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Evaluation of Antifungal, Antibacterial and Anti-insecticidal Activities of Three *Bacillus* Strains Produced by Protoplast Fusion from *Bacillus thuringensis.*

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Abstract

In this work, the protoplast fusion technique was evaluated for its efficiency in transferring various biological products from parental strains to fusion produced ones. *Bacillus thuringiensis* was the principal parent in three different protoplast fusion processes. Three new bacterial strains were produced previously from protoplast fusion; B18 [*Bacillus thuringiensis (Bt)* x *Bacillus subtilis 168* (*Bs*1)], C80 [*Bacillus thuringiensis* (*Bt*) x *Bacillus lichiniformis* (*Bl*)] and D27 [*Bacillus thuringiensis* (*Bt*) x *Bacillus subtilis subsp. Spizizinii* (*Bs*2)]. In this work, we aim to detect the non-ribosomal peptides (NRPS) encoding genes as important secondary metabolites involved in the biocontrol of several pathogenic diseases in plants in addition to protease, and chitinase known for their broad range of industrial applications. It was observed from the results that both fusions B18 and D27 revealed the highest number of products (two NRPs; surfactin and fengycin, protease and chitinase and two NRPs; surfactin and kurstakin, protease and chitinase) followed by C80 (two NRPs; lichenysin and kurstakin and protease), respectively. The produced strains showed moderate antagonism activity against *Aspergillus aflatoxinformans* and against *Erwinia carotovora*. Anti-insecticidal activity for the three Bacillus produced strains was evaluated against *Agrotis ipsilon*, whereas B18, D27 and B18 revealed a considerable toxic effect at higher tested concentrations (10%, 5%, and 2.5%), causing 60%, 30% and 23% larvae mortality respectively. Also, D27 showed 56.67%, 26.67% and 20% larval mortality with the same concentrations, while C80 showed lower mortality at higher concentration (10%, 5%, and 2.5%), causing 40%, 20% and 16.67% larvae mortality.

Keywords: Antifungal, Chitinase, Lepidopterous, NRPS, Protease, Protoplast fusion**.**

1. Introduction

Lepidopterous is one of the most harmful pests in agriculture. It is known by the black cutworm *Agrotis ipsilon* (Hufnagel), (Lep., Noctuidae) which has a wide host range. This species can feed on nearly all vegetables, such as alfalfa, clover, cotton, rice, sorghum, strawberries, sugar beet, tobacco, and occasionally grains and grasses. It can also feed on almost any fruit. Black cutworm often has a clear predilection for weeds, and it will not attack crops until the weeds have been eaten. Adults use floral nectar for nutrition. Moths are particularly drawn to deciduous trees and shrubs, including linden, wild plum, crabapple, and lilac. Since most of its feeding occurs below soil level, the black cutworm is not thought of as a climbing cutworm. However, until roughly their fourth instar, larvae will feed aboveground. During their development, larvae can eat more than 400 sq cm of leaves, but more than 80% of this occurs during the terminal instar, and only about 10% occurs in the instar just before the last (Boughton *et al*., 2001). Chemical pesticides are the major method for controlling *A. ipsilon*. There are significant issues with using synthetic insecticides to control agricultural pests in field crops, such as pesticide residues and insect pest resistance. Therefore, the search for chemical-free crops using environmentally friendly pest control techniques is critically required. The protoplast fusion is considered one of the most important genetic engineering techniques, which proved its efficiency in transferring some important biological microbial products (Mohamed *et al*., 2021).

Bacillus thuringiensis is widely used as a pesticide to produce toxins of specific insecticidal activity kills insects by binding to and creating pores in the midgut membranes of insects. (Zhang *et al*., 2020). *Bacillus spp*. like (*B. thuringiensis*, *B. subtilis*, *B. licheniformis*, etc,.) produce diversity of lytic enzymes, such as chitinases, glucanases, lipases, proteases and various antibiotics (Bhagwat *et al*., 2019). Proteases are biological macro-molecules known as simple destructive enzymes because of their broad range of catalytic, analytic, and applications in industry Neurath and Walsh, (2011). There are different types of proteases,

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such as Alkaline serine protease (Yang *et al*., 2020) and metalloprotease (Zhang *et al*., 2023). Proteases target the defense molecules of the insect (Mukherjee and Vilcinskas, 2018). In addition to the promising mechanism of non-ribosomal peptide synthesis which considers a source of alternatives to chemical substances for different plant pathogens. This NRPS mechanism is responsible for producing a wide spectrum of NRPs using a multi-enzyme function system (synthetases) (Marahiel, 1997). Among these NRPs products are lipopeptide families produced by Bacillus strains, one of which is the surfactin that is considered an extraordinarily powerful biosurfactant due to its capability to decrease the water surface tension in addition to its role as a biological membrane detergent (Carrillo *et al*., 2003). It has a wonderful activity as antiviral, anti-mycoplasma, emulsifying and foaming (Peypoux *et al*., 1999). Surfactin has an amazing number of applications in different fields; biocontrol of plant diseases (Ongena and Jacques, 2008), medicine (Kowall *et al*., 1998), food preservation (Bie *et al*., 2005), cosmetics (Kanlayavattanakul *et al*., 2010), enhanced oil recovery (Schaller *et al*., 2004) and the bioremediation (Mulligan *et al*., 2001). Also, the iturin role was proven as antifungal against various plant pathogens, so it is widely used in plant diseases biocontrol (Leclere *et al*., 2005). Kurstakin is also one of the lipopeptide families (Jacques, 2011) which was discovered recently in 2000 from *Bacillus thuringinesis* and showed antifungal activity against *Stachybotrys charatum* (Hathout *et al*., 2000). Kurstakins are found accompanying bacterial cells, especially on spores, and the kurstakin production was evaluated to 15-20 µg.mg^{-1} (Hathout *et al*., 2000; Abderrahmani *et al*., 2011; Béchet *et al*., 2012). Generally, Bacillus spp. lipopeptides are well known for their role in plant-pathogens biocontrol (Ongena and Jacques, 2008; Jacques, 2011). Also, (Yu *et al*., 2023) confirmed that kurstakin contributes to the control of the plant-pathogenic fungi; *Rhizoctonia solani*, *Ascochyta citrulline*, *fusarium graminearum* and *F. oxysporum*. The fengycin family shows antifungal activity and has induction defense specific to certain pathogen systems or plant species; for example, fengycin does not induce defence system in grapevine (Farace *et al*., 2015; Li *et al*., 2019; Deleu *et al*., 2008) but induces defense system in rice against *Rhizoctonia solani* (Chandler *et al*., 2015). This research aims to evaluate some biological products from three Bacillus strains produced by protoplast fusion to be used as alternatives to chemical substances in bicontrol.

