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DETECTION OF SYNTHETASES GENES INVOLVED IN NON RIBOSOMAL LIPOPEPTIDES (NRLPS) BIOSYNTHESIS FROM *BACILLUS* SPECIES BY BIOINFORMATICS AND PCR DEGENERATED PRIMERS AND ESTIMATION OF THEIR PRODUCTION

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ABSTRACT

Non Ribosomal Lipopeptides NRLPs produced by *Bacillus* spp. were firstly detected by bioinformatics in 54 sequenced genomes available from NCBI, which revealed the presence of the four lipopeptides families; surfactin, fengycin/plipastatin, iturin and kurstakin in 19 different strain, fusaricidin from (*Paenibacillus*) *B. polymyxa* SC2 and unknown PKS-NRPS lipopeptide from *B. safensis* FO-36b . Also the absence of NRLPs synthetases genes were detected in 14 different strain, while the 21 other strains harbor different NRP synthetases genes. Secondly, we used the degenerated primers approach to detect the synthetases genes involved in lipopeptides biosynthesis in 9 *Bacillus* strains. This technique detected non ribosomal synthetases genes for kusrstakin in *B. thrunigenesis israelienne* NRRL HD-522, surfactin and plipastatin synthetases genes in *B. subtilis* ATCC 21332 and unknown PKS-NRPS synthetases genes in (*Lysinibacillus*) *B. sphearicus* 23268 T. Thirdly, production levels were estimated for these 9 strains by HPLC. The production of *B. amyloliquefaciens* FZB42 were 294, 62 and 210 $mg.L^{-1}$ surfactin, fengycin and mycosubtilin, respectivelly. *B. amyloliquefaciens* S499 were 872, 103 and 103 $mg.L^{-1}$ surfactin, fengycin and bacillomycin respectivelly. *B. subtilis* ATCC 21332 were 1060 and 226 $mg.L^{-1}$ of surfactin and plipastatin respectivelly. While, *B. subtilis* 168 has no production. The production of *B. licheniformis* ATCC 14580 strain was 358 $mg.L^{-1}$ of lichenysin, and *B. pumilus* was produced 642 $mg.L^{-1}$ of pumilacidin. Also, the strains of *B. thuringiensis kurstaki* and *B. thuringiensis israelienne* NRRL HD-522 were produced 102 and 62 $mg.g^{-1}$ cells respectivelly of kurstakin.

KEY WORDS: ***Lipopeptides families, Bacillus spp., degenerated primers, NRLPs, lipopeptides production.***

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INTRODUCTION

Bacillus species produces a broad spectrum lipopeptides known as biosurfactants and has antifungal, antibacterial and antiviral activities. Firstly, three families of lipopeptides were identified in *Bacillus* species; surfactins, iturines, and fengycins or plipastatins families, while kurstakin family was detected recently in *Bacillus thuringiensis subsp. kurstaki* strain HD-1[1]. surfactin was discovered from the culture broth of *Bacillus subtilis*[2]. The surfactin operon consists of four openreading frames (ORFs) coding for four enzymatic subunits; SrfA, SrfB, SrfC and SrfD. Surfactin family harbor also four openreading frames (ORFs) coding for lichenysin synthetases; lchA, lchB, lchC and lchD, respectivelyand this heptapeptide is linked to a β-hydroxyl fatty acid chain (C12- C16).[3,4] The second lipopeptide family Fengycin and plipastatin which their discovery In 1986 was concomitant by German[5] and Japanese teams[6]. There was only a small structural difference between these two compounds when it's Tyr3 and Tyr9 residues are present in the L- and D-form and till now a doubt still exists about their structure and their biological activities. Plipastatin is a lipodecapeptide which the operon harbor five ORFs (ppsA, ppsB, ppsC, ppsD and ppsE or fenC, fenD, fenE, fenA and fenB)[7,8] and are linked to a β-hydroxyl fatty acid chain (C14-C18). Iturin family is different than both surfactin and plipastatin or fengycin families; it is synthesized by a hybrid of PKS-NRPS[9,10] and their is 38-40 kb in size harbor four ORFs; fenF, mycA, mycB and mycC for mycosubtilin or ituD, ituA, ituB and ituC for iturin[11] (Fig 1). This heptapeptide is linked to a β-amino fatty acid chain (C14-C17). The new lipopeptide family kurstakin operon contains three genes (krsA, krsB, and krsC) which encode three large multifunctional proteins (KrsA, KrsB, and KrsC) constituting the complete synthetase with the primary structure:

D-Thr_Gly/Ala/Gln/Glu_Ala_Ser_X_D-Gln_Gln

as mentioned[12,13,14] (Fig 2). The study of the genetic potential considered useful and important in the detection of lipopeptides as in the case of *B. amyloliquefaciens* S499 which was characterised for its ability to produce iturin and surfactin only and later shown to be a producer for another lipopeptide which is fengycin[15,16]. We firstly analyzed 54 *Bacillus* genomes by bioinformatics tools to get an overview about the Non Ribosomal Lipopeptides (NRLPs) potentially produced by these *Bacillus* strains and secondly used the approach of degenerated primers[17] to detect the presence of lipopeptide genes by PCR with degenerate primers based on intraoperon DNA sequence alignment of both adenylation and thiolation domains for lipopeptide biosynthesis enzymes in each family. The aim of this work is to detect synthetases genes involved in NRLPs biosynthesis in *Bacillus* species by bioinformatics and degenerated primers and then determine their production levels after fermentation process by HPLC.

