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IMPACT OF ENDOPHYTIC LIPOPEPTIDES USE AGAINST SOME PLANT PATHOGENIC MICROORGANISMS

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ABSTRACT: ***Twelve dissimilar plant associated bacterial colonies were isolated from five different tomato seedlings, the Non Ribosomal Lipopeptides genes were detected by degenerated primers technique in the twelve tomato endophytic isolates, only the long rode bacilli endophytic strains were found to harbour the lipopeptides synthetases genes, these three strains were given laboratory names of (BMG100, BMG101 and BMG102) for further pursuit. Partial sequencing of 16s RNA gene and protein profile analysis for the three bacilli isolates showed that all isolates belonged to Bacillus subtilis. The lipopeptides production by HPLC analysis revealed that all the endophytic isolates from tomato stems had the ability to produce lipopeptides except the third isolate named B. subtilis BMG102 showed no production. different levels of volumetric oxygen transfer coefficient (K_La) were used as cursors for lipopeptides production. The maximum concentration of surfactin type was about 1431, 529 and 437 $mg.L^{-1}$, the plipastitin (fengycin type) was 360 198 $mg.L^{-1}$ and zero while the Mycosubtilin (iturin type) was zero, 228 and 414 $mg.L^{-1}$ for the strains B. subtilis ATCC21332, BMG100 and BMG101, respectively. Surfactin production is visibly optimal at high oxygen transfer (k_La) and showed more than 0.07 S^{-1}, while the optimum production of the fengycin and iturin could be observed at mild values of O_2 transfer (k_La) which equal 0.01 and 0.015 S^{-1} respectively. The antibacterial - antifungal effects of the three B.subtilis extracts collected during lipopeptides productivity regimes showed inhibition activity against Pseudomonas syringae, Ralstonia solanacearum and Erwinia amylovora, as will as the antifungal activity against Aspergillus niger, Phanerochaete chrysosporium and Alternaria alternata. These results indicate potential use of B.subtilis strains as biocontrol agents, especially when considering commercial implementation. Studies regarding the lipopeptides applications, should also be considered as this will influence the efficacy of the lipopeptides against the target organisms.***

Key words: ***Endophytic; Lipopeptides; Plant pathogens; Biocontrol agents.***

INTRODUCTION

In Egypt, tomato (*Solanum lycopersicum*) are considered one of the most important vegetable crops and infected by numbers of different diseases. Studying the use of biological alternatives instead of chemical substances against plant diseases became necessary (Ongena *et al*., 2004). Beneficial microorganisms' endophytes can enhance plant resistance system to a wide range of pathogens, as bacteria, fungi, and viruses (Ongena and Jacques., 2008). Endophytes are considered groups of beneficial microorganisms which reside inside tissues of healthy and normal plant without any infectious

symptoms (Robert *et al*., 2007). These endophytes can promote plant growth and plant disease management by producing metabolite substances. These substances are considered novel natural products for agricultural, medicinal, and industrial applications (Sturz and Kinpinski., 2004). Several studies have reported that some endophytes has the ability to excrete antimicrobial agents; surfactin consider one of these compounds, which is cyclic lipoheptapeptide biosurfactant produced by *Bacillus subtilis* various strains. It has shown inhibitory activities against some phytopathogenic bacteria, fungi, viruses, and mycoplasma (Nissen-Meyer and Nes., 1997). Endophytes have several benefits to plants (Sturz *et al*., 2000) such as: promoting growth (Kang *et al*., 2007) reducing disease severity (Coombs *et al*., 2004; Kloepper *et al*., 2004; Senthilkumar *et al*., 2007), inducing plant defence mechanisms (Bargabus *et al*., 2002; Mishra *et al*., 2006; Bakker *et al.,* 2007), producing anti-herbivore products (Scott., 2001; Sullivan *et al*., 2007). Endophytes were investigated in the biological control of diseases on annual, perennial and biennial crops (Lodewyckx *et al*., 2002; Bargabus *et al*., 2002; Kloepper *et al*., 2004). Tomato bacterial wilts have some reports on their biological control (Li *et al*., 2003; Hu *et al*., 2006; Zhao *et al*., 2006; Almoneafy *et al*., 2012). Also, some reports have discussed the relationship between endophytes diversity and plant resistance (Araújo *et al*., 2002; Reiter *et al*., 2002). Moreover, the importance of tomato as a cultivated plant have risen the great role of the analysis of bacterial endophytes associated to these plants as a beneficial microorganisms. In the past, the control of post-harvest diseases has primarily been dependent on synthetic pesticides, however these substances have been shown to be harmful to both the environment as well as human health (Pimentel, 2005). Due to the undesired effects associated with synthetic pesticides, stricter rules and regulations are being enforced with regards to maximize residue limits (Holzmann, 2010). In addition, the high costs associated with the discovery and development of new synthetic pesticides (Glare *et al*., 2012) have created a need for a viable alternative control method. Biocontrol is a promising alternative due to its biodegradability (Coutte *et al*., 2010), low toxicity (Nitschke and Costa, 2007), environmental compatibility (Ongena and Jacques, 2008), as well as the fact that government regulations favor their use (Fahim, 2017). Biological lipopeptides, more commonly known as biosurfactants, are one of the examples of biocontrol that have shown great potential in various fields of study, including pharmaceutical, cosmetic and remediation. Biological surfactants have been found to be extremely effective in controlling phytopathogens, but the majority of biocontrol strategies currently available employ the micro-organism itself directly as a countermeasure to the pest. This is demonstrated by the use of products like Serenade (Marrone, 2002) and Bioshield™ (Jackson, 2007), where both products contain either living cells or endospores. Much of the research being carried out also includes the use of cells in the biocontrol agent (Yanez-Mendizabal *et al*., 2012), making the current study very valuable, as it focused on the use of the lipopeptides, i.e. the products produced by the micro-organisms. These are less sensitive to changes in pH or temperature compared to the organisms themselves, allowing for the production of a standardized product. An additional advantage to the use of lipopeptides instead of products containing living organisms is that there are fewer restrictions on importation and quarantine associated with these products. The main goal of this

manuscript was to study lipopeptides production, specifically the antibacterial surfactin, antifungal lipopeptides fengycin and iturin. Parameters associated with lipopeptides production were quantified and the production of the antifungal lipopeptides was optimized and the efficacy of the lipopeptides was tested against specific phytopathogens. Important factors in the study included the choice of organism to be used for lipopeptides production, growth medium composition as well as the process conditions under which production would be optimal. The aim of this work is to indicate the potential use of *B. subtilis* strains as biocontrol agents, especially when considering commercial implementation. Studies regarding the lipopeptides applications, should also be considered as this will influence the efficacy of the lipopeptides against the target organisms.

MATERIAL AND METHODES

1. Microbial and plant strains

Various strains of *Bacillus* genus were used in this work, wild type *Bacillus subtilis* ATCC 21332 was gathered from the culture collections of Microbial Biotechnology laboratory, Faculty of Agricultural, Menuofia University, Egypt. This strain was used as a model in order to estimate the conditions of lipopeptides production as surfactin and fengycin co-producer in aerobic condition (Fahim *et al*., 2012). The *Bacillus* strains named BMG100, BMG101 and BMG102 were isolated from tomato plant microbiome. Cultivar of super marmand tomato seedlings (*Lycopersicon esculentum*) were collected from the agriculture farm of Ain-Shams University, Egypt. The pathogenic strains which were used as a models in order to estimate the conditions of antimicrobial tests were collected from the plant pathology laboratory, Botany department, Faculty of Agricultural, Menuofia University, Egypt. The bacterial strains concluded *Pseudomonas syringae*, *Ralstonia solanacearum* and *Erwinia amylovora*. While, the fugal strains concluded *Aspergillus niger*, *Phanerochaete chrysosporium* and *Alternaria alternata*. This strains were recently proved to be a top plant pathogenic micro-organisms which affected on tissue culture and post harvest technologies.