2. Materials and methods

2.1. Bacterial strains

Four wild type parental strains and three fusions were used in this study; *Bacillus thurinigensis*, *Bacillus subtilis* 168, *Bacillus lichineformis* and *Bacillus subtilis* subsp. *spizizinii* (parental strains) and B18, C80, and D27 as fusions (Mohamed *et al*., 2016 and 2023) (Table 1).

Table 1. Parental strains and protoplast fusion produced strains

Parental strains	Produced strains by protoplast fusion	Reference
B. thurinigensis :: *B. subtilis* 168	B18	Mohamed *et al*., 2016 and 2023
B. thurinigensis :: *B. lichiniformis*	C80	
B. thurinigensis :: *B. subtilis* subsp. *spizizinii*	D27	

2.2. Bacterial culture

A single colony of each bacterium was cultured in 20 ml of LB in a 100 ml conical flask (Pyrex, United States of America) by shaking in an orbital shaker (Thermo Fisher Scientific, United States of America) at 120 rpm for 18 h at 30°C.

2.3. DNA isolation and PCR conditions

DNAs were prepared using GeneJET Genomic DNA Purification Kit (Thermo scientific, USA). Degenerate primers used in this study were previously designed according to (Tapi *et al*., 2010; Abdelrahmani *et al*., 2012) and all used primers are listed in Table (2). NRPS degenerate primers were designed by the alignment of conserved motifs of the nucleic acid sequences identified in the adenylation domain (A) and the thiolation domain (T). PCR conditions were performed by initial step of denaturation at 94 °C for 3 min, followed by 35 cycles of three steps; denaturation at 94 °C for 30 sec, annealing step at 43 °C, 44.4 °C and at 58 °C with surfactins, kurstakins and plipastatins, respectively. There was an extension step at 72 °C for 2 min with plipastatins and kurstakins except with surfactins for 45 seconds, in addition to the final extension step at 72 °C for 5 min. Protease detection PCR conditions are 95°C for 3 min; 35 cycles of 95°C for 30sec, 50°C for 30 sec, and 72°C for 1 min and a final extension of 72°C for 10 min. Detection of chitinase gene PCR conditions; 94°C for 3 min; 35 cycles of 94°C for 30sec, 48°C for 30 sec, and 72°C for 1 min and a final extension of 72°C for 10 min. PCR products were separated on a 1.2% agarose gel compared to Thermo Scientific Gene Ruler 100 bp DNA Ladder and photographed under Gel Doc™ XR+ Gel Documentation System.

Table 2. Degenerate primers used for non-ribosomal lipopeptides, protease and chitinase genes detection from fusion strains

Name	Sequence	Expected fragment size (bp)	NRLPs identified	References
AP1-F	AGMCAGCKSGCMASATCMCC	959, 929, 893	Plipastatin	Tapi *et al*., 2010
TP1-R	GCKATWWTGAARRCCGGCGG			
AS1-F	CGCGGMTACCGVATYGAGC	419, 422, 424, 431	Surfactin	Tapi *et al*., 2010
TS1-R	ATBCCTTTBTWDGAATGTCCGCC			
AKs-F	TCHACWGGRAATCCAAAGGG	1125, 1152, 1161, 1167, 1173	Kurstakin	Abderrahmani *et al*., 2011
TKs-R	CCACCDKTCAAAKAARKWATC			
Bspro-F	ATGGTGGATTACGAACGTG	1203 bp	Bacillus Protease	This study
Bspro-R	TTAACTGCCTAATTGGTCTG			
Bs ch-F	GAATTCATGCGCAAATTTAATAAACCGCT	1100 bp	chitinase	Berini *et al*., 2018
Bs ch-R	AAGCTTTTATTGAACGCCGGCGCT			

2.4. Fungi preparation

For the two fungi (*Aspergillus aflatoxiforman* and *Aspergillus flavus*), PDA liquid media was used and incubated 7 days at 30 ºC. The fungi were spread on PDA solid media using swab, and the discs supplemented with fusion bacteria were put on the petri dishes using three replicates. All plates were incubated for 3 days at 30 ºC.

2.5. Insect rearing

Agrotis ipsilon (Hufnagel) (Insecta: Lepidoptera: Noctuidae) was raised in a lab without the use of any insecticides for multiple generations. Hatched larvae are put in plastic jars and allowed to feed on the leaves of the caster bean plant *Ricinus communis* L. The newly emerging adult moths were moved to oviposition jars that included cotton tufts that had been wet with honey solution for the moths to feed on.