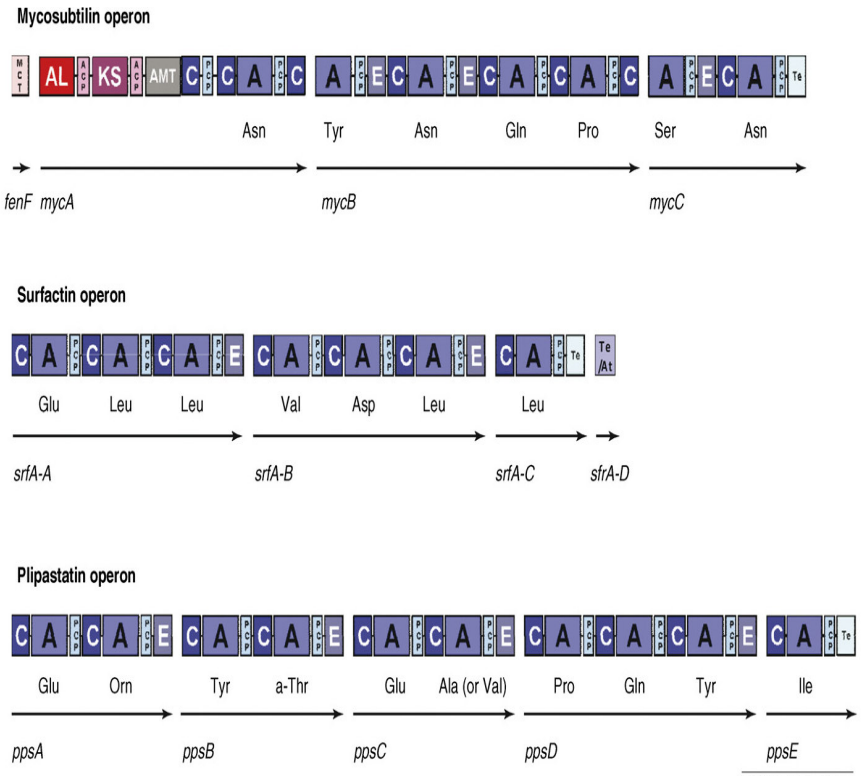

Figure 1
Operons of mycosubtilin, surfactin and plipastatin synthetases[18]

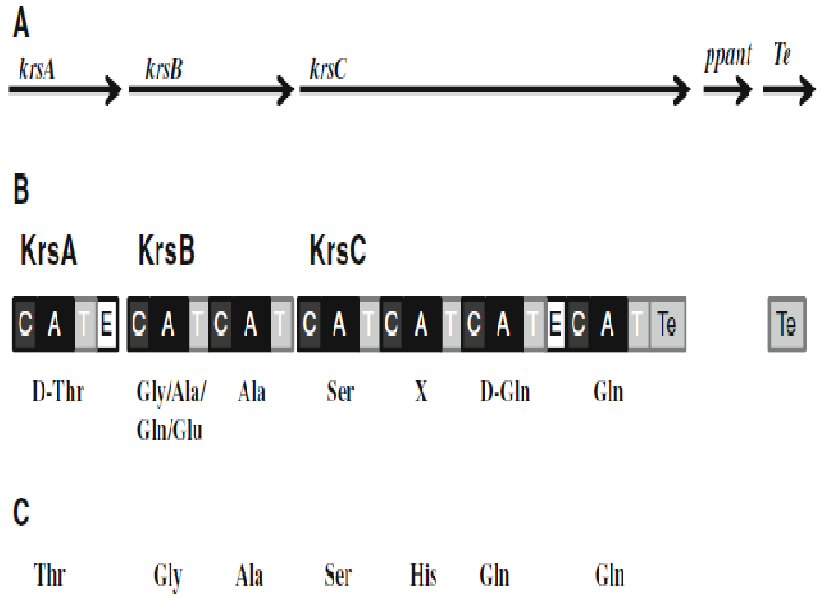

Figure 2
***Kurstakin operon synthetases genes*[19]**

MATERIALS AND METHODS

Microbial strains and culture media

All bacterial strains were grown aerobically in Luria-Bertani [LB] medium and Landy MOPS medium for lipopeptides production at 30°C. *Echerichia coli* JM109 strain was grown at 37°C in [LB] medium supplemented with 50 µg.ml^{-1} ampicillin[20]. All bacterial strains mentioned in (Table 1).

Table 1
Bacterial strains used in the study.

Name	Genotype	Source
Bacillus subtilis 168	trpC^{2}, sfp^{0}	*Bacillus* stock gentic center
Bacillus subtilis ATCC 21332	Wild type	ATCC
Bacillus amyloliquefaciens S499	Wild type	ProBioGEM
Bacillus pumilus	Wild type	NRC
Bacillus thrunigenesis israelienne NRRL HD- 522	Wild type	NRC
Bacillus licheniformis ATCC 14850	Wild type	ATCC
Bacillus sphearicus 23268T	Wild type	NRC
Bacillus thrunigenesis kurstaki	Wild type	AGERI
Bacillus amyloliquefaciens FZB42	Wild type	ProBioGEM
Escherichia coli JM 109	recA1, endA1, gyrA96, thi, hsdR17(rK−, mK+), relA1, supE44, Δ(lac proAB), [F′, traD36, proAB, lacIqZΔM15]	Promega

NRC: National Research Centre institute, Egypt.
AGERI: Agricultural Genetic Engineering Research Institute, Egypt.
ProBioGEM: Laboratoire des Procédés Biologiques, Génie Enzymatique et Microbien, France.

Bioinformatics analyses

Fifty-four sequenced genomes were obtaind from the National Center for Biotechnology Information (USA) NCBI (Table 2) and analyzed for the detection of NRLPs synthetases genes using some keywords as; non-ribosomal, synthetase, lipopeptide. The second step were to identify their NRPS genes, annotation, description of modules and domains, specificity prediction of A domains by PKS-NRPS website (http://nrps.igs.umaryland.edu/nrps), potential primary structure of the peptide.The last step was to compare the predicted peptides structures obtained by PKS-NRPS website with Norine database (http://bioinfo.lifl.fr/norine) which includes nonribosomal peptides and 1185 peptides to know the predicted peptide is existing molecule or new one[21].