2. Bacterial isolation and cultivation conditions

The strains used in this work were grown aerobically in Luria-Bertani (LB) medium at 30°C. While, for lipopeptides production, Landy modified Medium was used under 30°C and pH 7.0 using three oxygen transfer selective production regimes according to (Fahim, and Hussein 2016).

<u>Regime 1</u>: (flask size of 50 mL with proportional volume of 0.05 $mL.mL^{-1}$ flask) and shaking frequency of 250 r.p.m, in these conditions, k_La was higher than 0.06 s^{-1} and the ratio between the surfactins and others lipopeptides concentrations ($R_{surf/lipo}$) was reached 50.

<u>Regime 2</u>: (flask size of 50 mL with a proportional volume of 0.2 $mL.mL^{-1}$) flask) and shaking frequency of 150 r.p.m., these conditions k_La was less than 0.003 s^{-1}, ($R_{surf/lipo}$) was reached about 5.

<u>Regime 3</u>: (flask size of 1000 mL with a proportional volume of 0.4 $mL.mL^{-1}$ flask) and shaking frequency of 250 r.p.m, these conditions k_La was about 0.015 s^{-1} and ($R_{surf/lipo}$) was reached about 0.5. The lipopeptides production obtained in different production regimes were measured with standard deviation percentage between 3.34 and 9.96 % for surfactin and kurstakin values and between 2.03 and 59.55 % for iturin and fengycin values. This condition was

tested in each *Bacillus* strain, the cultures were performed during 48 h of fermentation (stationary phase) at 30°C in modified Landy MOPS medium with glutamic acid, the obtained results are means of three replicates with its standard deviations. For endophytic micro biome bacteria isolation process, the tomato stems surfaces were sterilized according to (Maurice *et al.*, 2009; Wen *et al.*, 2011). The attached dust and soil were removed from samples by washing with tap water, then 70% C_2H_5OH for 1 m and 5% NaOCl for 5 m were used to sterilize samples tissues. After that, samples were rinsed with sterilized distilled water three times, outer tissues were removed, and then were cut into fragments of 0.5 cm long. The plant fragments were sterilized one more time with 70% C_2H_5OH for 10 s and rinsed by sterilized water five times, then fragments were incubated on YPDA medium (pH 6.7) at 30°C until the appearance of colonies, which were purified and re-cultured on YPDA agar slants. At the same time, a negative control was performed without surface sterilization and a positive control of 100 µl of the last rinsed water were microbiological cheeked.

3. Equations and analytical methods

Microbial growth determination: The cell density was measured by various methods; (a direct method) by measuring the cell dry weight; (an indirect method) by measuring the density absorbance measurements. Cell dry weight (CDW): Shaken flasks during the stationery phase cultivation were incubated for 48 h, the serial dilution were applied. samples of 10 mL were collected from each dilution after filtration the bacterial cells pellet was discarded from the supernatant. A vacuum filtration (Buchner, Millipore® 0.2 µm, GR) were used for filtration, microbial filter disks were oven dried at 60°C for 24 h. After drying it were cooled in a glass desiccator, the filters were weighed to calculate the difference between the weight after and before filtration which represents as CDW. The wave length of 600 nm (visible light) were used for optical density measurements, this wave length were found to have the least amount of the growth medium. A free culture medium was used as standard blank for the cell absorbance measurements, the absorbance of the culture medium with growth was then measured at 600 *nm*. To avoid the false spectrophotometer sensitivity, samples cases of optical densities with absorbance exceeding 0.81 were decimal diluted to ensure that it were remained within the linear region.

(k_La) and (P_{VL}) quantifications: To achieve the volumetric oxygen transfer coefficient k_La in Erlenmeyer flasks, the investigated empirical equation was applied: $K_La = 6.67 \times 10^{-6}\ N^{1.16}\ VL^{-0.83}\ d^{1.92}\ d_0^{0.38}$ where (N) is the agitation rate by (min^{-1}), (V_L) the proportional volume (mL), (d_0) the shaker axe diameter (cm) and (d) the standard inner diameter of Erlenmeyer flask (cm). This latest equation was previously suggested by (Fahim and Hussein., 2016) for hydrophilic Erlenmeyer flasks, the axe shaker diameters of 5 *cm*, the proportional volumes of 0.05 to 0.4 $mL.mL^{-1}$, agitation rate within 150 to300 min^{-1}. Also, the power dissipation (P) was calculated according to the correlation proposed by (Fahim and Hussein., 2016) using the Reynolds number (Re) and modified Newton number (Ne): $P = Ne\ N^3\ \rho\ d^4\ V_L^{0.33}$; whereas $Ne = 70Re^{-1} + 25Re^{-0.6} + 1.5\ Re^{-0.2}$ While, the Reynolds number was calculated as $(Re) = Nd^2\ \rho/\mu$;... ρ ($kg.m^{-3}$) was water density and μ ($Pa.s$) the water dynamic viscosity at 30°C. the ratio between oxygen transfer coefficient k_La and the power dissipation (P) was calculated for each kind of

Erlenmeyer flask, for various proportional volumes (*Rv* from 0.05 to 0.4 $mL.mL^{-1}$) and shaking frequencies from 150 to 350 (min^{-1}) according to thee procedures developed by (Fahim, 2017).

4. Molecular characterization tools for bacterial isolates

Total proteins analyses and DNA isolation: The protocol of DNA Genomic isolation and purification kit was applied using the wizard of (Promega Co., Appl Microbiol Biotechnol Madison, USA). The concentration of DNA was measured by A_{260} direct method (1 ABS = 1 $mg.ml^{-1}$), the absorption at 260 nm was measured using NanoDrop UV-Vis spectrophotometer (Thermo Fisher Scientific ™). The purified DNA was directly used for PCR amplification or stored at -20 °C. For total protein analysis, Protein precipitation was carried out with salting technique by adding ammonium sulphate slowly to the cell-free culture (at 75% saturation), the frequently shaking using magnetic stirrer was done to precipitate the protein, the precipitate was collected by centrifugation at 10,000 ×*g* for 20 min and then dissolved in 50 *mmol* phosphate buffer (pH 8.0), dialyzed and stored at - 20°C (Switzer *et al.*, 1979). For molecular weight determination, the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles for extra-cellular degrading enzymes were preformed, the gel made of (10%) polyacrylamide and 5% stacking gels. After electrophoresis run, the gels were stained with Coomassie brilliant Blue R-250 staining method and overnight de-stained by 45% methanol and 10% acetic acid (Pant *et al.*, 2015). The gel was stained for 12 hr in 0.1% coomassie brilliant blur and distained until bands were clearly observed. Gel bands were scanned and analysed using (Gel Doc Bio-Rad system, USA).

Endophytic bacteria identification by 16S rDNA

The three long rod bacilli isolates were grown on YPDA medium, their total bacterial DNA were previously extracted by genomic DNA kits. The technique of bacterial 16S rDNA universal primers were used as described by (Teng *et al.*, 2006) 28 F: 5'-AGAGTTTGATCCTGGCTCAG-3' also 1493 R: 5'-TACCTTGTTACGACTT-3'.