2.6. Bioassay

To evaluate the efficacy of the three bacterial strains (B18, C80 and D27), they were generated at various concentrations (10, 5, 2.5, 1.25 %) by dilution with water. The third larval instar of *A. ipsilon* was used to examine the bacterial strains' ability to eliminate insects. Using a dipping technique, the toxic effects of the tested botanicals were investigated. Caster bean plant leaf discs measuring 10 cm in diameter were dipped in various concentrations for two minutes, allowed to dry at room temperature, and then provided to selected *A. ipsilon* larvae. The experiment was carried out in 10 replicates, each containing three larvae. Every day, the number of alive and dead larvae was counted after 25 days of feeding.

2.7. Statistical analysis

The LC50 values were determined by SPSS software using (Finney, 1971) Probity analysis approach. Duncan's test and one-way ANOVA were employed to analyze the statistical variance between groups.

3. Results

3.1. Detection of NRPs genes involved in the sequenced genome of Bacillus parental strains available on GenBank

Bacillus parental strain genomes were analyzed by Anti-smash version 7.0 which allows rapid detection and analysis of biosynthesis gene clusters responsible for secondary metabolite production of bacterial and fungal genomes. *Bacillus subtilis* 168 genome analysis revealed the presence of three non-ribosomal lipopeptide gene clusters for surfactin, fengycin and bacillibactin and one polyketide-NRPs hybrid bacillaene. Other secondary products have been detected in the *Bs* 168 genome of different types: sporulation killing factor, sublancin, pulchemiminic acid, subtilosin and bacilycin (Table 3). This result agrees with (Kunst *et al*., 1997; Barbe *et al*., 2009). *Bacillus subtilis* subsp spezizinii genome analysis showed the presence of three non-ribosomal lipopeptide gene clusters for surfactin, mycosubtilin and bacillibactin and one polyketide-NRPs hybrid bacillaene. Other secondary products have been detected for different types: subtilin, subtilosin and rhizocticin (Table 3) according to (Fan *et al*., 2011). By AntiSmash, two NRPs lipopeptide gene clusters were detected in *B. thurinigensis* genome, kurstakin and bacillibactin as reported by (Abderrahmani *et al*., 2011; Béchet *et al*., 2012).

Table 3. AntiSmash results for detecting NRPs clusters involved into genomes of Bacillus parental strains

Strain	Region	Type	From	To	Most similar known cluster	Similarity
***B. subtilis* str. 168 AL009126.3**	Region 2	NRPS	358,303	421,744	surfactin	82 %
	Region 4	Polyketide+NRP	1,763,763	1,869,009	bacillaene	100 %
	Region 5	NRPS, betalactone	1,940,625	2,017,738	fengycin	100 %
	Region 9	NRP-metallophore	3,260,519	3,312,296	bacillibactin	100 %
B. licheniformis CP000002.3	Region 2	NRPS	359,133	424,266	Lichenysin	100 %
	Region 9	NRP-metallophore	3,698,288	3,750,032	Bacillibactin	100 %
***B. spizizenii* str. W23 CP002183.1**	Region 2	NRPS	345,342	408,676	surfactin	86 %
	Region 4	TransAT-PKS NRPS	1,721,226	1,826,452	Bacillaene	100 %
	Region 5	NRPS- betalactone-TransAT	1,893,143	1,969,153	Mycosubtilin	100 %
	Region 8	NRP-metallophore,	3,043,946	3,096,063	Bacillibactin	100 %
***B. thuringiensis* BMB171 CP001903.1**	Region 3	NRP-metallophore,	2,184,326	2,236,074	Bacillibactin	100 %
	Region 5	NRPS	2,326,120	2,392,028		
	Region 6	NRPS- betalactone	2,409,777	2,435,015	Kurstakin	100 %

3.2. NRPs synthetase genes detection in Bacillus parental and fusion strains by degenerated primers

The detection of NRPs genes responsible for lipopeptides biosynthesis in the previously sequenced genome strains available on GenBank was expected. Kurstakin biosynthesis was detected in *B. thuringiensis* strain with an expected fragment size of 1167 bp, while plipastatin and surfactin biosynthesis genes were identified in *B. subtilis* 168 with expected fragment size of 958 and 424 bp respectively. The detection of lichenysin synthetase gene by both plipastatin and surfactin primers in *B. licheniformis* agrees with (Tapi *et al*., 2010; Abderrahmani *et al*., 2011) who established the kurstakin synthetase genes detection in *B. thuringiensis* using kurstakin AKs-F/TKs-R primers and the efficiency of using surfactin degenerate As1-F/Ts2-R primers to detect surfactin synthetase genes in *Bacillus subtilis*. The strain *B. subtilis* subsp. Spizizinii amplified three fragments; 424 bp, 350 bp and 300 bp respectively with surfactin primers, whereas the fragment of 424 bp belongs to surfactin synthetase gene (Figure 1).