Table 2
***Bacillus* genomes data analyzed for non ribosomal lipopeptides genes detection.**

No	Organisme	Genome Size (Mbp)	GenBank
1	*Bacillus amyloliquefaciens* DSM7	4.0	FN597644.1
2	*Bacillus amyloliquefaciens* FZB42	3.9	CP000560.1
3	*Bacillus amyloliquefaciens* S499	3.9	NZ_CP014700.1
4	*Bacillus anthracis str. 'Ames Ancestor'*	5.5	AE017334.2
5	*Bacillus anthracis str.* A0248	5.2	CP001598.1
6	*Bacillus anthracis str. Ames*	5.2	AE016879.1
7	*Bacillus anthracis str.* CDC 684	5.5	CP001215.1
8	*Bacillus anthracis str. Sterne*	5.2	AE017225.1
9	*Bacillus atrophaeus* 1942	4.2	CP002207.1
10	*Bacillus azotoformans* MEV2011	4.7	NZ_JJRY00000000.1
11	*Bacillus cellulosilyticus* DSM 2522	4.7	CP002394.1
12	*Bacillus cereus* 03BB102	5.4	CP001407.1
13	*Bacillus cereus* AH187	5.9	CP001177.1
14	*Bacillus cereus* AH820	5.6	CP001283.1
15	*Bacillus cereus* ATCC 10987	5.4	AE017194.1
16	*Bacillus cereus* ATCC 14579	5.4	AE016877.1
17	*Bacillus cereus* B4264	5.4	CP001176.1
18	*Bacillus cereus* E33L	5.8	CP000001.1
19	*Bacillus cereus* G9842	5.7	CP001186.1
20	*Bacillus cereus* Q1	5.5	CP000227.1
21	*Bacillus cereus biovar anthracis str. CI*	5.4	CP001746.1
22	*Bacillus clausii* KSM-K16	4.3	AP006627.1
23	*Bacillus coagulans DSM* 1 = ATCC 7050	3.3	CP009709.1
24	*Bacillus coahuilensis* p1.1.43	3.0	NZ_ABFU00000000.1
25	*Bacillus cytotoxicus* NVH 391-98	4.1	CP000764.1
26	*Bacillus endophyticus*	4.8	CP011974.1
27	*Bacillus endophyticus* 2102	5.1	NZ_ALIM00000000.1
28	*Bacillus flexus*	5.6	NZ_LFQJ00000000.1
29	*Bacillus halodurans* C-125	4.2	BA000004.3
30	*Bacillus licheniformis* ATCC 14580	4.2	CP000002.3
31	*Bacillus megaterium* DSM 319	5.1	CP001982.1
32	*Bacillus megaterium* QM B1551	5.5	CP001983.1
33	*Bacillus methylotrophicus* FZB42	3.9	CP000560.1
34	*Bacillus methanolicus* MGA3	3.4	CP007739.1
35	*Bacillus paralicheniformis* ATCC 9945a	4.3	CP005965.1
36	*Bacillus pseudofirmus* OF4	4.3	CP001878.2
37	*Bacillus pumilus SAFR-032*	3.7	CP000813.1
38	*Bacillus safensis* FO-36b	3.7	NZ_ASJD00000000.1
39	*Bacillus selenitireducens* MLS10	3.6	CP001791.1
40	*Bacillus smithii*	3.3	CP012024.1
41	*Bacillus sonorensis* L12	4.5	NZ_AOFM00000000.1
42	*Bacillus subtilis subsp. subtili sp. natto* BEST195	4.1	AP011541
43	*Bacillus subtilis subsp. subtili sp. spizizenii str.* W23	4.0	CP002183.1
44	*Bacillus subtilis subsp. subtili sp. subtilis str. 168*	4.2	AL009126.3
45	*Bacillus thuringiensis* BMB171	5.6	CP001903.1
46	*Bacillus thuringiensis serovar konkukian str.* 97-27	5.3	AE017355.1
47	*Bacillus thuringiensis str. Al Hakam*	5.3	CP000485.1
48	*Bacillus tusciae* DSM 2912	*3.4	CP002017.1
49	*Bacillus weihenstephanensis* KBAB4	5.8	CP000903.1
50	*Lysinibacillus sphaericus*	4.6	CP000817.1
51	*Lysinibacillus sphaericus* C3-41	4.8	CP000817.1
52	*Brevibacillus brevis*	6.3	AP008955.1
53	*Paenibacillus polymyxa* E681	5.4	CP000154.1
54	*Paenibacillus polymyxa* SC2	* 6.2	CP002213.1

Molecular biology methods

DNA isolation, primers and PCR conditions

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit from Promega. Primers were used[17] as described Table (3). Degenerated primers for surfactin , fengycin and mycosubtilin families were designed[17], while kurstakin was designed[19] by alignment of the nucleic acid sequences identified of adenylation and thiolation domains and using International Union of Pure and Applied Chemistry (IUPAC) code. The PCR conditions started with 94°C for 2 min as initial denaturation step, followed by 35 cycles of three steps; denaturation at 94°C for 30 s; annealing step for 30 s, at 43°C with As_1-F/Ts_2-R, at 45°C with Am_1-F/Tm_1-R; Af_2-F/Tf_1-R and at 44.4°C with Aks-F/Tks-R; an extension step of 45 s at 72°C except with Ap_1-F/Tp_1-R primers (75 s at 72°C) and Aks-F/Tks-R primers (2 min at 72°C). Then final extension step was lanced at 72°C for 5 min.

Cloning and sequencing conditions

PCR amplified bands were purified from gel with Zymoclean Gel DNA Recovery Kit (Epigenetics, USA). The PCR products were cloned into pGEM-T Easy Vector (Promega), and the heat shock method was followed to introduce plasmids into E. coli JM109 cells as described by the manufacturer's protocol. The transformants were selected on LB agar medium complemented with ampicillin, 5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-

D-thiogalactopyranoside (IPTG) at a concentration of 100, 200 and 200 µg.ml^{-1}, respectively. In LB medium complemented with ampicillin at final concentration of 100 µg.ml^{-1}, the white colonies were picked and grown at 37°C for 24 h. Then, Plasmid miniPREP Kit (GeneDireX) were used to isolate plasmids from the transformed cells. EcoR I (Fermentas) restriction digestion of the plasmids was performed to check the inserts. Cloned products were sequenced and sequences were compared with the GenBank databases using (BLAST) software provided online by the National Center for Biotechnology Information (USA) NCBI.

