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Bacteriocin Production by Newly Isolated *Lactobacillus* Strain

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Abstract: *An Egyptian local Lactobacillus strain was isolated from Cabbage and was identified to be more closely related to thetypestrains LactobacilluspentosusD79211and LactobacillusarizonensisAJ965482via 16S rRNA gene sequencing and analysis. The isolated strain was found to be a gram positive, rods and react negatively with catalase test. A Central Composite Design [CCD] algorithm was applied to determine the optimum pH and incubation temperature required for the growth of L-CbM101. The fatty acids profile of the local isolate was explored using Gas Chromatography/ Mass Spectroscopy [GCMS] methodology revealing that the main fatty acids to be; oleicacid, palmitic acid, myristic acid and stearic acid. The isolated strain was found to be able to produce bacteriocin with antimicrobial activity against a vast number of pathogens and food spoiling microbes. The activity and stability of the produced bacteriocin was studied at different pH, storage time and temperature via applying a Response Surface Methodology and the optimum activity was achieved at pH 4, after 2 days of incubation and 4℃. These results indicate that; the produced bacteriocin could be used as antimicrobial compound added to fresh food taking the advantage from protect fresh food from spoilage and disappear of the antimicrobial compound prior to consumption which is undesirable.*
Keywords*:Bacteriocin,Central Composite Design, Lactobacillus spp, MRS agar, 16s rRNA,*

I. Introduction

The genus *Lactobacillus* currently consists of over 180 species and encompasses a wide variety of organisms.*Lactobacillus* are gram positive, acid tolerant, cocco-bacilli, non-motile and catalase negative and having a DNA base composition of less than 50 mol % G + C.*Lactobacillus* are generally non motile and can survive in both aerobic and anaerobic environments. They are widely distributed in animal feeds, silage, manure and milk product (cheese ,yogurt …,etc) and they have an important role in the manufacture of fermented vegetables (pickles and sauerkraut), beverages (wine and juices), sourdough breads and some sausages.*Lactobacillus* is commensal inhabitant of animal and human gastrointestinal tracts, as well as the human mouth and vagina. *Lactobacillus* are able to ferment various carbohydrates producing acetate and lactate [1]. Many *lactobacilli* operate using homo fermentative metabolism (they produce only lactic acid from sugars), while other species use hetero fermentative metabolism where they can produce either alcohol or lactic acid from sugars. Lactic AcidBacteria (LAB) were identified and generally established to be Generally Regard as Safe (GRS's) [2]. It could be safely used as probiotic in food industry and several medical applications. In addition to beingidentified as GRAS, they were reported to have significant importance for human health including; lowering cholesterol, reducing irritable bowel syndrome, dietary supplement, immune-modulatory and antitumor activity [3].

Lactic acid bacteria act as useful bacteria because they have the ability to molder proteins, carbohydrates and fats in food and help in absorption of important elements and nutrients such as amino acids, minerals, vitamins and are required for the survival of humans and other animals [4]. *Lactobacilli* has applicationsin food industry with a long history, where the functions of the bacteria in the industrial setting have been well studied[5].Instead of chemical food additives such as nitrite, sulfite, propionic acid, sorbic acid, and benzoic acid are commonly applied in food preservation technology [6], using the natural additives that displayed by LAB strains may help to combat microbial contamination [7][8]. LAB produce several natural antimicrobials; including organic acids (lactic acid, acetic acid, formic acid, phenyl acetic acid, caproic acid), carbon dioxide, diacetyl, ethanol, bacteriocins, reuterin, and reutericyclin. Acetic acid, for instance, contributes to the aroma and prevents mold spoilage in sourdough [9]. Bacteriocins from LAB are low-molecular-mass peptides, proteins with an antibacterial mode of action restricted to related gram-positive bacteria. Bacteriocin-producing LAB could be applied for food preservation because of their microbiological, physiological and technological advantages[10]**.** For instance, bacteriocin-producing LAB can be used as an alternative to potassium nitrate to prevent late loss of cheese due to contamination by *Clostridia*[11]**.** Several studies indicated that; LAB starter strains are able to produce their bacteriocins in food matrices and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacterial strains.

The aim of this work is to isolate, characterize and identify an Egyptian local*Lactobacillus* strain that isolated from the Cabbage pickles and determining, via using Central Composite Design, the optimum pH and

temperature to attain the maximum growth. In addition, one of the main concerns is to test the capability of the isolated strain to produce antimicrobial compound which could be used to protect fresh food from rapid spoilage process.

II. Materials And Methods

2.1 Samples collection and Microbial Isolation

A number of local fermented foods including; cucumber, cabbage pickles, dairy product and yogurt, were collected and used to isolate *Lactobacillus* species. These food samples were collected in sterile box from local commercial producers.