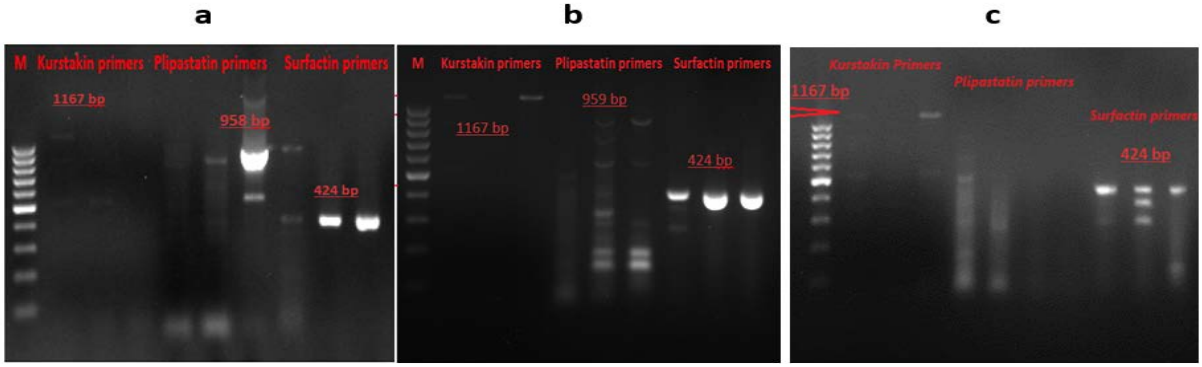

Figure 1. Degenerate primers amplification for; a. *B. thuringiensis*, *B. subtilis* 168, and B18; b. *B. thuringiensis*, *B. lichineformis*, and C80; c. *B. thuringiensis*, *B. subtilis subsp. spizizinii*, and D27 with kurstakin, plipastatin and surfactin primers respectively

The degenerate primers allowed us to detect the presence of both plipastatin and surfactin synthetase genes in B18 fusion, compared to their parental strains *B. thuringiensis* a harbouring kurstakin synthetase genes and *B. subtilis* 168 the surfactin and plipastatin harbouring synthetase genes (Figure 1. a). The fusion C80 was found to harbour the two synthetase genes of kurstakin and lichenycin (surfactin family), which have been transferred from both parental strains *B. thuringiensis* (kurstakin synthetase genes) and *B. licheniformis* (Lichenysin synthetase genes) (Figure 1. b). The fusion D27 was found to harbor kurstakin and surfactin synthetase genes which were transferred from their both parental strains *B. thuringiensis* (kurstakin synthetases genes) and *B. subtilis* subsp. Spizizinii (surfactin synthetases genes) (Figure 1c).

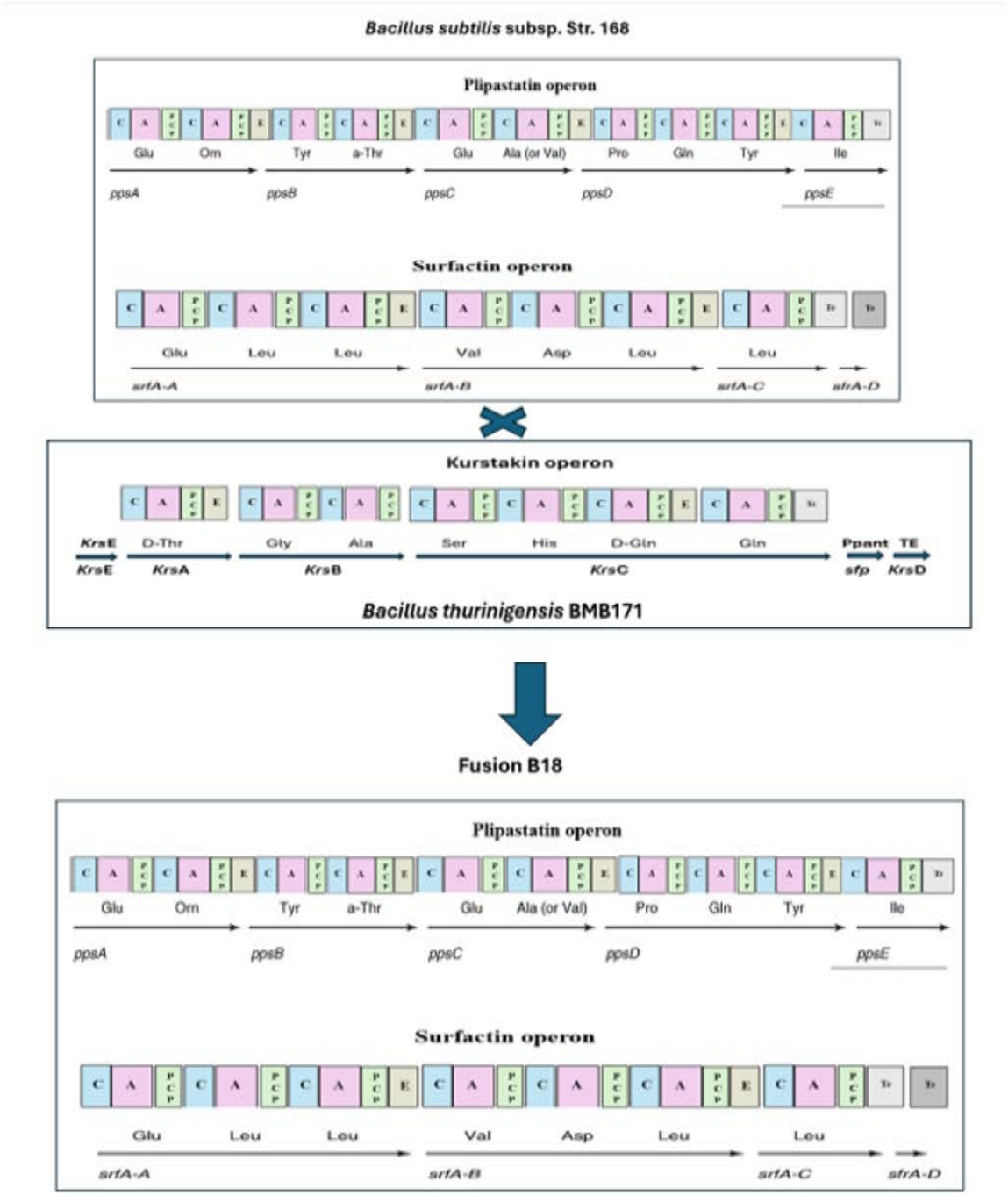

Figure 2. Schematic dendogram for NRPS synthetase clusters transferred by protoplast fusion from parental strains *B. thuringiensis* and *B. subtilis* 168 to fusion strain B18