Table 3
Degenerated primers for detection of lipopeptide biosynthesis genes.

Primers names	Sequence of primers	Expected fragment size (bp)	(NRLPs) identified	References
Ap1- F	AGMCAGCKSGCMASATCMCC	959, 929 ; 893	Plipastatin	17
Tp1- R	GCKATWWTGAARRCCGGCGG			
Af2-F	GAATAYMTCGGMCGTMTKGA	443, 452 455	Fengycins	17
Tf1-R	GCTTTWADKGAATSBCCGCC			
Am1-F	CAKCARGTSAAAATYCGMGG	416, 419	Mycosubtilins	17
Tm1-R	CCDASATCAAARAADTTATC			
As1-F	CGCGGMTACCGVATYGAGC	419, 422, 425, 431	Surfactins	17
Ts2-R	ATBCCTTTBTWDGAATGTCCGCC			
Aks-F	TCHACWGGRAATCCAAAGGG	1125, 1152, 1161, 1167, 1173	Kurstakins	19
Tks-R	CCACCDKTCAAAKAARKWATC			

Fermentation process and HPLC quantification

The produced lipopeptides were extracted after microbial batch fermentation in Landy Mops medium supplied by 0.03 s^{-1} of voulmetric oxygen transfer coefficient (k_La) at 30°C for 48 h. The bacterial cells were removed from 48 h fermented culture (stationary phase) by centrifugation at 15.000 rpm at 5°C for 20 min[20], kurstakin produced strains, cells were collected and sonication for 1 min in low temperture at 6 Watt (Ultrasonic processor, Cole-Parmer Instruments, Illinois, USA). The total yield was collected before analysis by high-performance liquid chromatography (HPLC) in C_{18} column (5 µm; 250 by 4.6 mm, VYDAC 218 TP, Hesperia, CA), the mobile phase was isocritical 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 famlies and fengycins or plipastatins, iturins famlies, respectively)[22,23]. Samples (20 µl) were injected and the compounds were eluted at a flow rate of 1 ml.min^{-1}. Purified surfacttin (Sigma), kurstakin, fengycins or plipastatins, iturins were purchased with purity of 98% as standards. The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules[24].

RESULTS AND DISCUSSION

Bioinformatics analyses

Fifty four *Bacillus* genomes were analyzed by PKS-NRPS analysis, NRPS predictor and NCBI web site Table (4). The presence of genes involved in the biosynthesis of members of the four families of lipopeptides were detected [surfactin, pumilacidin, lichenysin from surfactin family, fengycin or plipastatin from fengycin/plipastatin family, bacillomycin and mycosubtilin from iturin family and kurstakin from kurstakin family] and other lipopeptides fusaridin from (*Paenibacillus*) *B. polymyxa* and unknown lipopeptide from *B. safensis* FO-36b. Four *Bacillus* strains (*amyloliquefaciens* DSM7, *amyloliquefaciens* FZB42, *amyloliquefaciens* S499 and *methylotrophicus* FZB42) showed the presence of three lipopeptides families; surfactin, fengycin/plipastatin and iturin. Three other *Bacillus* strains showed the presence of two lipopeptides families; surfactin and plipastatin from *Bacillus subtilis* 168, surfactin and mycosubtilin from *Bacillus subtilis spizizenii* W23, truncated lichenysin and plipastatin from *Bacillus sonorensis* L12 in addition to; *Bacillus paralicheniformis* ATCC 9945a which harbor plipastatin seems different in the amino acid no 9 (Asp/Asn) and lichenysin. We also mentioned the presence of 14 *Bacillus* genomes harbor no NRLPs synthetases genes as described Table (5).

Table 4
Peptide sequence of each lipopeptide family from 19 Bacillus spp.

Lipopeptides	Predicted peptide structure	*Bacillus* spp.
Surfactin family		
Surfactin	L-Glu/ Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	*amyloliquefaciens* DSM7
	L-Glu/ Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	*amyloliquefaciens* FZB42
	L-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	amyloliquefaciens S499
	L-Glu/ Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	*subtilis subsp. natto* BEST195
	L-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	*subtilis subsp. spizizenii str*. W23
	L-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp-D-Leu-L-Leu/Ile/Val	*subtilis subsp. subtilis str*. 168
	D-Glu/Asp-L-Leu/Ile/Val-D-Leu-L-Val-L-Asp- D-Leu-L-Leu/Ile/Val	*methylotrophicus* FZB42
Pumilacidin	L-Glu/Asp-L-Leu-D-Leu-L-Phe/Trp-L-Asp-D-Leu-L-Leu	*pumilus SAFR*-032
Lichenysin	L-Gln-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	*licheniformis* ATCC 14580
	L-Gln-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	*Paralicheniformis* ATCC 9945a
Truncated lichenysin	L-Glu-L-Leu-L-Ile	*sonorensis* L12
Iturin family		
Iturin	L-Asx-D-Pro-D-Tyr/Trp-L-Asx-L-Gln-D-Asx-L-Ser	*amyloliquefaciens* DSM7
Bacillomycin	L-Asx-D-Glu/Asp-D-Asx-Tyr/Trp- L-Pro-D-Ser-L-Thr	*amyloliquefaciens* FZB42
	L-Asx-D-Glu/Asp- D-Asx- -L-Tyr/Trp- L-Pro-D-Ser-L-Thr	*methylotrophicus* FZB42
Mycosubtilin	L-Asx-D-Pro-D-Tyr/Trp-L-Asx-L-Gln-L-Ser-L-Asx	*atrophaeus* 1942
	L-Asx-D-Tyr/Trp-D-Asx-L-Gln- L-Pro-D-Asx-L-Ser	amyloliquefaciens S499
	L-Asx-D-Pro-D-Tyr/Trp-L-Asx-L-Gln-D-Ser-L-Asx	*subtilis subsp. spizizenii str*. W23
Fengycin family		
Truncated fengycin	L-X-D-Tyr-L-Ile	*amyloliquefaciens* DSM7
Fengycin	L-Glu-D-Orn-L-X-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Tyr-L-Ile	*amyloliquefaciens* FZB42
	L-Glu-D-Orn- L-X-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Tyr-L-Ile	amyloliquefaciens S499
	D-Glu-L-Orn- D-X-L-Thr-D-Glu-L-Val-D-Pro-Glu-L-Tyr-L-Ile	*methylotrophicus* FZB42
Plipastatin	L-Glu-D-X-L-Tyr-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Tyr-Ile	*subtilis subsp. subtilis str*. 168
	L-Glu-D-X-L-X-D-Thr-L-Glu-D-Val-L-Pro-L-Glu-D-Asp/Asn?-L-Ile	*Paralicheniformis* ATCC 9945a
Truncated plipastatin	L-Tyr-D-Thr-D-X	*sonorensis* L12
Kurstakin family		
Kurstakin	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Thr-L-Glu/Asp	*weihenstephanensis* KBAB4
	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Gln-L-Gln	*thuringiensis* BMB171
	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Gln-L-Gln	*cereus* G9842
Truncated kurstakin	D-Thr-L-Gln-L-Gly-L-X-D-Gln-L-Gln	*cereus* ATCC 14579
	D-Thr-L-Gln-L-Gly-L-Ser-L-X-D-Gln-L-Gln	*cereus* B4264
Other		
Fusaricidin	L-Thr-D-Val/Ala-Tyr_D-Thr-D-Asx-L-X	*polymyxa* SC2
Unknown	L-Leu- Pks- pks- D-X- L- Leu	*safensis* FO-36b