Half ml of each of the locally collected samples was added to 4.5ml of sterilized distilled water for the purpose of serial dilutions process. 100µl from each of the last dilutions were cultured on MRS agar (De Man Rogosa and Sharpe media). MRS is a selective media for *lactobacillus*spp growth and purchased from (Oxiod) that composed of g/l bactoproteose peptone 10.0gm,bacto beef extract 10gm, yeast extract 5gm, dextrose 20gm,sorbitan mono-oleate complex 1gm, ammonium citrate 2gm, sodium acetate 5gm, magnesium sulfate 0.1gm, manganese sulfate 0.05gm, potassium phosphate dibasic 2gm,bacto agar 15gm, distilled water 1000ml, final pH was adjusted at 6.4 +/- 0.2 [12]. The inoculated plates were incubated at 37°C for 24 hours.
The grown colonies were picked and separated where each single colony was streaked onto fresh agar to obtain pure cultures of each isolate and then preserved in glycerol stocks for at -80°C for using later.

2.2 Characterizationand identification of the local *Lactobacillus* strain

The identification of the isolated local strain was performed based on their morphology, cultural characteristics and 16S rRNA gene sequence analysis**.**Gram stainwas carried out using[13]; while Catalase test was performed using [14] method.

2.2.1 Molecular Identification of the local*Lactobacillus*strain by 16S rRNA

The kits used for sequence analysis was the MicroSeq® 500 16S rRNA Bacterial Identification Kits Protocol (PN 4346298), which provides all reagents necessary to sequence the 16S rRNA bacterial gene. The resulting DNA sequence is analyzed and compared to a library of 16S rRNA bacterial gene sequences using MicroSeq®ID analysis software.
The genomic DNA was used as a template for PCR amplification of the 16S rRNA gene using forward primer 5'- AGTTTGATCATGGTCAG-3' and reverse primer 5' GGTTACCTTGTTACGACT 3'.The PCR product of the isolate was purified and sequenced. The generated sequences were analyzed by Finch TV software and the phylogenetic tree was generated via Seaview software using the closest published type strains sequences.

2.2.2 Determination of the Optimum Cultivation Conditions

Central Composite Design (CCD) algorithm was applied to determine the optimum level of pH and temperature controlling the isolated*Lactobacillus* strain growth. The CCD matrix included five levels for each variable, six center points andstar points to estimate the curvature. The CCD provided an indication of the main effect of each factor in addition to the interactionamong them [15]. The values used to create the CCD matrix are shown in Table (1) in which the temperature degree represented by low degree (26°C) and high degree (48°C), while the minimum pH value was (pH 3.2) and maximum value is (pH 8).

Table 1: The variables and their levels used for the CCD experiment for pH and temperature optimization

	Levels				
Variables	**-2**	**-1**	**0**	**+1**	**+2**
pH	3.2	5.2	6	7.2	8
Temperature (°C)	26	30	37	45	48

In this study, the experimental plan consisted of 13 trials and the variables were studied at three different levels, low (-1, -2), medium (0) and high (+1, +2). Thirteen experimentswere prepared in 250mL Erlenmeyer flask, each containing100mL of MRS broth medium and incubated according to the generated CCD matrix.Flasks were incubated at different conditions as proposed by the design. The calculated response was cell weight (g/L) 3D response surface plots were generated to understand the interaction among the tested variables and reveals the optimum pH and temperature for the growth of the investigated microorganism.

2.2.3 Fatty acid methyl ester (FAME) preparation and Analysis

Twentymg of freeze dried cells were suspended in 2 ml of 5% methanolicHCl and heated at 70°Cin a water bath for 2 hours in sealed glass tubes. The tubes were cooled at room temperaturefor 30 minutes and to extractthe FAME 1ml of hexane was added and vigorously vortexes. The tubes were kept till twolayers were formed. The upper layer was transferred into a clean tube and dried in desiccator. A known volume of hexane was added in addition to a known volume of the internal standard [16].The extract was then methylated and the resulting Fatty Acid Methyl Esters(FAME) was analyzed using gas chromatography.

GC-MS analysis was performed on an Agilent 7890A GC in split mode, injector at280℃) linked to a Agilent 5975C MSD with the electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V,interface temperature 310°C, controlled by a HP Compaq computer using Chemstation software .The sample (1µl) in hexane was injected using HP7683B auto sampler with the split injection mode. After the main solvent peak had passed the GC temperature program and data acquisition commenced. Separation was performed on an Agilent fused silicacapillary column (30m x 0.25mm) coated with 0.25 µm dimethyl poly-siloxaneHP-5) phase. The GC was temperature programmed from 30-130°C at 5°C/ min thento 300°C at 20°C/min and held at final temperature for 5 minutes with helium as thecarrier gas (flow rate of 1ml/min, initial pressure of 50kPa, split at 10ml/min).

2.3 Activity and Stability of the produced Bacteriocin

2.3.1 Determination of Bacteriocin Activity:

The local isolate *Lactobacillus* strain was grown in 500ml MRS broth at 37°C for 48 hours. The culture was centrifuged at 5000rpm for 15 minutes at 4°C. The resulting cell free supernatant fluids were adjusted to pH 7.0 with 1N NaOH[17]. The crude supernatant was further purified by treating with 40% ammonium sulfate for 6 hours at 4ºC with gentle stirring, bacteriocin was then precipitated by centrifugation at 5000 rpm for 20 minutes at 5°C [18], the surface pellet (viscous and dark color not purified) were collected and dialysis (purification) was performed in a tubular cellulose membrane.The extracted bacteriocin was poured in cellulose membrane tube against distilled water for 24 hours at 4°C (in ice) so as to remove impurities that might be present in the extracted bacteriocin.