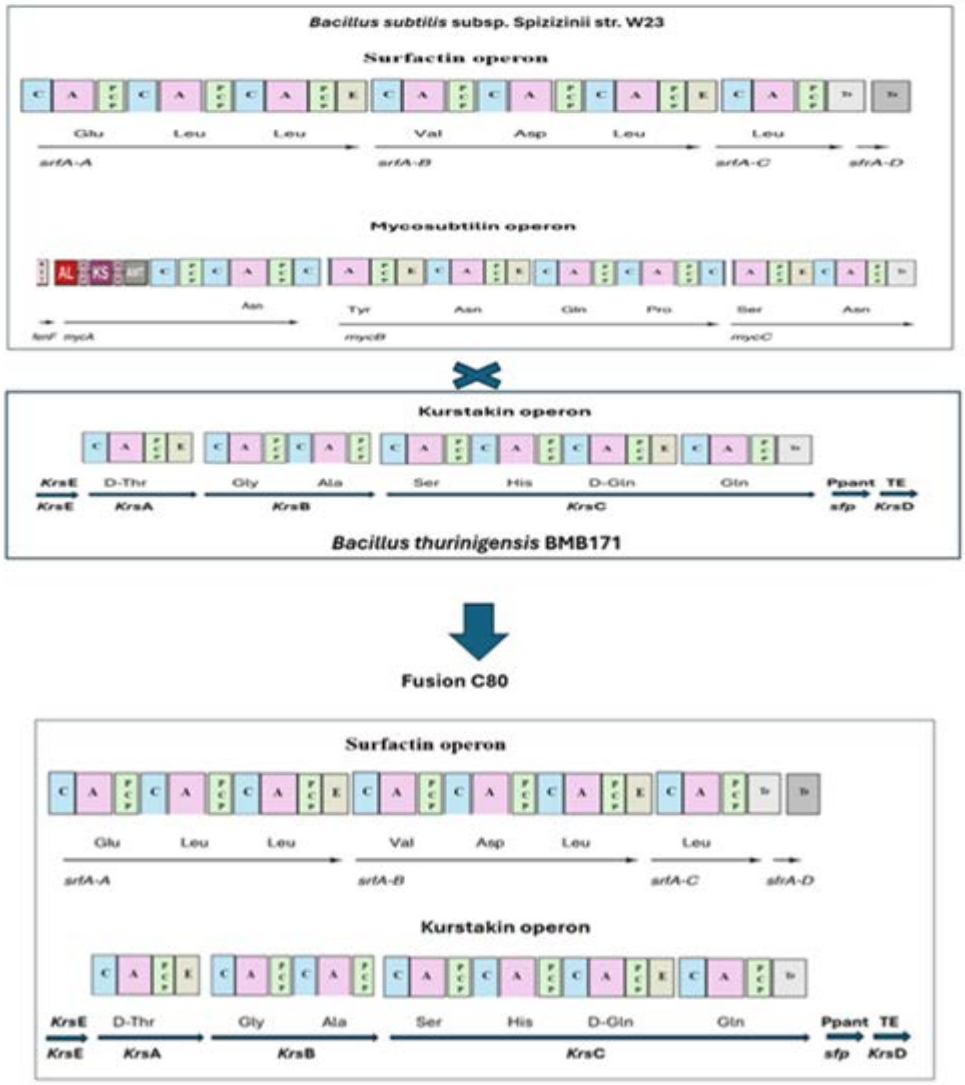

Figure 3. Schematic dendogram for NRPS synthetase clusters transferred by protoplast fusion from parental strains *B. thuringiensis* and *B. subtilis* spizizinii to fusion strain C80

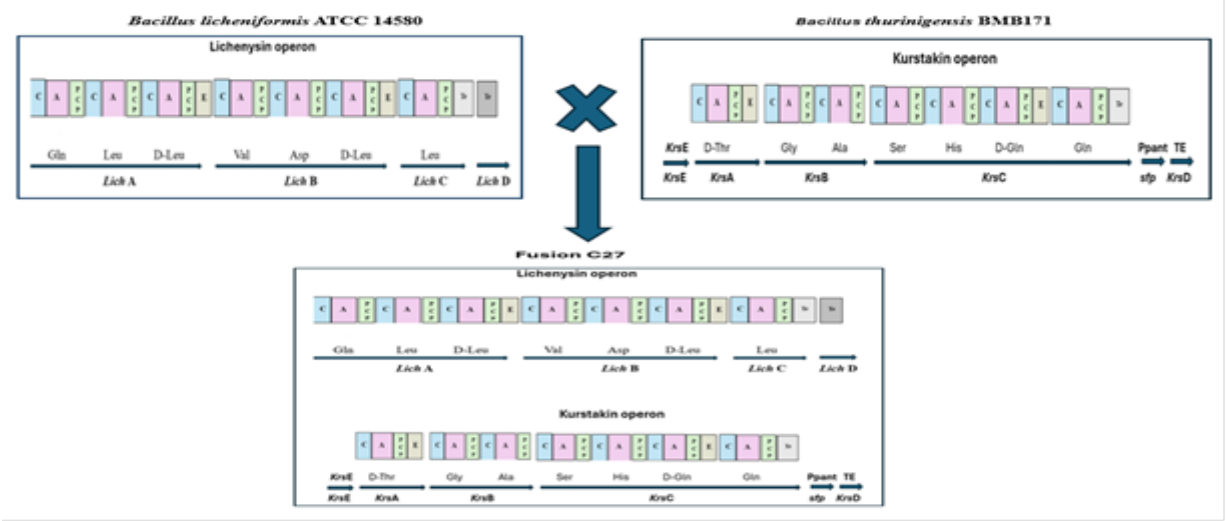

Figure 4. Schematic dendogram for NRPS synthetase clusters transferred by protoplast fusion from parental strains *B. thuringiensis* and *B. licheniformis* to fusion strain D27