Table 5
***Bacillus* strains with no NRLPs synthetases genes.**

Bacillus azotoformans MEV2011	*Bacillus megaterium* DSM 319
Bacillus cellulosilyticus DSM 2522	*Bacillus megaterium* QM B1551
Bacillus coahuilensis p1.1.43	*Bacillus methanolicus* MGA3
Bacillus coagulans DSM 1 = ATCC 7050	*Bacillus pseudofirmus* OF4
Bacillus clausii KSM-K16	*Bacillus selenitireducens* MLS10
Bacillus flexus	*Bacillus smithii*
Bacillus halodurans C-125	*Bacillus tusciae* DSM 2912

Table 6
Nucleotide sequence alignment for partial synthetases genes of NRLPs detected in three Bacillus strains by degenerated primers.

Sequence Origins	Srf primers	Pps primers	Fen primers	Myc primers	Krs primers	Similarity with	Similarity %
B. amyloliquefaciens FZB42	+	+	+	+	-	Surfactin, fengycin and iturin synthetases genes from *B. amyloliquefaciens* FZB42	100
B. amyloliquefaciens S499	+	+	+	+	-	Surfactin, fengycin and iturin synthetases genes from *B. amyloliquefaciens* S499	99
B. licheniformis ATCC 14580	+	+	+	-	-	Lichenysin synthetases genes from *B. licheniformis* ATCC 14580	100
B. pumilus	+	-	-	-	-	Pumilacidin synthetases genes from *B. pumilus*	100
B. thuringiensis kurstaki	+	-	-	-	+	Kurstakin synthetases genes from *B. thuringiensis kurstaki*	100
B. thuringiensis isra NRRL HD-522	+	-	-	-	+	Non-ribosomal peptide synthetase from *B. thuringiensis serovar israelensis str.* AM65-52	99
B. subtilis ATCC 21332	+	+	+	-	-	Surfactin and plipastatin synthetases genes from *B. subtilis* 168	98 ; 96
Bacillus subtilis subsp. subtilis str. 168	+	-	+	-	-	Surfactin and plipastatin synthetases genes from *B. subtilis* 168	100
B. sphaericus 23268T. (*Lysinibacillus sphaericus*)	+	-	-	+	-	Surfactin and mycosubtilin synthetases genes from *B. subtilis subsp. spizizenii str.* W23	93 ; 89

Primers pairs were first tested with *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499, *B. licheniformis* ATCC 14580, *B. pumilus*, *B. thuringiensis kurstaki* and *B. subtilis* 168, which are completely sequenced and are known to harbor NRPS synthetases genes involved in the biosynthesis of the four families of lipopeptides. Srf primers amplified fragments for surfactin, lichenisyn and pumilacidin in *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499, *B. licheniformis* ATCC 14580, *B. pumilus*, and *B. subtilis* 168 with similarity percentage of 99-100%, while Fen and Pps primers were amplified fragments for fengycin and or plipastatin family in *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499 and *B. subtilis* 168. Myc primers amplified fragments for iturin family in *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499. Primers pairs were tested with the unsequenced strains; *B. thuringiensis israelienne* NRRL HD-522, *B. subtilis* ATCC 21332 and (*Lysinibacillus*) *B. sphaericus* 23268T. Srf and Krs primers amplified fragments for kurstakin family which were 450 and 1152 bp of length as reported[19] and was showed a 99% similarity with kurstakin synthetase gene from *B. thuringiensis israelienne* AM65-52 on Blast alignment (Fig. 3). In *B. subtilis* ATCC 21332, Srf, Fen and Pps primers amplified three fragments of (431, 449 and 893 bp) length, similar to the surfactin and plipastatin synthetases genes of *B. subtilis* 168 (98 and 97%), respectively (Fig. 4). This was confirmed before[17] and this strain was characterised befor as surfactin and fengycin producer strain[9, 25, 26]. Srf and Fen primers amplified two fragments (416 and 425 bp) from (*Lysinibacillus*) *B. sphaericus*, which have a partial similarity with surfactin and mycosubtilin synthetases genes, respectively from *B. subtilis* subsp. *Spezizenii*. *Str* W23. This was confirmed before[17,19]. These fragments may due to unknown polyketide-non ribosomal peptide molecule encoding by four synthetases genes, which were detected by bioinformatics in *Bacillus (Lysinibacillus*) *sphaericus* genome sequence, these four synthetases genes two annotated as polyketide synthase WP_036221991.1, WP_036222373.1, one annotated as non ribosomal peptide synthase WP_036221997.1, and the last annotated as hypothetical protein WP_036222000.1, respectively (Fig. 5). It is interesting to note that the four genes are adjacent, spanning a locus of about 22718 nt. Fen primers was described before that are more degenerated than the other primers pairs, and this could explain their lower specificity compared to the others. Also, when these genes are not present, other genes containing adenylation and thiolation domains are detected as observed by primers from other species.