The bacteriocin activity was detected by using Agar well diffusion method against a number of standard indicator pathogenic organisms including *Erwiniacarotovora, Staphylococcus epidermis, MRSA (Methicillin-resistant Staphylococcus aureus), Candida albicans, E.coli* and *Bacillus subtilis*. The tested indicator strains were swapped on LB agar medium then wells were made in agar (5mm diameter) by sterile cork borer. 100µl of filter sterilized extracted bacteriocinwas added in the generated wells and the plates were incubated at 37°C for 24h. The inhibition zones were noted (mm) and recorded.

2.3.2 Bacteriocin Stability

The stability of bacteriocin was studied under different storage conditions including (pH, temperature and storage time) by using Central Composite Design (CCD). The tested values used to create the CCD matrix are shown in Table (2).Bacteriocin pH was adjusted using 1M HCl and 1M NaOH.

Table 2: Variables and their tested levels upon Bacteriocin stability

Variable	Levels				
	-2	**-1**	**0**	**+1**	**+2**
pH	2	4	6	8	10
Temp (°C)	-10	4	30	56	80
Incubation Time (Days)	1	2	3	4	5

The CCD provided an indication of the main effect of each factor in addition to the interactionamong them. The values used to create the CCD matrix are shown in Table (2), in which the temperature degree represented by low degree (-10°C) and high degree (80°C). Also, pH low value was (2) and high value was (10) and incubation time in range of (1-5days).

In this study, the experimental plan consisted of (20) trials and the variables were studied at three different levels, low (-1, -2), medium (0) and high (+1, +2). Subsequently incubated according to design then assayed for activity using treated bacteriocin against the proposed indicator organisms [19, 20].

III. Results

3.1 Morphological and molecular identification of the local isolate.

3.1.1 Morphological and microscopic characteristics

Several different morphological types of bacterial colonies were isolated. All the obtained isolates were screenedby gram stain and the result was observed by light microscope. Among all the tested colonies, one type of colony from Cabbage pickles after serial dilutions then culturing and purification on MRS media, was found to be more related to *Lactobacillus* and was named CbM101, in which observed to be creamy white, round, raised, opaque, smooth and their size of colony (diameter in range) 1-2mm. They are gram positive bacteria, rod shaped. In performing catalase test, no bubblewas observed indicating that the isolated bacteriumis catalase negative.

3.1.2 Molecular Identification of local isolate strain by 16S rRNA

The 16S rRNA PCR product was sequenced and the obtained sequence was exposed to Basic Local AlignmentSearch Tool (BLAST) and Seq Match analysis. The 16S rRNA genesequence was used to build a phylogenetic tree, in comparison with the closest type strains, using Seaview software.

The 16S rRNA sequence was submitted to the GenBank and assigned an accession number (**KU170607)** and was used to build a phylogenetic tree using Seaview software, by performing automated BLAST searches, to determine the closest type strains to the isolate under investigation that illustrated in Fig (1).