PCR conditions started with initial denaturation step at 95°C for two minutes and 35 cycles of 45 sec at 95°C and then the annealing step at 50°C for 45 s sec and followed by elongation step at 73°C for 1.5 min and the final extension at 73°C for 10 min.

Plant associated bacterial 16S rDNA and PCR amplification. The obtained PCR fragments were amplified with universal bacterial 16S rDNA primers Table (1), the PCR products were extracted and purified using ZymocleanTm Gel DNA recovery kit (Epigenetics Company). The purified PCR products were ligate by using pGEMR - TEasy vector and then, the transformation into E. coli DH5α was completed. The transformants E. coli DH5α colonies were grown on LB plates supplemented with 55 mg.L-1 of ampicillin antibiotic. White colonies were selected for extracting their plasmid DNA by using plasmid miniPREP Kit (GeneDireX). Plasmids were double digested with EcoRI to verify the insertion of interested fragments then, amplified fragments were migrated on 1.2% agarose gel electrophoresis. Cloned products were sequenced and aligned with the databases available in GenBank (BLAST) online software of NCBI.

Detection of lipopeptides production genes.

The sequence published for ***Bacillus subtilis*** 168 lipopeptides operon were used to primers design as accession number of AL009126. Known DNA sequences of ***Bacillus subtilis*** which are involved in lipopeptides synthesis operons were compared with online Needle software (Needleman *and Wunsch.,* 1970). Lipopeptides genes detection by degenerated primers: degenerated primers for surfactin, fengycin, iturin, and kurstakin families were applied to investigate the gene absence or presence of NRPS, the PCR conditions were performed as described (Tapi *et al*., 2006; Hussein and Fahim., 2017).

5. Lipopeptides extraction and purification and detection.

Microbial fermentation was lanced in Landy modified medium for 48 h (stationary phase), the bacterial cells were discarded from fermented media by centrifugation with 14.000 r.p.m for 20 min at 5°C according to (Fahim, 2017). For lipopeptides extraction, 0.5 ml supernatants samples were passing through clean C_{18} cartridges (protocol of Alltech, Fr). Lipopeptides were washed with 3 ml of methanol grade; Sigma alderch, India). The elution was cold dried at 5 °C, the residues in glass tube bottom were re-dissolved in 200 µl of methanol grade. And then samples were injected on high-performance liquid chromatography (HPLC) using a C_{18} column (5.1 µm; 255 by 4.5 mm; VYDAC 218 TP, Hesperia, GE). The concentrations of lipopeptides families were determined by the spectrum of (HPLC) reverse phase, the produced lipopeptides were extracted after microbial batch fermentation. kurstakin produced strains, cells were collected and sonicated for 1 min at low temperature at 6 Watt (Ultrasonic processor, Cole-Parmer Instruments, Illinois, USA). The total yield was gathered before analysis by (HPLC) using a C_{18} column (5.1 µm; 255 by 4.5 mm; VYDAC 218 TP, Hesperia, GE). The mobile phases were iso-critical acetonitrile-water-trifluoroacetic acid solvent system (80:20:0.5, 55:45: 0.5, 45:55:0.5 and 40:60:0.5 [vol/vol/vol] for surfactin; kurstakin; fengycins or plipastatins; iturins families, respectively). (20 *µl*) of collected samples were injected through (HPLC) C_{18} column at a flow rate of 0.5 $ml.min^{-1}$. Surfactin (Sigma), kurstakin, fengycins or plipastatins, iturins were purchased with purity of 98% as standards. The spectrum of second derivatives of UV-visible and the retention time of each peak was used to identify the lipopeptides molecules (Waters integrated PDA 996 diode array detector; Millenniums Software) according to (Fahim, 2017).

Table (1). Degenerated primers for detection of lipopeptide biosynthesis genes.

Primers Names	Sequence of primers	Expected Fragment size (bp)	(NRLPs) identified	References
Ap1- F	AGMCAGCKSGCMASATCMCC	893/929	Plipastatin	Tapi *et al*., 2010
Tp1- R	GCKATWWTGAARRCCGGCGG			
Am1-F	CAKCARGTSAAAATYCGMGG	416/419	Mycosubtilins	
Tm1-R	CCDASATCAAARAADTTATC			
As1-F	CGCGGMTACCGVATYGAGC	422/425/431	Surfactins	

Ts2-R	ATBCCTTTBTWDGAATGTCCGCC			
Aks-F	TCHACWGGRAATCCAAAGGG	ND	Kurstakins	Abderrahmani *et al*., 2011
Tks-R	CCACCDKTCAAAKAARKWATC			

6. Lipopeptides antagonistic and antimicrobial testing.

Primary antimicrobial testing was performed using Kirby-Bauer technique according to the disk diffusion protocol described by the clinical laboratory standards institute (CLSI) guidelines using following standard lipopeptides discs: surfactin, iturin and fengycin with doses of 50, 150 and 350 µg for each lipopeptides susceptibility disk. The microbial isolates were inoculated onto a Muller-Hinton agar (Oxoid) plate and lipopeptides discs were added to the surface of the solid medium. The prepare disk diffusions plates were incubated at 30°C for 20 h. After microbial growth, the inhibition zones diameters were measured (by millimetres) to assess susceptibility or non-susceptibility according to the interpretation criteria for each fungal and bacterial strains established by the (CLSI) guidelines prepared by (Cockerill ., 2012).

Antibacterial activity test was determined by using Mueller-Hinton agar plates and carried out according to the protocol described by (CLSI) guidelines as follows: The overnight bacterial cultures grown on Mueller-Hinton broth (Oxoid) were adjusted to the density of 0.5 standard McFarland turbidity unit. The inoculation of the tested micro-organisms were placed streaky on to Mueller-Hinton medium (Oxoid) using a cotton swab. Whatman sterile discs with 6 mm diameter (Whatman-N°:(1), England) impregnated with (20 µl/disc) from purified produced lipopeptides mixtures (surfactins, iturins and fengycins), were added on the used solid culture medium, distilled water was used as negative control. After microbial growth, the inhibition zones diameters were measured (by millimetres) to assess susceptibility or non-susceptibility according to the interpretation criteria for each fungal and bacterial strains established by the (CLSI) guidelines. The experiments were carried out in three replicate and the inhibition zones diameters empirical means were noted with its standard divisions. While, the minimal inhibitory concentrations (MICs) µg.mL^{-1} were calculated as the lowest lipopeptides concentrations of the mixtures that completely inhibited the microbial growth of each model fungal or bacterial strain. The lipopeptides cultures media (supernatant) were purified and injected within the culture medium (Mueller-Hinton broth with decimal dilutions to investigate the final concentrations from zero to 350 µg.mL^{-1}, the examined tubes were inoculated by 100 colony (cfu.mL^{-1}) and incubated at 30°C for 20 h (Cockerill ., 2012). The controlling of microbial growth consisting comparing with growth culture and clear media (positive or negative control) to achieve the dilutions which were used in the applications treatments. In addition, the minimum bacterial concentration (MBCs) and (MFCs) were applied to detect the test model microbial cells which were killed or only inhibit their growth. The poured plates of Mueller-Hinton solid medium were autoclaved at 110°C for 30 minutes. The recorded MBCs or MFCs decimal dilutions were sub-cultured onto other prepared culture medium, the plates were incubated at 30°C for 20 h, after microbial growth, the lowest lipopeptides concentrations without any microbial growth were noted as the MBCs or

(MFCs) according to CLSI guidelines (Cockerill ., 2012).