3.3. PCR detection of protease gene in the modified Bacillus strains

Detection of protease gene was conducted in the parental strains *B. thuringiensis* (*Bt*), *B. subtilis* 168 (*Bs*1), *B. subtilis* subsp. Spizizinii (*Bs*2), and *B. licheniformis* (*Bl*) compared to the fusion B18 (Bt::Bs1), C80 (Bt::Bl) and D27 (Bt::Bs2). PCR has been implemented to detect the absence or presence of the protease gene in the three fusion strains and a fragment of 1203 bp of expected size was amplified. The protease gene was detected in the three fusion produced strains; B18 and parent 1(Bt::Bs1), C80 (Bt::Bl) and D27 (Bt::Bs2) (figure 5a).

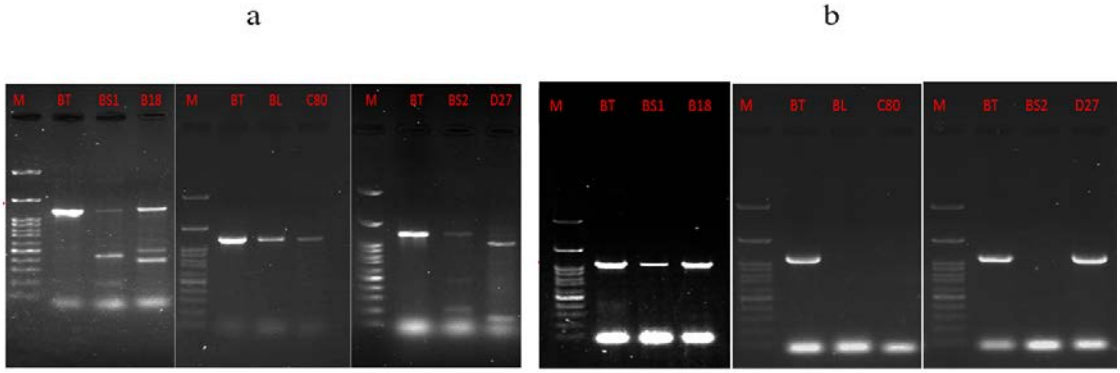

Figure 5. a. Protease; b. chitinase primers amplification for *B. thuringiensis*, *B. subtilis* 168, and B18, *B. thuringiensis*, *B. lichineformis*, and C80, *B. thuringiensis*, *B. subtilis* subsp. spizizinii, and D27.

3.4. PCR detection of chitinase gene in the modified Bacillus strains

Detection of chitinase gene in fusion strains was carried out by PCR and a fragment of 1100 bp was amplified. The chitinase gene appeared in the produced fusion strain B18 and their parents (Bt::Bs1), while it disappeared in the fusion strain C80 (Bt::Bl) and one of their parental strains (*Bl*) and appeared in the other parent Bt. Finally, chitinase gene was detected in the fusion strain D27 (Bt::Bs2) and one of their parental strains (*Bt*), while it was absent in the other parent (*Bs*) (Figure 5b).

Table 4. NRPs genes, protease and chitinase detected by PCR in Bacillus parental and fusion strains

Parental strains and fusions	***B.subtilis* 168**	***B. lichenyformis***	**B. *subtilis* subsp. *spizizinii***	**B. thuringensis**	***B.subtilis* 168+ *B. thuringensis* B18**	***B. lichenyformis* + *B. thuringensis* C80**	***B.subtilis* Spizizini+ *B. thuringensis* D27**
Surfactin	Surfactin		Surfactin		Surfactin		Surfactin
Fengycin or plipastatin	Fengycin or plipastatin				Fengycin or plipastatin		
Lichenysin		Lichenysin				Lichenysin	
Kurstakin				Kurstakin		Kurstakin	Kurstakin
Mycosubtilin			Mycosubtilin				
Protease	Protease	Protease	Protease	Protease	Protease	Protease	Protease
Chitinase	Chitinase			Chitinase	Chitinase	Chitinase	Chitinase
	4	2	3	3	4	4	4

All detected NRPs products, protease and chitinase are summarized in Table 4 for the parental and fusion produced strains. It was observed that the three fusions B18 (surfactin, fengycin, protease and chitinase), D27 (surfactin, kurstakin, protease and chitinase) and C80 (lichenysin, kurstakin and protease) revealed the same number of products.

3.5. Antifungal activity of parental and fusion produced strain against Aspergillus aflatoxinforman and Aspergillus flavus

The three fusion bacteria showed moderate antagonism activity against *A. aflatoxinforman*, the strains E (B18 and D27) and G (B18, C80 and D27) (++) followed by C (D27), and F (C80 and D27) (+), while A (B18), B (C80) and D (B18 and C80) (-) compared to control (Figure 6A).

These results may refer to a synergistic relationship between the strains (B18 and D27) and between (B18, C80 and D27) and also refer to the effective role of the strain D27 compared to the other strains. On the other hand, no anti-fungal activity was detected whether with the parental strains or the fusion produced strains against *Aspergillus flavus* (Figure 6B). The two types of fungi secrete fungal toxins (aflatoxin) cause great harm to humans. Therefore, we resort to finding a solution for combating these fungi with beneficial bacteria capable of producing various non-ribosomal peptides (NRPS) that have antifungal activity, such as (fengycin or plipastatin).