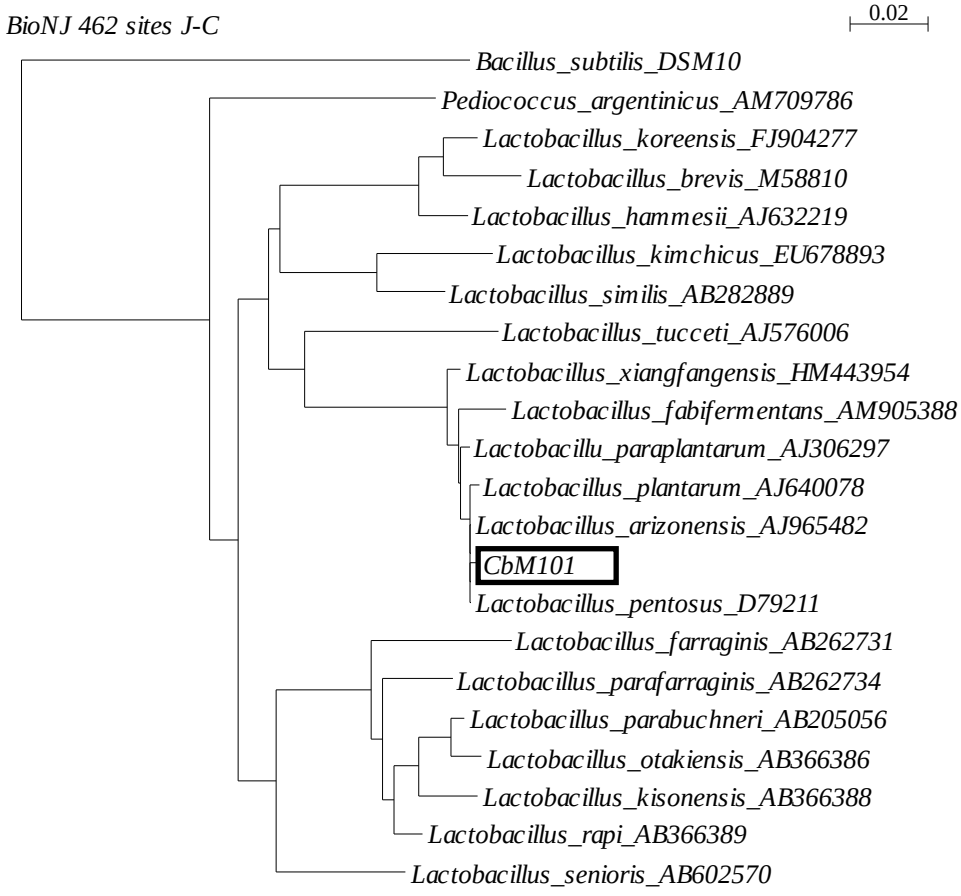

Fig 1: Phylogenetic tree showing the relative positions of strain *CbM101* and related *Lactobacillus* species by using the neighbor-joining method of complete 16S rRNA gene sequences. *Bacillus subtilis* is used as an out group as external reference.

The sequence analysis showed that the locally isolated (*CbM101*) was found to be closely related to the *Lactobacillus* type strain *Lactobacillus*pentosusD79211 and *(Lactobacillus*arizonensisAJ965482 (99.8%) with 1 gap difference in both of them for all sequence. DNA sequencing was proved to be a standard, faster and easiest molecular method used for microbial characterization than the biochemical-based conventional methods.

3.1.3 Determination of the best cultivation conditions

Temperature and pH are expected to be an important factors that promoting the bacterial growth andtheir metabolites production. To detect the best temperature and pH that controllingthe growth of the local isolate, A Central Composite Design matrix was performed as previously mentioned atsection (2.2.2), biomass as cell weight was used as target parameter to be enhanced. The generated matrix and responses of the design were summarized in Table (3);

Table 3: Matrix and response for the CCD experiment to determine the optimum cultivation conditions including pH and Temperature in coded units

Run	Variables		Response
	pH	Temperature	Cell weight (g/L)
1	-1	+1	23.2
2	-2	0	0
3	0	-2	9
4	0	0	9
5	0	+2	0
6	-1	-1	9.1
7	+1	-1	24.1
8	0	0	24.6
9	0	0	24.6
10	+1	+1	10.6
11	+2	0	11.6
12	0	0	24.6
13	0	0	24.6

Table (3) clearly showed that the highest amount of biomass was obtained when applying the central values for each of the tested variables (Temperature 37°C and pH 6). Some trials completely reduced the microbial growth especially trials number 5 and 2 with extremely high temperature (trial number 5) and extremely low pH (trial number 2).

Analysis of variance of the linear ,quadratic effects and the interaction among factors are given in Table (4). At 90 % level of confidence, any factors with P-value less than 0.1 could be considered as statistically significant. The smallest P-value, the highest significance of the corresponding variable and a variable is with negative effect sign (-), it indicates that the variable is effective on the response at the low concentration or less and the significant variable with positive effect sign (+), it indicates that it is effective at the high concentration or more.

Table (4): Analysis of variance by Multi way ANOVA of the CCD experiment for cell weight (g/L) versus pH and temperature for the local isolate (*CbM101*) growth,analysis of variance of the linear and quadratic effects and the interaction among factors with P-value<0.1 were considered as significant.

Term	Coefficient	SE Coefficient	T-value	P-value
Constant	0.14440	0.02517	5.737	**0.001**
A: (pH)	0.09669	0.01990	4.859	**0.002**
B: (Temperature)	-0.08645	0.01990	-4.344	**0.003**
A*A	-0.02832	0.02134	-1.327	0.226
B*B	-0.01517	0.02134	0.711	0.500
A*B	-0.04950	0.02814	-1.759	0.122

The ANOVA analysis of the CCD experiment for cell weight shows that both linear effect of pH (0.002) and temperature (0.003) were significant for growth of the local isolate (*CbM101*) due to P-values were found to be less than 0.1. Both thequadratic effects and interaction between the tested variables were found to be statisticallyinsignificant, although the interaction between the two tested variables was found to be very close to being considered as significant indicating that this item should not be ignored for any future interpretation and optimization.The interaction among the tested variables could be summarized using 3Dsurface plots and contour plot as show in Fig (2) to determine the optimum range graphically.

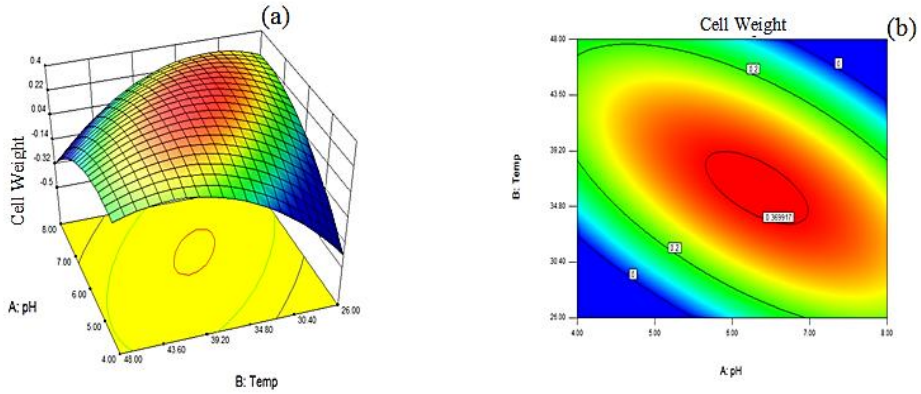

Fig (2): 3D Surface plot (a) and counter plot (b) showing the interaction between pH and temperature on a bacterial growth, where the optimum levels were shown as red color.