RESULTS AND DISCUSSION

1. Isolation and identification of lipopeptides producers

1-1. Endophytic isolation from tomato plants

Twelve dissimilar plant associated bacterial colonies were isolated aerobically on YPDA medium from five different tomato seedlings of super marmand cultivars. Three bacterial isolates were belonged morphologically to spore forming long rode bacilli, as showed a positive result with Gram and spore staining. The other endophytic bacterial isolates, seven of them were belonged to short bacilli and two isolates were small cocci. These isolates revealed a negative result with gram and spore staining. Several reports have discussed the relationship between endophytes diversity and plant resistance (Araujo *et al.*, 2002). Moreover, the tomato importance as a cultivated plant have risen the great role of the analysis of bacterial endophytes associated to these plants as a beneficial microorganisms (Reiter *et al.*, 2002). Also, a lot of studies have reported that some endophytes have the ability to excrete antimicrobial agents; surfactin consider one of these compounds, which is cyclic lipoheptapeptides biosurfactant produced by *Bacillus* various strains, it has shown inhibitory activities against some phytopathogenic bacteria, fungi, viruses, and mycoplasma (Phae *et al.*, 1990).

1-2. Non ribosomal Lipopeptides genes detection

The Non Ribosomal Lipopeptides genes were detected by degenerated primers technique in the twelve tomato endophytic isolates, four genes were amplified; *Srf* (surfactin-surfactin), *Pps* (plipastatin-fengycin), *Myc* (mycosubtilin-iturin) and *Krs* (kurstakin-kurstakin) by degenerated primers, respectively. The endophytic short rode bacilli and coccii forms strains had no fragments amplified with the four degenerated primers. While, only the long rode bacilli endophytic strains were found to harbour the lipopeptides synthetases genes, these three strains were given laboratory names of (BMG100, BMG101 and BMG102) for further pursuit. The observed fragment length from used degenerated primers for the standard lipopeptides producer, *Bacillus subtilis* ATCC 21332 strain, and tomato associated bacilli isolates were mentioned in Table (2).

Bacillus subtilis ATCC 21332 strain was given two fragments sizes of 431 and 893 bp length for amplified surfactins and fengycins primers, respectively. While, the endophytic strain BMG100 was given three fragments sizes of 425, 893 and 419 bp length for amplified surfactins, fengycins and iturins primers, respectively. The endophytic strain BMG101 was given two fragments sizes of 431 and 416 bp length for amplified surfactins and iturins degenerated primers respectively. Also, the endophytic strain BMG102 was given two fragments sizes of 422 and 929 bp length for amplified surfactins and fengycins degenerated primers respectively. As mentioned above, no fragments were amplified with kurstakin degenerated primer for all examined strains. The use of *Pps* primers could amplify fragment length about 893 bp from *Bacilli* sp. BMG100 belongs to plipastatin synthetases genes, which was confirmed before in *Bacillus subtilis* ATCC 21332 strain by (Hussein and Fahim., 2017). In general, degenerated primers approach led to achievement of NRPs genes presence in tomato associated bacilli

isolates, this was confirmed before by (Tapi *et al.*, 2010) who reported that the use of degenerated primers are helpful in screening the various non-ribosomal synthesis genes harbor by *Bacillus spp.* which supported to detect a new non-ribosomal synthesis molecules and it facilitate the study and genetic potential knowledge of lipopeptides molecules biosynthesis.

Table (2): NRPs fragment detected by degenerated primers for lipopeptides producing strains.

Sequence origins	Degenerated primers sizes			
	Srf primer	*Pps* primer	*Myc* primer	*Krs* primer
Bacillus subtilis 21332	431	893	ND	ND
Bacilli sp. BMG100	425	893	419	ND
Bacilli sp. BMG101	431	ND	416	ND
Bacilli sp. BMG102	422	929	ND	ND

ND: Not detected

1-3. Identification of endophytic bacteria by 16S rRNA gene

The amplified fragment of 16S rDNA gene of the three endophytic *Bacilli* isolates were 600 bp in length as shown in Fig (1). While, their sequences were compared and aligned with Blast databases which were found to belong to *Bacillus* sp.

The sequence of 16S rRNA gene of BMG 100 showed similarity to the *Bacillus subtilis* strain MZS1 sequence (accession N°: KJ882376.1) by (85%) Also, it has similarity to *Bacillus amyloliquefaciens* strains SRM sequence (accession N°: KY401501.1) by 84%. Also, the sequence of 16S rRNA gene of the isolated BMG101 have similarity to *Bacillus subtilis* strain MZS1(accession N°: KJ882376.1) sequence by (85%). While was similar to *Bacillus pumilus* strain SR110 (accession N°: KY111721.1) sequence by (84%). The sequence of third *Bacilli* BMG102 strain, was similar by (81%) with *those of Bacillus subtilis* strain EBs5(accession N°: JQ289292.1) In general, the previous data of 16S rRNA gene similarity refer to the strong probability of belonging the three *bacilli* endophytic isolates to the species of *Bacillus subtilis.*

1-4. Phylogenetic tree based on 16S rRNA partial gene sequence

The phylogenetic tree based on alignment of 16S rRNA gene sequence of tomato associated *Bacilli* strains with other *Bacillus* strains indicated that all *Bacillus* sp could be classified into two major clusters from the same node as shown in Fig. (2).

The first cluster includes the associated *Bacillus* strain named BMG102, while the second cluster divided into some sub-clusters which divided into sub groups. One of these subgroups includes endophytic strain *Bacilli* BMG100 and the second subgroup includes the endophytic strain *Bacilli* BMG101 which they appears to be related to *B. subtilis* strain MZS1.

1-5. Total proteins SDS-PAGE profile analysis

SDS-PAGE for total extracted proteins from the endophytic strains *Bacilli* BMG100 BMG101 and BMG102 showed 28 polymorphic bands. Both *Bacilli* BMG100 and BMG101 were showed ten bands patterns, while *Bacilli* BMG102 showed pattern of 12 bands, Fig (3).

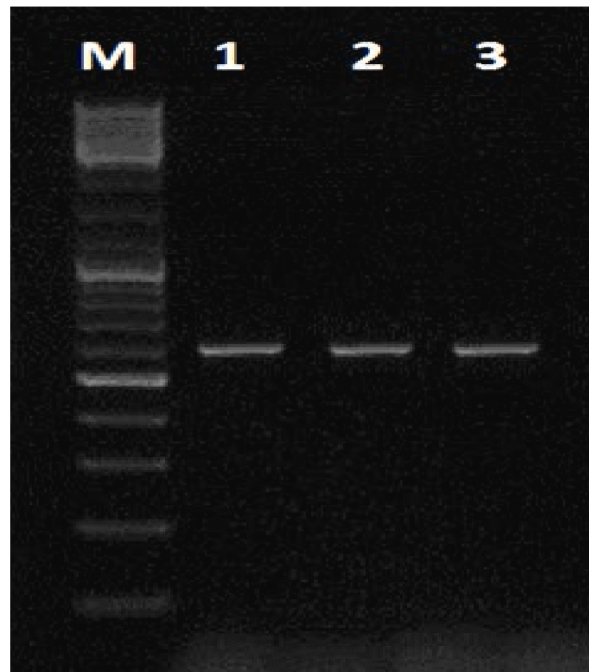

Figure (1). PCR amplification of 16S rDNA genes of the three endophytic isolates.