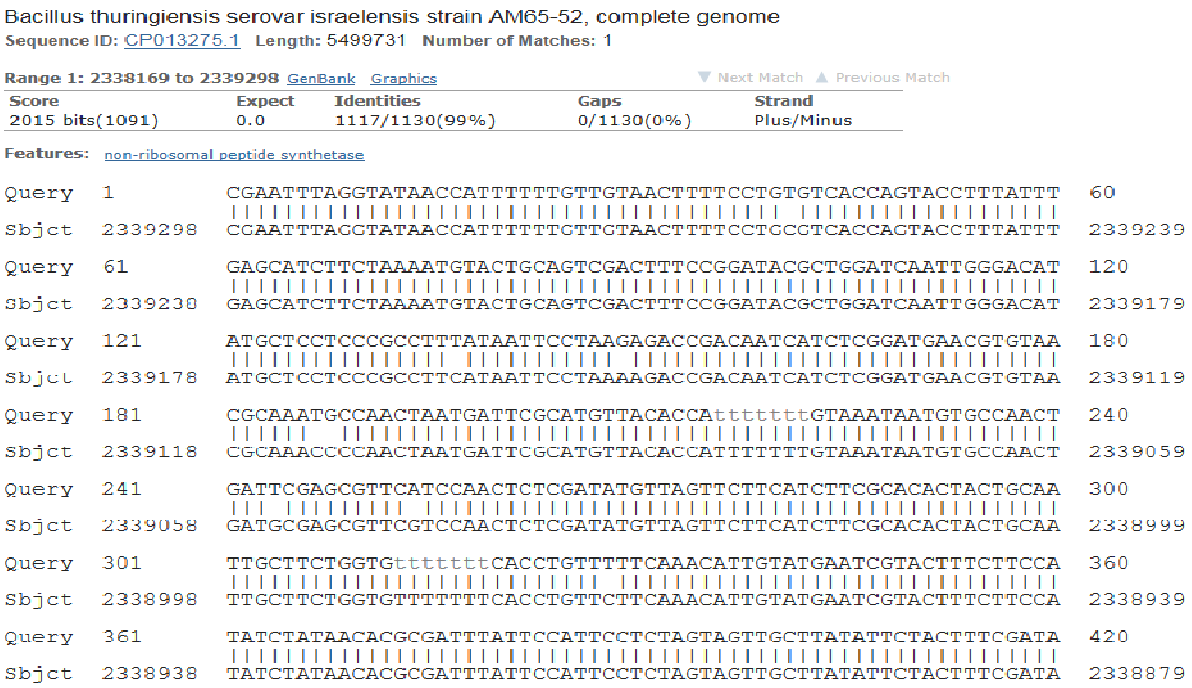

Figure 3
Kurstakin fragment BLAST alignment of B. thuringiensis isra. NRRL HD-522.