3.6. Antibacterial Activity

The three bacterial fusions (B18, C80 and D27) have revealed anti-bacterial activity against *Erwinia carotovora* whereas the highest activity was shown with A, (B18) B (C80), D (B18 and C80) and E (B18 and D27) (+++) followed by F (C80 and D27) and G (B18, C80 and D27) (++), while C (D27) (+) compared with control as shown in figure (6C).

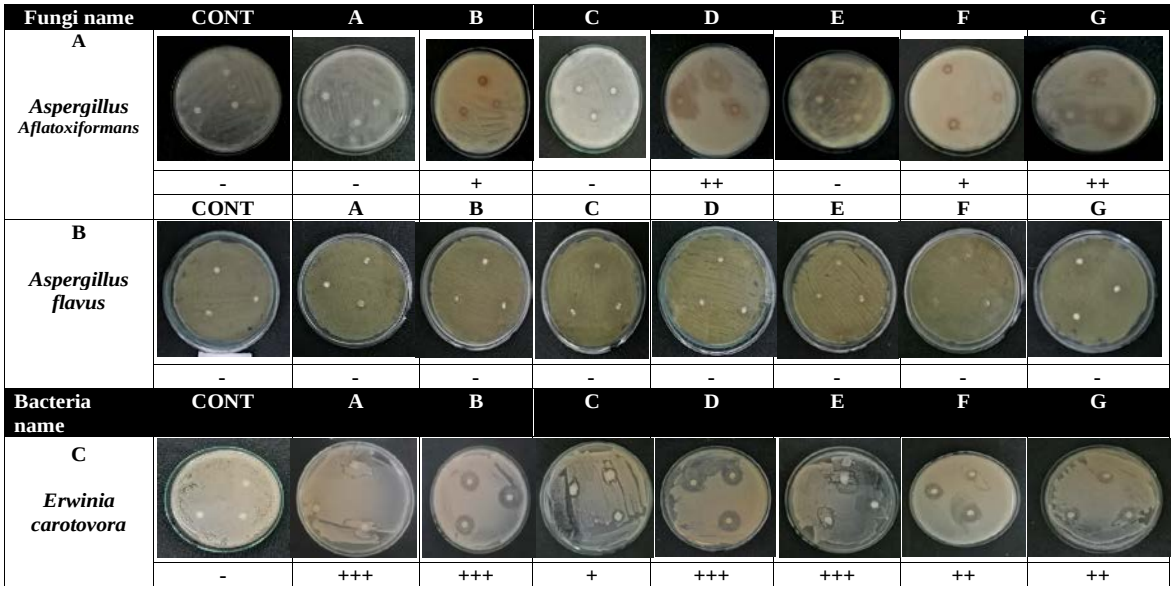

Figure 6. Antifungal activity of the three bacterial fusions against; a. *Aspergillus Aflatoxiformans* and *b*. *Aspergillus flavus* and c. Antibacterial activity against *Erwinia carotovora*, A= (B18), B=(C80), C=(D27), D= (B18+C80), E= (B18, D27), F= (C80+D27), G= (B18, C80, D27)

3.7. Anti-insecticidal activity of the three Bacillus producing strains

The data in Table 5 demonstrated the three tested bacterial strains have harmful effects on *A. ipsilon* third larval instar. B18 revealed a considerable toxic effect at higher tested concentrations (10%, 5%, and 2.5%), causing 60%, 30% and 23% larvae mortality respectively, although D27 showed 56.67%, 26.67% and 20% larval mortality with the same amount of concentration, C80 showed lower mortality at higher concentrations (10%, 5%, and 2.5%), causing 40%, 20% and 16.67% larvae mortality.

Table 5. Mortality (mean ± SE) of *A. ipsilon* 3rd larval instar after 25 days of treatment with three bacterial strains

Concentrations	B 18				C 80				D27			
10.00%	60.00	±	10.00	a	40.00	±	5.77	a	56.67	±	3.33	a
5.00%	30.00	±	5.77	b	20.00	±	5.77	b	26.67	±	3.33	b
2.50%	23.33	±	3.33	b	16.67	±	6.67	b	20.00	±	5.77	b
1.25%	6.67	±	3.33	c	3.33	±	3.33	b	6.67	±	3.33	c
control	0.00	±	0.00	c	3.33	±	3.33	b	0.00	±	0.00	c
F	22.10				8.54				36.50			
Sig.	0.00				0.00				0.00			

4. Discussion

In this work, we exhibited the effectiveness of using the degenerated primers in detecting NRPs synthetase genes, which allowed us to get a near view into bacterial genomes side by side with bioinformatics tools, such as AntiSmash and PKS-NRPS analysis websites. The degenerated primers utilization for NRPs genes detection from un-sequenced genomes became widely applied in several studies. (Marahiel, *et al*., 1997) designed primers based on the highly conserved motif A2 (KAGGAY) LV P for peptide synthetases. Another degenerate primer was designed by (Neilan *et al*., 1999; Vizcaino *et al*., 2005)