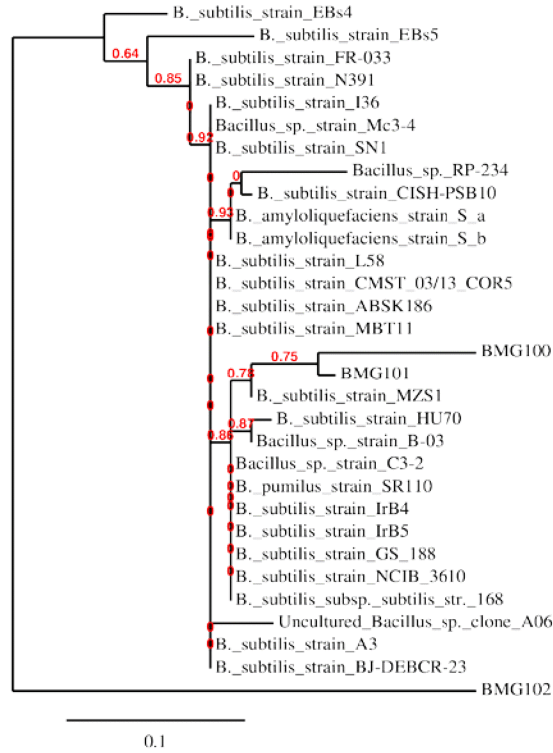

Figure (2): Phylogenetic tree based on alignment of 16S rRNA gene sequence of tomato associated *Bacilli* strains with other *Bacillus* strains.

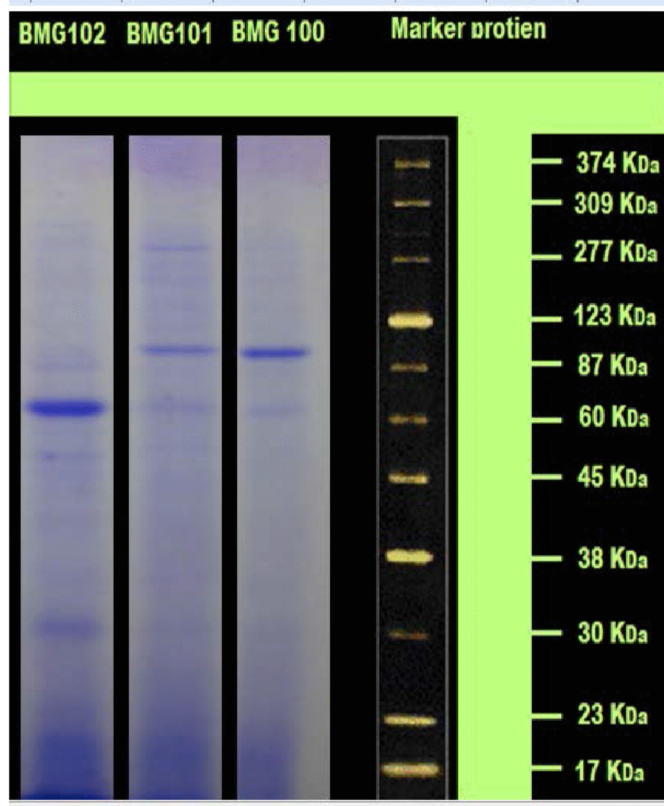

Figure (3): SDS-PAGE total proteins profiles extracted from the isolated *Bacilli* endophytic strains

On the other side, genetic similarity coefficient grouped the three strains into one main cluster of *Bacilli* BMG102 that include both *Bacilli* BMG100 and BMG101. Genetic distance ranged from 0.2 for *Bacilli* BMG102 to 0.36 for both *Bacilli* BMG100 and BMG101 as mentioned in Fig (4).

2. Production of Non Ribosomal Lipopeptides

2-1. Primarily lipopeptides production investigation

In this study lipopeptides production by *B. subtilis* ATCC 21332 and the isolated endophytic *B. subtilis* BMG100, BMG101and BMG102 which isolated in laboratory of Microbial Biotechnology laboratory, Faculty of Agricultural, Menuofia University, Egypt were investigated. Previous study showed that, *B. subtilis* 21332 strain was thought to be lipopeptides co-producer for both surfactin and plipastatin (Gancel *et al*., 2009). While, the primarily lipopeptides production experimentations under investigation condition showed different type of lipopeptides production by the endophytic strains. The evolution of lipopeptides production of each endophytic strains was carried out in modified Landy medium at pH 7.2 and 30 °C, the results details presented in Fig (5).

The production quantification of lipopeptides by HPLC revealed the ability of first isolate strain *B. subtilis* BMG100 to produce three types of lipopeptides, (surfactin, plipastatin and Mycosubtilin) which belonging to (surfactin, fengycin and iturin families) by 293, 105 and 178 mg.L^{-1}, respectively. The second isolate strain *B. subtilis* BMG101 was found to co-produce surfactin and mycosubtilin (surfactin and iturin families) by 385 and 236 mg.L^{-1}, respectively. Although, the third isolate strain *B. subtilis* BMG102 showed no lipopeptides production although it showed presence of lipopeptides production genes. These results proved that, all the endophytic isolates from tomato seedling had the ability to produce lipopeptides except the third isolate named *B. subtilis* BMG102 which showed no lipopeptides production by HPLC, it was also found that endophytic isolates are co-producers of three families of lipopeptides; surfactin, fengycin and iturin which known as inducers of systemic resistance system in plant (Hussein *and Fahim*., 2016).