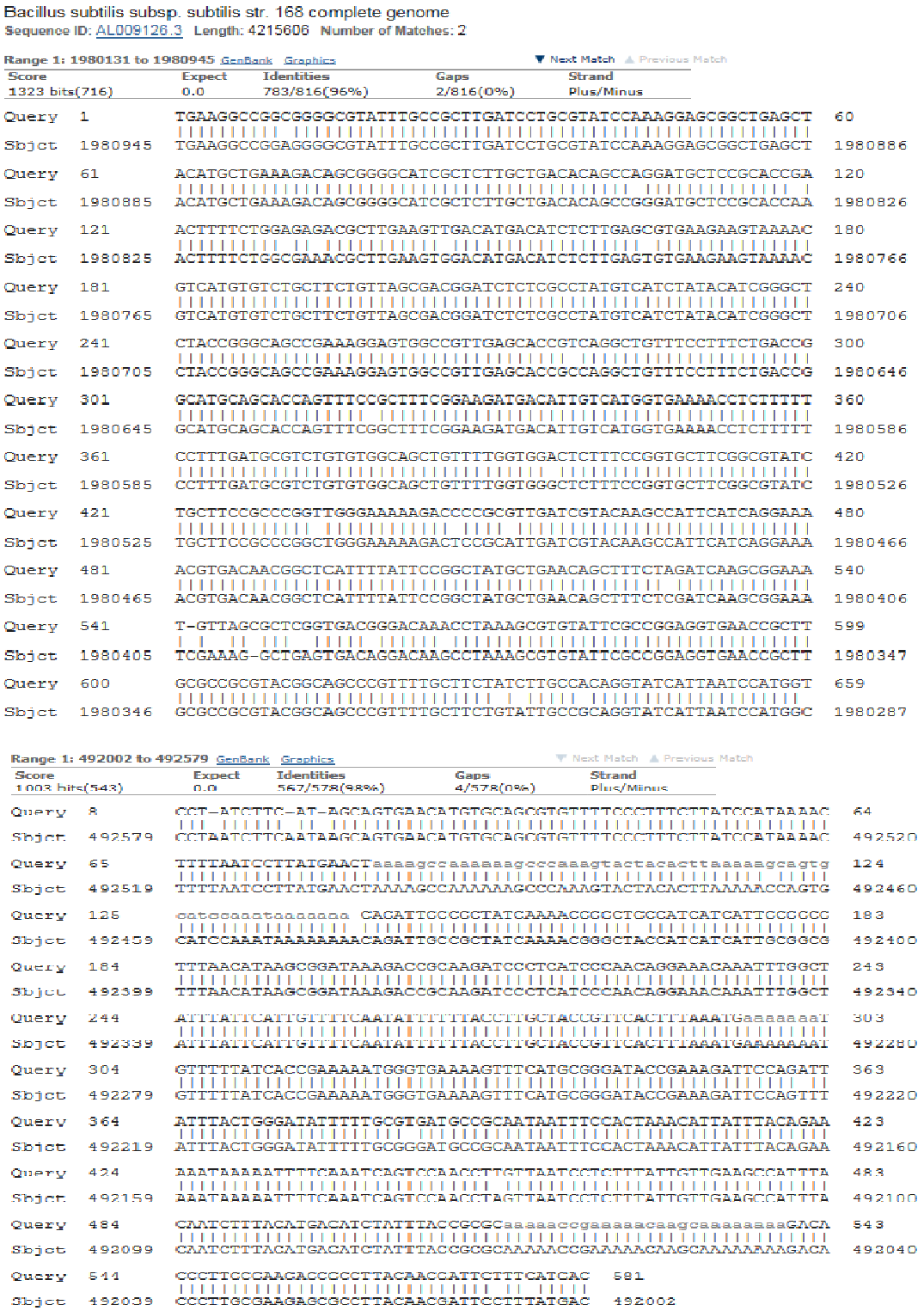

Figure 4

Plipastatin and Surfactin fragments BLAST alignment of B. subtilis 21332.

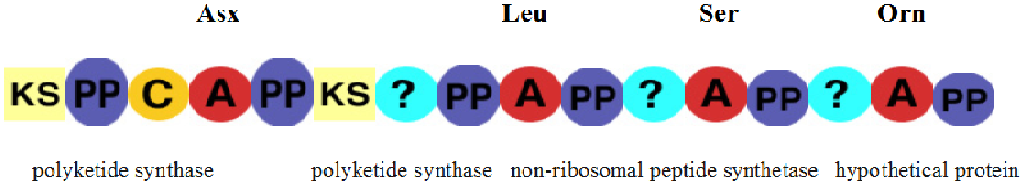

Figure 5

Unknown PKS-NRPS genes prediction from B. sphaericus (Lysinibacillus).

Lipopeptides production definition

The obtained results show clearly that, at the studied conditions, in all cases, strains produced much more surfactins types than iturin and fengycin and low amount of kurstakin. *B. amyloliquefaciens* strains produced three families of lipopeptides; surfactin, fengycin and iturin (mycosubtilin type). *B. subtilis* strains produced two families of lipopeptides surfactin and fengycin (plipastatin type) or no production. While, strains of *B. licheniformis* and *B. pumilus* produced only surfactin family with two types of lipopeptides (lichenysin and pumilacidin, respectively). The two strains belongs to *B. thuringiensis* were found to produce one lipopeptides family (kurstakin) as mentioned (table 7).

Table 7
***Bacillus* strains harbor lipopeptides synthetases genes with their production.**

Bacillus strains	Produced Lipopeptides families							
	Surfactin types	Production mg. L^{-1} ± SD	Fengycin types	Production mg. L^{-1} ± SD	Iturin types	Production mg. L^{-1} ± SD	Kurstakin types	Production mg. g^{-1} ± SD
B. thuringiensis kurstaki	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	Kurstakin	102 ± 28.12
B. thuringiensis isra. NRRL HD-S22	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	Kurstakin	62 ± 19.42
B. pumilus	Pumilacidin	642 ± 13.52	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
B. licheniformis ATCC 14580	Lichenysin	358 ± 17.09	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
B. subtilis str. 168	Surfactin	0.0	Plipastatin	0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
B. subtilis ATCC 21332	Surfactin	1060 ± 7.31	Plipastatin	226 ± 9.54	-	0.0 ± 0.0	-	0.0 ± 0.0
B. amyloliquefaciens S499	Surfactin	872 ± 12.23	Fengycin	103 ± 9.46	Bacillomycin	589 ± 14.17	-	0.0 ± 0.0
B. amyloliquefaciens FZB42	Surfactin	294 ± 8.03	Fengycin	62 ± 11.34	Mycosubtilin	210 ± 14.22	-	0.0 ± 0.0

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

The production of *B. amyloliquefaciens* FZB42 were 294 ± 8.03, 62 ± 11.34 and 210 ± 14.22 mg.L^{-1} surfactin, fengycin and mycosubtilin respectivelly. *B. amyloliquefaciens* S499 were 872 ± 12.23, 103 ± 9.46 and 103 ± 9.46 mg.L^{-1} surfactin, fengycin and bacillomycin respectivelly. *B. subtilis* ATCC 21332 were 1060 ± 7.31 and 226 ± 9.54 mg.L^{-1} of surfactin and plipastatin respectivelly. While, *B. subtilis* 168 has no production. The production of *B. licheniformis* ATCC 14580 strain was 358 ± 17.09 mg.L^{-1} of lichenysin, and *B. pumilus* was produced 642 ± 13.52 mg.L^{-1} of pumilacidin. Also, the strains of *B. thuringiensis kurstaki* and *B. thuringiensis israelienne* NRRL HD-522 were produce 102 ± 28.12 and 62 ± 19.42 mg.g^{-1} cells, respectivelly of kurstakin. The regulation of surfactin biosynthesis has been well described in the literature[27] but little information is available concerning fengycin iturin and kurstakin. Our results clearly show that the regulations of the four famlies of lipopeptides are different; molecular analyses should be performed in further studies to characterize the regulation mechanism of lipopeptides families biosynthesis.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Hathout Y, Ho YP, Ryzhov V, Demirev P, Fenselau C. Kurstakins: A New Class of Lipopeptides Isolated from *Bacillus thuringiensis*. J Nat Prod. 2000; 63:1492-1496
2. Arima K, Kakinuma A, Tamura G. Surfactin, A Crystalline Peptidelipid Surfactant Produced by *Bacillus subtilis*: Isolation, Characterization and its Inhibition of Fibrin clot formation. Biochem Biophys Res Commun. 1968; 31:488-494
3. Yakimov MM, Kroger A, Slepak TN, Giuliano L, Timmius KN, Golyshin PN. A putative Lichenysin A synthetase Operon in *Bacillus licheniformis*: initial characterization. Biochim Biophys Acta. 1998; 1399:141–153
4. Peypoux F, Bonmatin JM, Wallach J. Recent Trends in the Biochemistry of Surfactin. Appl Microbiol Biotech. 1999; 51:553–563
5. Wakayama S, Ishikawa F, Oishi K. Mycocerein, A Novel Antifungal Peptide Antibiotic Produced by *Bacillus cereus*. Antimicrob Agents Chemother. 1984; 26: 939-940
6. Volpon L, Besson F, Lancelin JM. NMR Structure of Antibiotics Plipastatins A and B from *Bacillus subtilis* inhibitors of phospholipase A2. FEBS Lett. 2000; 485: 76-80.
7. Kunst F et al. The Complete Genome Sequence of the Grampositive Model Organism *Bacillus subtilis* (strain 168). Nature. 1997; 390:249–256
8. Steller S, Vollenbroich D, Leenders F, Stein T, Conrad B, Hofemeister J, Jacques P, Thonart P, Vater J. Structural and Functional Organization of the Fengycin Synthetase Multienzyme System from *Bacillus subtilis* b213 and A1/3. Chem Biol. 1999; 6:31–41
9. Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J. The Mycosubtilin Synthetase of *Bacillus subtilis* ATCC 6633: A Multifunctional Hybrid between a Peptide Synthetase, An Amino Transferase, and A Fatty acid Synthase. Proc Natl Acad Sci USA. 1999; 96:13294-13299
10. Tsuge K, Akiyama T, Shoda M. Cloning, Sequencing and Characterization of Iturin A Operon. J Bacteriol. 2001; 183:6265-6273