The three dimension (3D) surface plot and color contour plots showed the optimal levels of the variables and the relationship between factors. Theoptimum combination of factors for maximum growth was estimated as pH6and temperature 37℃which found in the red area that consider the optimum pH and temperature.

3.1.4 Fatty Acid Methyl Ester Profile of the Local Isolate

Gas Chromatographic for fatty acids methyl ester resulted in different peaks at different retention time, each peak represents the probability of different compounds, and this is clear in Fig (3).

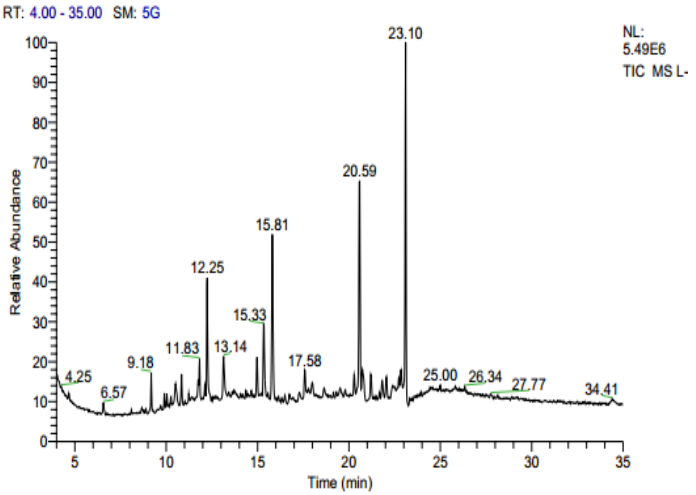

Fig 3: Gas Chromatographic (GC) separations for the local isolate(*CbM101*) showing fatty acids profile.

As shown in Figure (3) there were 11 peaks, four of them were found to be the major fatty acids contents of the isolate *Lactobacillus (CbM101)*.The highest probability of mass spectrum of these major fatty acids is summarized in Table (5).

Table 5: Identification of the major Fatty acids in the local isolate CbM101 profile

Name	%of area under peak	Chemical formula	Retention time
tetra-decanoic acid	6.39	$C_{14}H_{28}O_2$	15.35
9-Hexadecenoic acid	20.87	$C_{16}H_{32}O_2$	20.30
cis-9-octadecenoic acid	2.31	$C_{18}H_{34}O_2$	23.10
9-Octadecenoic acid	14.45	$C_{18}H_{34}O_2$	20.59

3.2 Activity and stability of the produced bacteriocin.

3.2.1 Determination of Bacteriocin Activity:

The Bacteriocin produced by the local isolate was tested against a number of microbes including (*Erwiniacarotovora, Staphylococcus epidermis, MRSA, Candida albicans, E- coli* and *Bacillus subtilis*)via measuring the generated clear zone (mm) as an indication of the inhibition of the microbial growth. The produced bacteriocin was found to be active against *Candida albicans* (15mm) and the least activity against *E-coli* was (3mm) as shown in Table (6).

Table 6: Antimicrobial activity of bacteriocin from *L-CbM101* against pathogenic microbes through clear zone (mm).

Indicator organisms	***Candida albicans***	***Staphylococcus epidermidis***	***Bacillus subtilis***	***MRSA***	***E-coli***	***Erwinia carotovora***
Clear zone (mm)	15	13	10	10	3	7

3.2.2 Bacteriocin Stability

The effect of pH, temperature and storage time on bacteriocin activity, produced by the *LactobacillusCbM101* , was performed via Statistical optimization which subsequently exposed to statistical analysis of the responses (clear zone as a representative of the antimicrobial activity) in Minitab 16 environment to determine the best conditions affecting thebacteriocin activity as shown in Table (7).

Table 7: Central composite design matrix and responses for bacteriocin stability, testing the stability of the produced bacteriocin under different storage conditionsthrough clear zone (mm) assay

Run	**Variables**			**Responses clear zones (mm)**		
	pH	**Temperature**	**Storage time**	***MRSA***	***Candida albicans***	***Streptococcus pyogenes***
1	0	0	+2	0	0	0
2	-1	+1	-1	4	5	3
3	0	0	0	0	0	0
4	-1	-1	-1	10	15	7
5	-2	0	0	7	10	5
6	0	0	0	0	0	0
7	+1	+1	+1	0	0	0
8	0	0	0	0	0	0
9	0	+1	0	0	0	0
10	0	0	0	0	0	0
11	-1	+1	+1	2	4	1
12	0	0	0	0	0	0
13	0	0	+1	0	0	0
14	+2	0	0	0	0	0
15	-1	+2	-2	9	12	6
16	+1	-1	+1	0	0	0
17	+1	-1	-1	0	0	0
18	+1	+1	-1	0	0	0
19	0	-2	0	0	0	0
20	0	0	0	0	0	0

Table (7) clearly showed that the highest clear zone was obtained against indicator pathogenic organisms were (temperature 4°C and pH 4 and storage time 2 days) and also at 80℃ at pH 4, the highest clear zone against *Candida albicans* (12mm). The results of ANOVA of the main effects of each factor, the interactions among them and the quadratic effects againstthree indicator organisms were illustrated in Table(8).