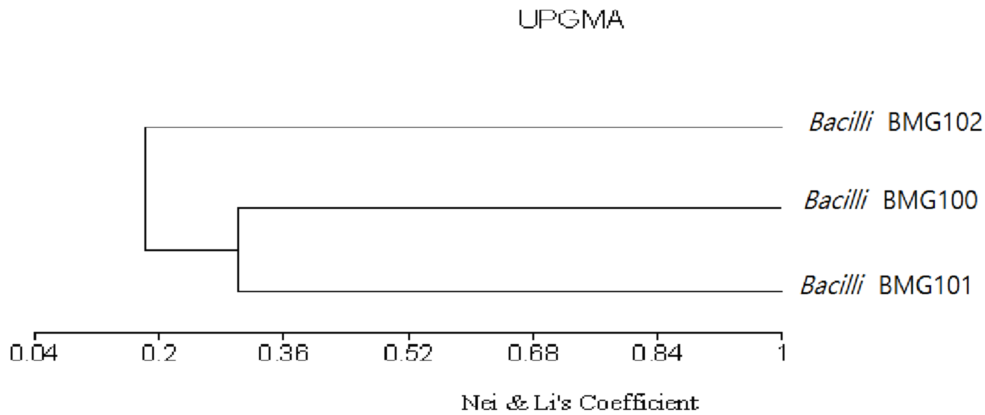

Figure (4): SDS-PAGE based cluster formation of the *Bacilli* endophytic strains

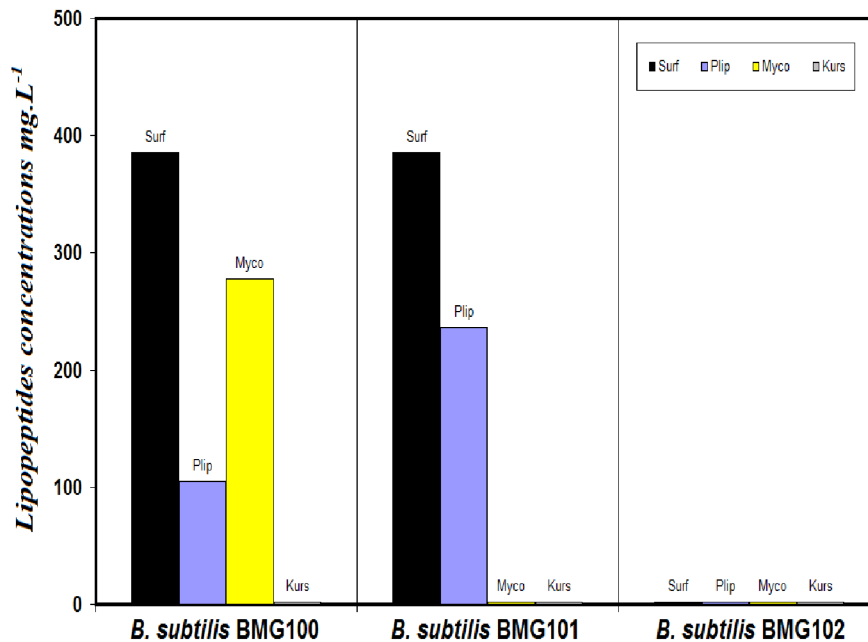

Figure (5): The evolution lipopeptides production of tomato endophytic strains

2-2. Kinetic of NRPs lipopeptides productivity

The kinetic study of the evolution of biomass and lipopeptides production of each endophytic strains was determined during 96 h of fermentation, the results of various experiments indicated that the fengycins or iturins had complete contrary behaviour with oxygen transfer (K_La) function. While, there was a positive correlation between surfactins productivity and increasing, K_La, this phenomena behind the increase of surfactin and decrease of fengycin or iturin concentrations levels. The maximum productivity of lipopeptides families were noted during various period of fermentation depending on the lipopeptide type and cultivation condition, thus leading to different lipopeptides and its family type

production at various K_La levels. These levels of K_La were used as cursors for surfactins, fengycins and iturins production from the various *B. subtilis* strains. At the many studies conditions, *Bacillus amyloliquefaciens* strains produced three families of lipopeptides; surfactin, fengycin and iturin (mycosubtilin type) according to Fahim, (2017). The standard *B. subtilis* ATCC21332 and the endophytic *B. subtilis* BMG101 strain produced two families of lipopeptides surfactin and fengycin (plipastatin type). While, strains of *B. subtilis* BMG100 produced the three lipopeptides families surfactin with the two types (plipastitin and mycosubtilin), the strain *B. subtilis* BMG102 showed no detectable for lipopeptides production as summarized in Table (3).

In all cases, *B. subtilis* strains was more surfactins productivity than iturin or fengycin types. Adjusting the shaking frequencies and the Erlenmeyer flask sizes led to adjust K_La levels which controlled the lipopeptide concentration and the selectivity of family type, the most suitable conditions for increasing surfactins types production was flask size minimization and increasing shaking frequency, while the fengycin and iturins types were maximized at low shaking frequency and biggest flask size, The obtained maximum concentration of surfactin type using 50 *mL* flask at *Rv* = 0.05 $mL.mL^{-1}$ and shaking frequency of 250 min^{-1} was about 1431, 529 and 437 $mg.L^{-1}$ for the strains *B. subtilis* ATCC21332, BMG100 and BMG101, respectively. A different behaviour was observed for fengycin type (plipastatin) and iturin (mycosubtilin), the concentrations levels weren’t the same with adjusted relative filling volume and shaking frequency for the various flasks size. The smaller ones (50 *mL*) enhanced the maximum plipastitin production with *Rv* increasing from 0.05 to 0.4 $mL.mL^{-1}$ which was 360 and 198 $mg.L^{-1}$ for *B. subtilis* ATCC21332, BMG100 and BMG101, respectively. The same thing was happened with mycosubtilin production with *Rv* increasing from 0.05 to 0.4 $mL.mL^{-1}$ which was 228 and 414 $mg.L^{-1}$ for *B. subtilis* BMG100 and BMG101, respectively. The shaking frequencies effect on fengycin and iturin production is very complicated since the increasing of oxygen supply of the culture media which is favoured for iturins and fengycins production could be observed by combined the agitation , filling volume and the size of flask. Many authors reported that, *Bacillus subtilis* is known to produce lipopeptides aerobically and able to grow under micro-aerobic conditions, the K_La values can thus had a strong effect of *Bacillus* metabolism which have a complex regulatory system according to (Fahim *et al*., 2012). Other investigation found that the production of lipopeptides by *B. subtilis* ATCC 6633 was determined based on the oxygenation levels (Chtioui *et al*., 2010).

TABLE 3

3. Behavior of *B. subtilis strains* under lipopeptides productivity regimes

The comparison of the lipopeptides productions obtained by the three oxygen transfer regimes for the *B. subtilis* ATCC21332 and the endophytic Bacillus isolated was studied Regarding lipopeptides production kinetics, surfactin in every case more produced by ATCC21332 than BMG100 and BMG101,and the regime one was the best for total productivity than the other regimes, but the situation is more complex for plipastatin and mycosubtilin production which is slightly higher in regime 2 or 3, respectively. However at regime 1, ATCC21332 produced surfactin two times more plipastatin or mycosubtilin than BMG100 and BMG101, the surfactin/plipastatin or mycosubtilin ratios are identical for these strains in the

different regimes which well illustrated in the Figure (6).

B. subtilis ATCC 21332 showed a big difference in the productivity comparing with the two endophytic strains in the maximum lipopeptides yield observed during the kinetic and production profiles of the three *B. subtilis* strains by the different production regimes. The *B. subtilis* strains showed positive correlation between the microbial growth and increasing of Oxygen Transfer Rate (OTR) in the different regimes. Reduced pH values were observed with the decreasing (OTR) and the pH values were more reduced for the endophytic strains comparing with the strain ATCC21332, these results confirm that endophytic strains are probably different in a pleitropic regulator production gene which is directly or indirectly involved in lipopeptides regulation biosynthesis (Fahim, 2017).

4. potentials of lipopeptides antimicrobial properties.

4-1. Lipopeptides antibacterial properties

In the present study the effects of primary antibacterial susceptibility testing was performed against three plant pathogenic bacterial strains which were *Pseudomonas syringae*, *Ralstonia solanacearum* and *Erwinia amylovora*. The effect of the three bacterial extracts from the lipopeptides productivity regimes for *B. subtilis* ATCC21332, BMG100 and BMG101 were examined, respectively. The extracted bacterial lipopeptides containing different concentrations of surfactin, plapastitin or/and mycosubtilin mixtures were evaluated as measured inhibition zones. The minimal inhibitory concentrations (MICs) and the Minimum bacterial concentration (MBCs) $\mu g.mL^{-1}$ of the net concentrations of lipopeptides were tested and summarized in Table (4).