11. Meena, KR and Kanwar, SS. Lipopeptides as the Antifungal and Antibacterial Agents: Applications in Food Safety and Therapeutics; BioMed Research International. 2014; 2015: 3-9
12. Ansari MZ, Yadav G, Gokhale RS, Mohanty D. NRPS-PKS: A knowledge-based Resource for Analysis of NRPS/PKS Megasynthases. Nucleic Acids Res. 2004; 32:405–413
13. Rausch C, Weber T, Kohlbacher O, Wohlleben W, Huson DH. Specificity Prediction of Adenylation Domains in Nonribosomal Peptide Synthetases (NRPS) using Transductive Support Vector Machines (TSVM). Nucl Acids Res. 2005; 33: 5799–5808
14. Bachmann BO, Ravel J. Methods for in Silico Prediction of Microbial Polyketide and Nonribosomal Peptide Biosynthetic Pathways from DNA Sequence Data. Methods Enzymol. 2009; 458:181–217
15. Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, Pauw E, Thonart P. Optimization of Biosurfactant Lipopeptide Production from *Bacillus subtilis* S499 by Plackett-Burman Design. Appl Biochem Biotechnol. 1999; 77:223–233
16. Ongena M, Jacques P, Toure Y, Destain J, Jabrane A, Thonart P. Involvement of Fengycin-Type Lipopeptides in the Multifaceted Biocontrol Potential of *Bacillus subtilis*. Appl Microbiol Biotech. 2005; 69:29–38
17. Tapi A, Imbert MC, Scherens B, Jacques P. New Approach for the Detection of Non-ribosomal Peptide Synthetase Genes in *Bacillus* Strains by Polymerase Chain Reaction. Appl Microbiol Biotechnol. 2010; 85:1521-1531
18. Ongena M, Jacques P. *Bacillus* Lipopeptides: Versatile Weapons for Plant Disease Biocontrol. Trends Microbiol. 2008; 16:115-125
19. Abderrahmani A, Tapi A, Nateche F, Chollet M, Leclère V, Wathelet B, Hacene H, Jacques P. Bioinformatics and Molecular Approaches to Detect NRPS Genes Involved in the Biosynthesis of Kurstakin from *Bacillus thuringiensis*. Appl Microbiol Biotechnol. 2011; 92:571-581
20. Hussein W, Fahim S. Modification of Wild Type *Bacillus subtilis* 168 Strain for Single Surfactin Production. Int J Cuur Micobiol Appl Sci. 2015; 4 (11): 177-184
21. Caboche S, Pupin M, Leclère V, Fontaine A, Jacques P, Kucherov G. NORINE: A Database of Nonribosomal Peptides. Nucleic Acids Res. 2008; 36:D326-D331
22. Hussein W, Awad H, Fahim S. Systemic Resistance Induction of Tomato Plants Against ToMV Virus by Surfactin Produced from *Bacillus subtilis* BMG02. Americ J Microbiol Res. 2016; (4)5: 153-158
23. Fahim S, Dimitrov K, Gancel F, Vauchel P, Jacques P, Nikov I. Impact of Energy Supply and Oxygen Transfer on Selective Lipopeptide Production by *Bacillus subtilis* BBG21. Biores technol. 2012; 126: 1-6
24. Fahim, S, Dimitrov K, Vauchel P, Gancel F, Delaplace G, Jacques P and Nikov I. Oxygen Transfer in Three Phase Inverse Fluidized Bed Bioreactor During Biosurfactant Production by *Bacillus subtilis*. Biochem Engineer J. 2013; 76: 70-76
25. Gancel F, Montastruc L, Liu T, Zhao L, Nikov I. Lipopeptide Overproduction by cell Immobilization on Iron-enriched Light Polymer Particles. Process Biochem. 2009; 44:975–978
26. Hussein W, Fahim S. Expression of *pps* and *fen* Promoters in *Bacillus subtilis* under Optimal Production Condition. Res J Pharma Biol Chem Sci. 2016; 7(2) : 1114-1121
27. Jacques P. Surfactin and other Lipopeptides from *Bacillus* spp. In Biosurfactants. Microbiology Monographs, Soberon-Chavez G, ed., Volume 20, Chapter 3, 2011; 57-91, Springe.

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