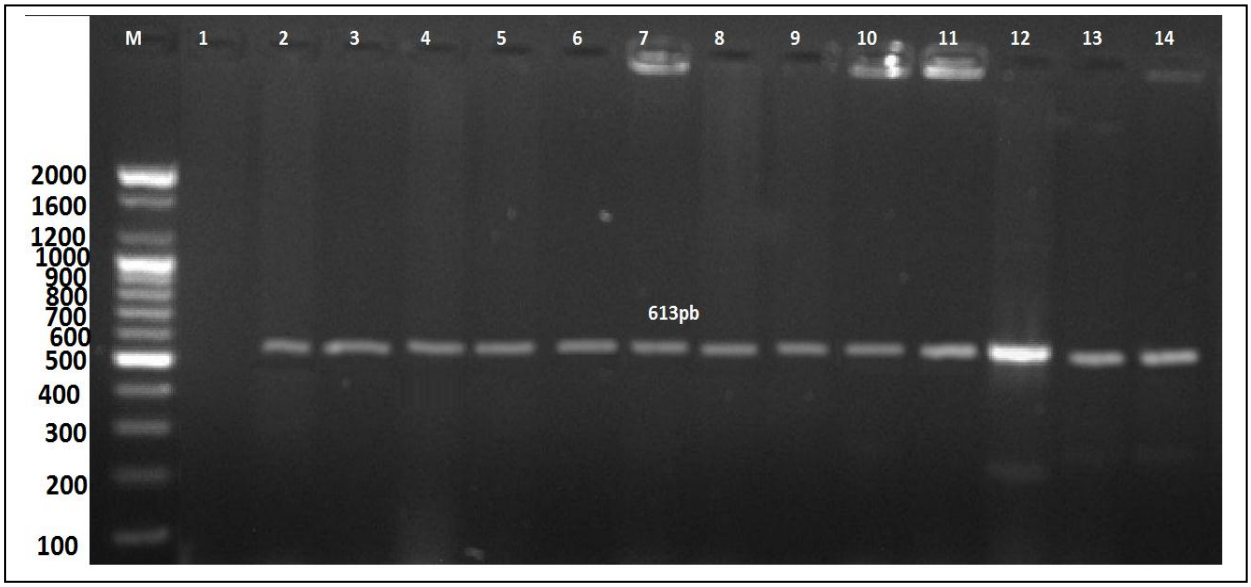

Fig. 3-Ethidium bromide-stained agarose Gel Electrophoresis (1%) of PCR Amplification products of *gyrB* Gene (613)bp of *B. licheniformis* Isolates for (120) min at (65) volt.,M.: DNA Molecular size marker(100-2000)bp Ladder from Bioneer(Korea),Lan1: negative for *gyrB*(control),Lan2-13:show positive result for *gyrB* gene.

Conclusions

The conclusions are extracted from the present study were *Bacillus licheniformis* widespread in nature of all samples of these bacteria in spite of the different areas, and Not all isolates heat-loving there are thermophilic or facultative thermophilic strain. And the last conclusion are efficiency of vitek-2 system for identification.

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