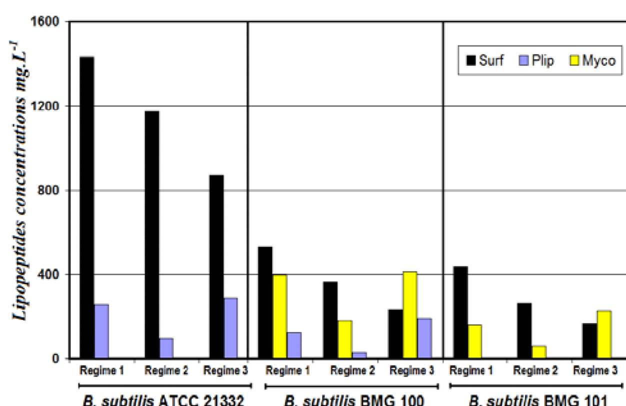

Fig (6): The comparison of the lipopeptides productions obtained by the three oxygen transfer regimes

TABLE 4

The plates treated with 20 µl of various lipopeptides types notably inhibited all test plant pathogenic bacterial growth, the strong inhibition zone was 19.1 *mm* for *P. syringae* by the lipopeptides mixture produced by *B. subtilis* ATCC21332 from regime 1, which also inhibit the growth of *R. solanacearum* and *E. amylovora* strains with inhibition zones of 18.9 and 18.2 *mm*, respectively. The relatively inhibitory concentrations 280 and 310 *µg.mL*$^{-1}$ of (MICs) and (MBCs) for all strains. Also, the plates treated with the lipopeptides produced from *B. subtilis* ATCC21332 regime 2 given 16.4, 16.3 and 16.8 *mm* with 290 and 320 *µg.mL*$^{-1}$ of (MICs) and (MBCs). While, the

lipopeptides produced from regime 3 given 13.3, 13.1 and 13.7 *mm* with 300 and 320 $\mu g.mL^{-1}$ of (MICs) and (MBCs) for *P. syringae, R. solanacearum* and *E. amylovora*, respectively. The lipopeptides produced from *B. subtilis* BMG100 regime 1 given 11.4, 11.2 and 11.8 *mm* with 320 and 350 $\mu g.mL^{-1}$ of (MICs) and (MBCs). Also, the plates treated with the lipopeptides produced from regime 2 given 8.2, 8.4 and 8.9 *mm* with 360 and 390 $\mu g.mL^{-1}$ of (MICs) and (MBCs). While, the lipopeptides produced from regime 3 had no inhibition. effects with 480 and 520 $\mu g.mL^{-1}$ of (MICs) and (MBCs) for *P. syringae, R. solanacearum* and *E. amylovora*, respectively. In addition, the lipopeptides produced from *B. subtilis* BMG101 regime 1 given 10.8, 10.2 and 10.9 *mm* with 340 and 370 $\mu g.mL^{-1}$ of (MICs) and (MBCs). While, those treated with the lipopeptides produced from regimes 2 and 3 had no inhibitions effects with 480 and 520 then 620 and 670 $\mu g.mL^{-1}$ of (MICs) and (MBCs) of (MICs) and (MBCs) for *P. syringae, R. solanacearum* and *E. amylovora*, respectively.

4-2. Lipopeptides antifungal properties

The effects of primary antifungal susceptibility testing performed against three plant pathogenic fungal were shown in *Aspergillus niger*, *Phanerochaete chrysosporium* and *Alternaria alternata*, The antifungal effect of the three bacterial extracts from the lipopeptides productivity regimes for *B. subtilis* ATCC21332, BMG100 and BMG101 were examined, respectively. The extracted bacterial lipopeptides containing different concentrations of surfactin, plapastitin or/and mycosubtilin mixtures were evaluated as measured inhibition zones. The minimal inhibitory concentrations (MICs) and the Minimum fungi concentration (MFCs) $\mu g.mL^{-1}$ of the net concentrations of lipopeptides were tested and summarized in Table (5)

In the plates treated with 20 µl of various lipopeptides types notably inhibited all test plant pathogenic fungi growth, the strong inhibition zone was 21.3 *mm* for *A. niger* by the lipopeptides mixture produced by *B. subtilis* BMG100 from regime 3, which also inhibit the growth of *R. solanacearum* and *E. amylovora* strains with inhibition zones of 20.4 and 20.8 *mm*, respectively. The relatively inhibitory concentrations 260 and 300 $\mu g.mL^{-1}$ of (MICs) and (MFCs) for all strains. Also, plates treated with the lipopeptides produced from *B. subtilis* BMG100 regime 1 given 18.1, 17.3 and 17.1 *mm* with 300 and 320 $\mu g.mL^{-1}$ of (MICs) and (MFCs). While, the lipopeptides produced from regime 2 given 19.6, 19.1 and 19.2 *mm* with 280 and 310 $\mu g.mL^{-1}$ of (MICs) and (MFCs) for *P. syringae, R. solanacearum* and *E. amylovora* respectively. The lipopeptides produced from *B. subtilis* ATCC21332 regime 1 given 18.2, 18.3 and 17.9 *mm* with 290 and 320 $\mu g.mL^{-1}$ of (MICs) and (MFCs). Also, plates treated with the lipopeptides produced from regime 2 given 13.7, 13.4 and 12.8 *mm* with 360 and 390 $\mu g.mL^{-1}$ of (MICs) and (MFCs). While, the lipopeptides produced from regime 3 given 17.6, 17.1 and 17.4 *mm* with 320 and 360 $\mu g.mL^{-1}$ of (MICs) and (MFCs) for *P.*

Table 5

syringae, R. solanacearum **and *E. amylovora*, respectively. In addition, the lipopeptides produced from *B. subtilis* BMG101 regime 1 given 14.5, 14.6 and 14.5 *mm* with 340 and 380 *μg.mL*$^{-1}$ of (MICs) and (MFCs). The plates treated with the lipopeptides produced from regime 2 given 12.9, 12.2 and 11.8 *mm* with 380 and 420 *μg.mL*$^{-1}$ of (MICs) and (MFCs). While, the lipopeptides produced from regime 3 given 16.3, 16.1 and 15.8 *mm* with 310 and 340 *μg.mL*$^{-1}$ of (MICs)**

and (MFCs) for *P. syringae, R. solanacearum* and *E. amylovora*, respectively. A positive correlation between the maximum lipopeptides production levels and maximum inhibition zone were found. In this regard, Fahim., (2017) confirmed the bactericidal and bacteriostatic of surfactin especially against various bacterial strains and multidrug resistant bacteria. Moreover fungicidal of mycosubtilin and plipastatin against various fungal strains. Also, the efficiency of surfactin is moderated by comparison of antibiotics especially in low concentration but without chance to produce drug resistant strain of this lipopeptides type (Ongena and Jacques, 2008). Consequently, the occurrence potential of lipopeptides types drug resistant strains is very low due to its specific natural composition that might function as a barrier to bioactive compounds that are reason for antibacterial activity (Jacques, 2008; Fahim., 2017).

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تأثير استخدام الليبوبيبتيدات بكتريا الاندوفيت ضد الميكروبات الممرضة للنبات

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[3] قسم الوراثة وعلم الخلايا ، قسم الهندسة الوراثية والتكنولوجيا الحيوية ، المركز القومي للبحوث (رقم الانتساب: 60014618) ، الدقي ، مصر.