Table 8 :Analysis of variance by Multi Way ANOVA for CCD to determine the most significant factors affected on bacteriocin stability against three pathogenic microbes MRSA ,*streptococcus pyogenes* and *Candida albicans* at 90% level of confidence , p value<0.1 the factors more significant.

Terms	MRSA				*Streptococcus pyogenes*				*Candida albicans*			
	Coefficient	SE Coefficient	T-value	P-value	Coefficient	SE Coefficient	T-value	P-value	Coefficient	SE Coefficient	T-value	P-value
Constant	-0.06	0.6362	-0.096	0.926	-0.04	0.4288	-0.093	0.927	-0.08	0.9090	-0.097	0.925
(pH)A	-2.69	0.4221	-6.379	**0.000**	-1.86	0.2845	-6.539	**0.000**	-3.86	0.6031	-6.413	**0.00**
(Temperature)B	-0.95	0.4221	-2.255	**0.048**	-0.65	0.2845	-2.316	**0.043**	-1.31	0.6031	-2.185	**0.054**
(Storage time)C	-0.21	0.4221	-0.520	0.614	-0.21	0.2845	-0.772	0.458	-0.29	0.6031	-0.486	0.638
AA	1.63	0.4109	3.981	**0.003**	1.14	0.2770	4.137	**0.002**	2.34	0.5871	3.993	**0.003**
BB	0.39	0.4109	0.969	0.355	0.26	0.2770	0.946	0.367	0.57	0.5871	0.982	0.349
CC	0.39	0.4109	0.969	0.355	0.26	0.2770	0.946	0.367	0.57	0.5871	0.982	0.349
AB	1.62	0.5515	2.947	**0.015**	1.12	0.3717	3.026	**0.013**	2.25	0.7880	2.855	**0.017**
AC	0.37	0.5515	0.680	0.512	0.37	0.3717	1.009	0.337	0.50	0.7880	0.635	0.540
BC	-0.12	0.5515	-0.227	0.825	-0.12	0.3717	-0.336	0.744	0.25	0.7880	0.317	0.758

A: means (pH) B: means (Temperature)
C:means (incubation period), A*A:means (quadratic effect of pH),
B*B:means (quadratic effect of temperature) (A*B)(A*C)(B*A):means interaction between them.

At the 90 % level of confidence, any factor with a P-value less than 0.1 could be considered as statistically significant. The model T-values for each of the response variables and the model P-values versus three pathogenic indicator microorganisms *MRSA (Methicillin-resistant Staphylococcus aureus), Candida albicans* and *Streptococcus pyogenes*were calculated and used to determine the significance effect of each variables. The results showed that the main effect of both pH and temperature were significant importance for bacteriocin stability against all the tested microbes. In addition, the interaction between pH and temperature and the quadratic effect of pH was reported to be significant on the stability of bacteriocin.
According to the generated mathematical model, the best conditions for bacteriocin activity were reported to be pH 4, temperature 4℃ and Storage time of 2 days.

IV. Discussion

The Egyptian local strain *(CbM101)* was isolated from Cabbage and identified as *Lactobacillus*species from microscopic, biochemical and cultural characteristics, where it was reported to be a gram positive, rods under microscope with creamy white colonies upon growing on specific MRS medium. Cells were reported to be catalase negative with incapability to decompose hydrogen peroxide. This agrees with [21], [22]who reported that *Lactobacillus acidophilus* is a gram positive, rod shaped coccobacilli, occurring singly or in chains and catalase negative. The isolated local strain was subsequently identifiedat molecular level via 16S rRNA gene sequencing and analysis. The 16S rRNA genesequencing was used as a tool to study bacterial phylogeny and taxonomy asbeing the most common housekeeping genetic marker used for a number ofreasons: it present in almost all bacteria as a multigene family, or operons; it isfunction over time proceeded at a constant rate; and its size is around 1500bp which has a length enough for informatics analysis [23]. One of the most crucial uses of 16S rRNA gene sequence informatics is theidentification of isolates by providing the genus and species, for strains it generated only a "low chance "or "acceptable" identification according to commercial systems, or for taxa that are diseases rarely related to human infection .

In this study the bacterial strain isolated from the local Egyptian cabbage was identified by the 16S rRNA gene sequence and the obtained sequence was submitted to theGenBank with the accession number of **(KU170607)**. The 16S rRNAgene sequence analysis revealed that; the local*Lactobacillus* strain*CbM101* had the highest similarities to the *lactobacillus* type strains*Lactobacilluspentosus*D79211 and *Lactobacillusarizonensis*AJ965482 (99.8%) as showed in the phylogenetic tree, Fig (1), with one gap difference with both of them for the full 16S sequence. RNA sequencing has converted to be a standard, faster and easiest molecular method, used for microbial characterization than the biochemical-based traditional methods.