based on the adenylation domain conserved motif from various fungi and bacteria. Recently, the designation of these degenerated primers became more specific depending on bacteria genera. *Bacillus* degenerate primers for their NRPs encoding genes were designed by the alignment of the conserved nucleic acids sequence of both the adenylation and the thiolation domains (Tapi *et al*., 2010; Chen *et al*., 2006). In this work, *Bacillus* degenerate primers (Tapi *et al*., 2010; Chen *et al*., 2006) proved their effectiveness in the detection of NRLPs clusters of surfactin, (As1-F/Ts2-R primers), plipastatin (Ap1-R/Tp1-R primers) in fusion B18, lichenysin (As1-F/Ts2-R primers) and kurstakin (Aks-F/ Tks-R) from C80, surfactin (As1-F/Ts2-R primers) and kurstakin (Aks-F/ Tks-R) from strain D27. These results agree with (Tapi *et al*., 2010) who confirmed the amplification of fragments of 99% similarity with surfactin As1-F/Ts2-R primers from *B. subtilis* 168 and lichenysin from *B. licheniformis* ATCC 14580 respectively. He also confirmed the amplification of fragment with plipastatin (Ap1-R/Tp1-R) primers from *B. subtilis* 168 similarly to plipastatin (99%). (Tapi *et al*., 2010) also indicated that the Af2-F/ Tf1-R primers amplified a fragment similarly to bacillaene synthase of *B. amyloliquefaciens* FZB42 from *B. subtilis* ATCC6633, and of *B. subtilis* 168 (Chen *et al*., 2009; Al-shebly, 2022). Insect defense compounds are the focus of proteases, which break them down (Mukherjee and Vilcinskas 2018). Moreover, *B. thuringiensis* protoxin is implicated in the activation of these protoxins into dangerous forms; in this active state, the toxins bind to receptors on the intestinal epithelium in several insect groups as a result of the creation of oligomeric pores in the gut cell membrane of the larvae. Furthermore, the chitinase gene improved the behavior of the *B. thuringiensis* strain (Bravo *et al*., 2017) However, in response to *R. Speratus*, *Bacillus licheniformis* secretes hydrolytic enzymes called protease and chitinase, which break down the cuticle layers of insect pests' exoskeletons of subterranean worker termites (Moon *et al*., 2023). Notably, repurposing chitinase to restrict or eliminate pests can prevent soil contamination that could harm the ecosystem. As an example, Rostami et al. demonstrated chitinase on the spore as a possible biopesticide by fusing it with the B. subtilis spore coat protein CotG (Hosseini *et al*., 2016; Rostami *et al*., 2017). Protease used in plant defense against herbivory offers particular promise for the production of insect-resistant transgenic plants in the future. In the era of genomes and transcriptomics, a more efficient knowledge of the biology of virulence factors may facilitate the discovery of prospective proteases for application in pest control (Negi *et al*., 2023). It was discovered that the BG strains carry the genes for the ChiA and ChiB belonging to the GH18 (glycoside hydrolase) family and exert endochitinase activity (Drewnowska *et al*., 2020). Chitinases play a role in the pathophysiology of *B. thuringiensis* in insects since they break down the peritrophic membrane, which is composed of chitin and protects the insect gut from hazardous substances and toxins. In addition, chitinases are used to strengthen the defenses of genetically modified crops against pests (Berini *et al*., 2018). Therefore, the presence of genes encoding chitinolytic enzymes in BG strains increases insecticidal activity. On the other hand, Zhang *et al*., 2019, found that 558 midgut genes and 65 midgut genes are differently expressed in Vip3Aa11-M-A and Vip3Aa39-M-A respectively. *Agrotis ipsilon* midgut BBMV can be competitively bound by the Vip3Aa protein due to its trypsin sensitivity and binding specificity. However, Yan *et al*. in 2020 evaluated the effectiveness of three (C010, C009 and C008) GM maize juveniles expressing Vip3Aa19 toxins against BCW and evaluated the susceptibility of BCW neonates to 11Bt toxins, namely, Cry1Ab, Cry1Ac, Cry1Ah, Cry1F, Cry1Ie, Cry1B, Cry2Aa, Vip3_ch1, Vip3_ch4, Vip3Ca2, and Vip3Aa19. Vip3Aa19 was the most active protein against BCW (LC50 = 0.43 µg/g) according to the bioassay data of toxin diet. Less toxic chimeric proteins were Cry1F (LC50 = 83.62 µg/g), Vip3_ch1 (LC50 = 5.53 µg/g), and Cry1Ac (LC50 = 184.77 µg/g). The biological activity of six various heterologous pesticidal proteins—Cry1Aa, Cry1Ca, Cry1Ia, Cry2Ab, Cry9Ea, and Vip3Aa produced by *B. thuringiensis* have been evaluated against *A. exclamationi* histopathologically (Baranek *et al*., 2023b). Among the examined pest species, only Cry9Ea and Vip3Aa exhibited considerable mortality, with LC50 values of 950 and 140 ng/cm2 respectively. It was established how Cry9Ea and Vip3Aa affected *A. exclamationis* histopathologically. However, most currently used *B. thuringiensis*-based biocontrol agents (including the commercial strains tested in this work) primarily contain Cry1- and Cry2-type toxins as their active molecules. These toxins only cause varying degrees of growth inhibition in the target insect, not death. (Baranek *et al*., 2023a) demonstrated the presence of genetic determinants encoding the chitinolytic enzymes ChiA and ChiB in the examined entomopathogens. The examined strains exhibit insecticidal activity against two different, economically significant pest insects: *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) and *Cydia pomonella* L. (Lepidoptera: Tortricidae). When it comes to both pests, however, the BG12 and BG15 strains are noticeably more active than the BG11 strain. The strains BG12 and BG15 have the potential to be utilized in the development of novel lepidopteran-active bioinsecticides that will enhance current biocontrol approaches.

5. Conclusion

The protoplast fusion is considered one of the most important genetic engineering techniques, which proved its efficiency in transferring some important biological microbial products. In this study, we checked the transferring of various secondary metabolites, such as protease chitinase and NRPS, using bioinformatics and PCR techniques. The newly produced fusions proved their antifungal, antibacterial and anti-insecticidal activities.

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