الملخص العربي:

تم عزل اثني عشر عزلة بكتيرية متباينة مرتبطة بالنبات من سيقان خمسة شتلات طماطم مختلفة، كما فحص وجود جينات الليبوببتيدات اللاريبوزومية بواسطة تقنية ال PCR مع إستخدام البادئ الجينى في الأثني عشر عزلة وأظهرت النتائج وجود ثلاث عزلات ذات شكل عصوي طويل تملك الجينات المسئوله عن إنتاج الليبوببتيدات اللاريبوزومية. أعطيت هذه السلالات أسماء معملية باسم بي أم جي مائة 100 و 101 و 102 وبتعريف هذه العزلات عن طريق تحليل تتابعات قواعد الحمض النووي الريبوزومي من نوع 16 أس للعصويات الثلاثة المعزولة وكذلك التحليل المظهري للبروتين وجد ان العصويات الثلاثة تنتمي لنوع الباسلس سيبتلس وبدراسة قدرة عزلات الباسلس علي إنتاج الليبوبيتيدات بواسطة جهاز الفصل الكروماتوجرافي وجد ان لديها القدرة علي إنتاج الليبوبيبتيدات باستثناء العزلة الثالثة بي أم جي 102 والتي لم تظهر أي إنتاج. وقد تم دراسة تأثير إستخدام مستويات مختلفة من انتقال الأوكسجين الحجمي علي نوع وكمية الليبوبيتيد المنتجة حيث تم تطبيق ثلاث مستويات مختلفة من الأكسجين للتحكم في نظم إنتاج الليبوبيتيدات وهي مستوي مرتفع لنقل الأكسجين بأكثر من 0,07 وحدة في الثانية والذي اعطي اقصي تركيز من السيرفكتين حوالي 1431 و 529 و 437 مجم لكل لتر واكبر تركيز من البلباستاتين كان 360 و 198 مجم لكل لتر واكبر تركيز من الميكوسابتلين 228 و 414 مجم لكل لتر بالنسبة للسلالات الباسيلس سيبتلس (القياسية) إي تى سي سى 21332 وسلالات الباسلس المعزولة بي أم جي مائة 100 و 101 على التوالي. ويمكن ملاحظة إنتاجية فنجيسين وإيتورين المثلى عند قيم منخفضة علي التوالي اما المستويين الأخرين ذات القيم المنخفضة من نقل الأكسجين (0,01 و 0,015 وحدة في الثانية) على التوالي. فاعطت إنتاجية مثلي من الليبوبيتيدات من النوع فينجسين وإيتورين تم دراسة تاثير الليبوبيتيدات المنتجة من الستخلصات البكتيرية الثلاثة من أنظمة الأنتاج المختلفة كمواد مضادة للبكتريا الممرضة للنبات وهي *Ralstonia solanacearum – Erwinia amylovora – Pseudomonas syringae* وكمواد مضادات للفطريات الممرضة التالية *Alternaria –Phanerochaete chrysosporium –Aspergillus niger alternata* والتي أظهرت تثبيط لنمو هذه الممرضات ، وهذه النتائج تشير إلى إمكانية استخدام الليبوبيبتيدات في المكافحة الحيوية وخاصة عند النظر في التنفيذ على المستوي التجاري. كما ينبغي أيضًا الاهتمام بدراسة النتائج المتعلقة بطرق ونوعية الإنتاج حيث سوف تؤثر ذلك على فعالية الليبوبيبتيدات ضد الكائنات الحية الممرضة المستهدفة.

السادة المحكمين

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Table (3): *Quantification of* lipopeptides families produced by Bacillus isolates.

B. subtilis Strains	Produced Lipopeptides families							
	Surfactin types	Production *mg. L^{-1}* M ± SD	Fengycin types	Production *mg. L^{-1}* M ± SD	Iturin types	Production *mg. L^{-1}* M ± SD	Kurstakin types	Production *mg. L^{-1}* M ± SD
ATCC21332	Surfactin	1431 ± 9.11	Plipastatin	360 ± 17.35	-	0.0 ± 0.0	-	0.0 ± 0.0
BMG100	Surfactin	529 ± 13.77	Plipastatin	198 ± 16.13	Mycosubtilin	228 ± 9.53	-	0.0 ± 0.0
BMG101	Surfactin	437 ± 10.17	-	0.0 ± 0.0	Mycosubtilin	414 ± 14.21	-	0.0 ± 0.0
BMG102	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0

Results are means (M) of triplicate experiments ± standard deviation (SD).

Table (4): Antibacterial activity of lipopeptides against plant pathogenic bacteria

Treatments		*P. syringae*			*R. solanacearum*			*E. amylovora*		
Production Types	*B. subtilis* strains	Inh Zone *mm* M± SD	MICs *µg.mL^{-1}*	MBCs *µg.mL^{-1}*	Inh Zone *mm* M± SD	MICs *µg.mL^{-1}*	MBCs *µg.mL^{-1}*	Inh Zone *mm* M± SD	MICs *µg.mL^{-1}*	MBCs *µg.mL^{-1}*
Regime 1	ATCC 21332	19.1 ± 0.5	280	310	18.9 ± 0.4	280	310	18.2 ± 0.7	280	310
	BMG 100	11.4 ± 0.9	320	350	11.2 ± 0.7	320	350	11.8 ± 0.2	320	350
	BMG 101	10.8 ± 0.4	340	370	10.2 ± 0.7	340	370	10.9 ± 0.6	340	370
Regime 2	ATCC 21332	16.4 ± 0.7	290	320	16.3 ± 0.2	290	320	16.8 ± 0.6	290	320
	BMG 100	8.2 ± 0.4	360	390	8.4 ± 0.3	360	390	8.9 ± 0.5	360	390
	BMG 101	0.0 ± 0.0	480	520	0.0 ± 0.0	480	520	0.0 ± 0.0	480	520
Regime 3	ATCC 21332	13.3 ± 0.6	300	320	13.1 ± 0.3	300	320	13.7 ± 0.8	300	320
	BMG 100	0.0 ± 0.0	480	520	0.0 ± 0.0	480	520	0.0 ± 0.0	480	520
	BMG 101	0.0 ± 0.0	620	670	0.0 ± 0.0	620	670	0.0 ± 0.0	620	670

Results are means(M) of various experiments ± standard division (SD).

Table (5): Antifungal activity of lipopeptides against plant pathogenic fungi

Treatments		*A. niger*			*P. chrysosporium*			*A. alternata*		
Production Types	*B. subtilis* strains	Inh Zone *mm* M ± SD	MICs *µg.mL⁻¹*	MFCs *µg.mL⁻¹*	Inh Zone *mm* M ± SD	MICs *µg.mL⁻¹*	MFCs *µg.mL⁻¹*	Inh Zone *mm* M ± SD	MICs *µg.mL⁻¹*	MFCs *µg.mL⁻¹*
Regime 1	ATCC 21332	18.2 ± 0.4	290	320	18.3 ± 0.6	290	320	17.9 ± 0.4	290	320
	BMG 100	18.1 ± 0.2	300	320	17.3 ± 0.3	300	320	17.1 ± 0.8	300	320
	BMG 101	14.5 ± 0.9	340	380	14.6 ± 0.7	340	380	14.5 ± 0.2	340	380
Regime 2	ATCC 21332	13.7 ± 0.6	360	390	13.4 ± 0.4	360	390	12.8 ± 0.5	360	390
	BMG 100	19.6 ± 0.3	280	310	19.1 ± 0.2	280	310	19.2 ± 0.6	280	310
	BMG 101	12.9 ± 0.5	380	420	12.2 ± 0.7	380	420	11.8 ± 0.2	380	420
Regime 3	ATCC 21332	17.6 ± 0.4	300	320	17.1 ± 0.5	300	320	17.3 ± 0.5	300	320
	BMG 100	21.3 ± 0.5	260	300	20.4 ± 0.7	260	300	20.8 ± 0.4	260	300
	BMG 101	16.3 ± 0.6	310	340	16.1 ± 0.6	310	340	15.8 ± 0.6	310	340

Results are means(M) of various experiments ± standard division (SD).