To determine the optimum cultivation conditions for the isolated strain, the optimum initial pH and incubation temperature was determined via applying a Central Composite Design (CCD) algorithm. Upon analyzing the obtained results, the optimum initial media pH was found to be 6; while the optimum incubation temperature was reported to be37℃. By performing the ANOVA analysis, Table (4), it was found that both pH and temperature are significant parameters that affected the growth of *lactobacillus* strain (*CbM101*). The 3D surface plot and colored counter plot showed the optimum pH and temperature in red area, as displaying in Fig (2), to be 37°C and pH= 6. This may be due to the enzymatic reactions are controlled by pH and temperature.Thus, the decrease of pH results in decrease of enzymatic reactions and concomitantly in slower growth rate. Also the lower growth rate at lower pH due to production of lactic acid [24]. This agrees with [25]who reported that, the maximum growth by *lactobacillus acidophilus* was achieved at 37°C incubation temperature and pH 5.8. Another observation were recorded with earlier research by [26] who stated that *lactobacillus curvatus L442* and*Leuconostocmesenteroides L124* showed that the optimum pH for growth occurred between pH 6 and 6.5.At a relatively Lower pH (pH4) microbial growth was dramatically decreased and thegrowth of the cell stopped. The results obtained in this study agrees with[27] who reported that the

optimum growth cultivation conditions of the isolate *Lactobacillus plantarum* were observed at 37.2°C and pH 5.6. The optimum incubation temperature of the *Lactobacillus* strain *CbM101* weredisagreed with the optimum temperature of the isolate *Lactobacillus delbrueckiisspbulcaricus*[28], which showed the maximum biomass and specific growth rate and acid production at 44°C. As a conclusion, the results obtained from this study showed that the efficiency of growth of the microbial cell was significantly affectedby the manipulation of incubation temperature and initial pH.

Fatty acids are one of the mainvaluablecriteria that applied in the field of microbial taxonomy. The bacterial membrane of *Lactobacilli* are typically composed of straight chain saturated and unsaturated fatty acids [29]. Fatty acids composition of the strain examined in this study via Gas Chromatographic (GC) as presented in Fig(3).

The pioneering investigations [30] on the lipids of *Lactobacillus* spp constitutethe first systematic chemical investigation of a*Lactobacillus*genera. The crude lipids derived from*L-CbM101* were found to contain the neutral fat afforded a mixture of saturated fatty acids as myristic acid (Tetra-decanoic acid $C_{14}H_{28}O_2$), palmitic acid (Hexadecanoicacid $C_{16}H_{32}O_2$), and stearic acids (Octadecanoic acid $C_{18}H_{36}O_2$) in addition to minor unsaturated fatty acid including; oleic acid ((omeg-9) cis-9-Octadecenoic acid) with molecular formula $C_{18}H_{34}O_2$ due to the *lactobacillus CbM101* was cultured on the MRS media which contain Tween 80 that contained about 10 mg of oleic acid, which is potentially transported into the bacterial cells. The most dominant fatty acid in the local isolate GC-MS profile was Hexa-decenoic acid with molecular formula of $C_{16}H_{32}O_2$ (palmitic acid) that have an important role in our life such as; treatment of schizophrenia, suppression of the body's natural appetites and suppression signal of insulin. Oleic acid, with molecular formula $C_{18}H_{34}O_2$, is monounsaturated omega-9 fatty acid with many health benefits. Oleic acid reduces blood pressure, increases fat burning to help with weight loss, protects cells from free radical damage, may prevent type 2 diabetes, prevents ulcerative colitis and generates brain myelin so, it mean that it extracted from natural source. The results of this workagrees with [31] who reported that*Lactobacillus buchneri* R1102 and *lactobacillus fermentum*probably had tetra-cosanoic acid, while the unsaturated fraction contained both C16 and C18 acids. Another observation, the hydrophobic fraction of the rhamnolipid produced by *Pseudomona putida*, which is mainly composed of palmitic, stearic, oleic and myristic fatty acids with similar carbon chain length that those also found in the *Lactobacillus. pentosus* was similar to thefatty acids chain of the local isolate *L- CbM101*[32].
Lactic acid bacteria synthesize antimicrobial agents that vary in their spectra of activity. One of these agents was bacteriocin with a proteinaceous active moiety, while others are non-protein agents[33, 34]. *Lactobacillus* bacteria inhibits cell invasion by entro-virulent bacteria due to protenious substances [35]. The anti-bacterial activity of bacteriocins against food borne pathogenic as well as spoilagebacteria has raised considerable interest for their application in food preservation. Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eatand lightly preserved. In this study, *lactobacillus (CbM101)* was found to be able to produce a type of bacteriocin which potentially could act as an antimicrobial agent against six different indicator pathogenic organisms, showing the highest activity against *Candida Albicans*while theleast activity was observed against *E-coli.*This agrees with [36], [37], [38], [39] who reported that the inhibitory activities showed on bacteriocins against selected microorganisms.[40] reported that *L. plantarum*and *L. sake* strains, isolated from meat and meat products, had inhibitory effects against several pathogenic and spoilage bacteria. In addition, its similar to[41] who indicated that *L. paracasei* and *L. acidophilus* strains, isolated from infant feces, had weak antibacterial activity on *Escherichia- coli*. Different conditions such as change in pH, storage time and temperature were expected to affect up on the activity of the produced bacteriocin. In this work, these factors were tested on the activity of bacteriocin produced by the *L.CbM101*, where a Central Composite Design (CCD) was introduced to determine the optimum pH, temperature and storage time, in addition to explore the potential interaction among these variables upon the bacteriocin activity. The obtained results showed that; the best conditions to attain bacteriocin activity were 4℃, pH4 and storage time of 2 days.The results of ANOVA analysis of the main effects of each factor, interactions among them and the quadratic effects was noted Table (8) versus three pathogenic indicator organism *MRSA (Methicillin-resistant Staphylococcus aureus) ,Candida albicans and Streptococcus pyogenes* showed that, bacteriocins of *LactobacillusCbM101*was reported to work actively at pH range of (2-5) and reduced upon increasing the pHup to 10. This could be due to the fact that most of the studied bacteriocins are more resistance to acidic pH more than alkaline pH which is related to the protein status of bacteriocins.This agrees with [18]who reported that, *Lactobacillus fermentum* showed a maximum activity at an initial pH of 2 and 4 against *Staphylococcus aureus.*[42] illustrated that, bacteriocinof *Lactobacillus casei*showed a maximum activity at an initial pH of 2, 4 and 6 against *Klebsiella pneumoniae*. Also agrees with other study by[43, 44] who studied two bacteriocins, namely bulgarican and lactobulgarican, isolated from *L. bulgaricus* were shown to have the highest activity and stability at pH 2.2 and 4.0 respectively against a range of pathogenic and spoilage bacteria. However, the optimum pH of bacteriocins lower than 5.0 this similar finding in the report by [45].

On the other side, the obtained result showed that; the relatively low temperature ranging from 4℃ to -20℃ are the best preservation temperature for storing bacteriocins when comparing with the control due to inactivation of enzymes such as protease enzymes. The bacteriocins which are more stable during prolonged storage in cold temperaturemake them excellent and have an inhibitory effect on the pathogenic organisms.

Another study for testing the heat stability of bacteriocins was produced by*lactobacillus* strains,[46][47] who reported that, heating stability is the best characteristic in the using bacteriocin as a food storage, because many food processing procedures involve heating step [48]. In this study, the bacteriocin that produced from the local isolate *(CbM101)* withstand at 80℃ and pH= 4 against *Candida albicans*.The optimum growth conditions of the *Lactobacillus*spp at 37℃ for 24hrsbut the optimum activity of bacteriocin after 48hrs during the growth of *Lactobacillus* strain[49]. So that the temperature of the growth play an important role in bacteriocin production, it means that the bacteriocin more stable and can be bear high temperature. The results of the present work show that, bacteriocin produced from *L-CbM101* retained its activity at 80°C and pH4,this agrees with finding in [50]*L. plantarum J-51* bacteriocin remained stable at storage temperatures (4, –20°C) and room temperature, and under strong heating conditions (100°C for 60 min). Also [19] reported that the activity of enterocin BFE 900 retained its activity after heating at 100 °C and 121°C. This study refer to possession of bacteriocin in *Lactobacilli* strains indicates their probiotics potentials. In conclusion, bacteriocin production was strongly dependent on pH, nutrients source and temperature as claimed by [51]. Various physicochemical factors seemed to affect bacteriocin production as well as its activity. From the results proved that it could be used in acidic foods like pickle or yogurt.

The Bacteriocin produced by the local isolate was found to be unable to tolerate both high temperature and long term incubation which could be advantageous from the marketing point of view. The unstable bacteriocin could be desirable as it degrade at high temperature which confirms the total elimination of that compound from the food prior to consumption by the customers. The fast (short incubation time) rate of degradation limits the usage of the produced bacteriocin in the preservation of fresh food only (maximum of 5 days).

V. Conclusions

The local isolate named CbM101, which identified as *lactobacillus* depending on16s- rRNA gene sequencing and analysis, via using Central Composite Design [CCD] algorithm show the optimum pH and incubation temperature were 6 at 37℃ . The fatty acids profile of the local isolate was explored using Gas Chromatography/ Mass Spectroscopy [GCMS] methodology revealing that the main fatty acids to be; oleicacid, palmitic acid, myristic acid and stearic acid. The isolated strain has ability for bacteriocin production with antimicrobial activity against wide range of food spoiling microbes. The activity and stability of the produced bacteriocin was studied at different pH, storage time and temperature by using a Response Surface Methodology and the optimum activity was achieved at pH 4, 2 days of incubation and 4℃. This result noted that; the produced bacteriocin could be used as antimicrobial compounds added to fresh food taking the advantage from protect fresh food from spoilage and disappear of the antimicrobial compound prior to consumption which is undesirable.

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