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# Production of levansucrase from novel honey *Bacillus subtilis* isolates capable of producing antiviral levans

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ABSTRACT

Six *Bacillus* strains were isolated from different honey sources as levansucrase producers. The isolates were identified as *Bacillus* sp. based on both phenotypic and molecular identification using sequencing of 16S rRNA gene method. To distinguish them on species level other genes such as *rec*A and *ytc*P genes have been employed. The molecular identification methods revealed that the six strains K, M, A, C, E, and G belong to unique *Bacillus subtilis*. Levan production by the isolates was dependent mainly on commercial sucrose media, the use of beet molasses glucose, lactose and fructose led to noticeable reduction in levan synthesis. Chemical hydrolysis of levan products showed that the polysaccharide consisted entirely of fructose. Levan produced by isolates K, M, A, C, E, and G had different molecular masses (40.938, 71.887, 43.487, 154.638, 77.753 and 14.200 kDa, respectively). The levan yield using sucrose medium for isolates M, K, A, C, E and G were 11.00, 16.25, 6.60, 1.81, 1.74, and 6.6 g/L, respectively. The biological activity of levan produced by strains K, M and E had antiviral activity against pathogenic avian influenza HPAI, H5N1 while levan produced by strains A and M had antiviral activity against adenovirus type 40.


## 1. Introduction

Most of the researches in the honey field focused on its antimicrobial, antioxidant and anticancer activities, also the identification of the dormant endospore inside it (Sabaté, Carrillo, & Audisio, 2009). None till now paid attention to the enzymatic products of these dormant endospores. Osmophilic microorganisms survive environmental extremes of desiccation, pressure and acidity, it is expected that their biopolymers will also have some unique properties to adapt to such extreme conditions. The genus *Bacillus* sp. is ubiquitous, endospore-forming Gram-positive bacteria, their primary habitat is the soil. According to their resistant spores, they can colonize different environments such as honey, food, animals, insects, etc. (Sanders, Morelli, & Tompkins, 2003; Urdaci, Bressollier & Pinchuk, 2004). Extracellular polysaccharides produced by these species offer a variety of useful and potentially low-cost bioactive products. Levans are polyfructans linked by B-(2–6) linkages, and found in many plants and microbial products. Despite their widespread occurrence, they are little known and poorly understood (Pontis & Del Campillo, 1985).

*Bacillus subtilis*, known as the hay bacillus or grass bacillus, is a Gram-positive, catalase positive bacterium commonly found in soil (Madigan & Martinko, 2005). A member of the genus *Bacillus*, *B. subtilis* is rod-shaped and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. It may contaminate food but rarely causes food poisoning. *B. subtilis* produces the proteolytic enzyme subtilisin. Those spores can survive the extreme heat during cooking. *B. subtilis* is responsible for causing ropiness, a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough (Ryan & Ray, 2004).

Potential applications of levan as an industrial gum, a blood plasma extender, a sweetener, an emulsifier, a formulation aid, a stabilizer, a thickener, a surface-finishing agent, an encapsulating agent and a carrier for flavor and fragrances have also been proposed (Beine et al., 2008; Shih, Shieh, Yu, & Hsieh, 2005). In addition, *in vitro* anti-tumor activity of levan produced from *Microbacterium laevaniformans*, *Rahnella aquatilis* and *Zymomonas mobilis*, has been shown against eight different tumor cell lines (Yoo, Yoon, Cha, & Lee, 2004). Accordingly, interest in commercial production has recently intensified. Levansucrase is responsible for levan formation during sucrose fermentation of different bacterial species.

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Levansucrase was produced from various microorganisms (Abdel-Fattah, Mahmoud, & Esawy, 2005; Ben Ammar et al., 2002; Esawy, Mahmoud, & Abdel Fattah, 2008; Hernandez et al., 1995; Kojima, Saito, Iizuka, Minamiura, & Ono, 1993; Lizuka et al., 1991).

The aim of this work was to isolate strains from honey, which represented new source for levansucrase production from dominant spores in honey and identify the selected strains on species level by molecular identification methods. The antiviral activity of levan products on both respiratory virus (HPAI, H5N1) and enteric virus (adenovirus type 40) was tested.

## 2. Experimental

### 2.1. Sources of honey and microorganisms

Three different honey samples were purchased; local honey bee collecting nectar from clover flower; Kashmiry honey, honey bee collecting nectar from desert flower (Saudi Arabian); and Gably honey, a honey bee collecting nectar from desert flower (Libya).

### 2.2. Isolation of bacterial strains from honey samples

One hundred micro liters of honey samples was spread on nutrient agar plates (g/L): beef ext., 1.0; yeast ext., 2.0; peptone, 5.0 and agar, 25.0. After drying for 20 min in a laminar flow hood, the plates were incubated at 50 °C to avoid the growth of any pathogenic spores for 24 h or until the colonies size was sufficient (approximately larger than 3–5 mm in diameter). The bacterial isolates were streaked onto agar plates and preserved at 4 °C. The purity of the isolates was assessed by colony morphology and microscopy.

### 2.3. Chromosomal DNA and plasmid extraction

Chromosomal DNA was prepared from overnight culture in LB, using AxyGEN Biosciences DNA extraction kit, according to manufacturer's instructions. Plasmid extraction was performed using Wizard mini prep. extraction kit (Promega) according to manufacturer's instructions with slight modification, where 50 μL of lysozyme (200 mg/mL) were added to the resuspended buffer and incubated at 37 °C for 1 h then the protocol was carried on as described in the kit (O'Sullivan & Klaenhammer, 1993; Sambrook, Fritsch, & Maniattis, 1989).

### 2.4. PCR amplification for molecular identification

To amplify the 16S rRNA gene, a primer pair hybridizing to two conserved regions in 16S rRNA genes from *Bacillus* sp. was used: (bac-F and bac-R) (Ash, Farrow, Dorsch, Stackebrandt, & Collins, 1991; Kwon et al., 2009). For the amplification of the 16–23S intergenic region, a primer pair was used: L516SF and L523SR. While for *rec*A gene, a primer pair corresponding to conserved regions in *rec*A genes from *Bacillus* sp. was used: recA-F and recA-R. Species-specific primer set for *B. subtilis* corresponding for *ytc*P gene was used: ytcP-F and ytcP-R. All polymerase chain reaction amplifications were performed with the Taq DNA polymerase kit (Promega). Reaction mixtures consisted of 20 mm Tris–HCl (pH 8.4), 50 mm KCl, 3 mm $MgCl_2$, 50 mm of each of the four deoxynucleoside triphosphates (dNTP), 1 U Taq polymerase, 5 pmol of each primer and 1 μL of template DNA in a final volume of 50 μL. Samples were amplified in a GeneAmp polymerase chain reaction system 2700 (Applied Biosystems) programmed as follows: initial denaturation of DNA for 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C. Polymerase chain reaction products were quantified by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. Polymerase chain reaction products obtained from the selected isolates were purified, using QIA quick polymerase chain reaction purification KIT (Qiagen) and then sequenced commercially by Sigma-Egypt. The sequencings were performed and manually aligned, using DNAMAN software (version 4.0). Sequence homologies were examined by comparing the obtained sequence with those in the NCBI database and the NEB cutter V 2.0 database. NEB cutter V 2.0 is an on-line DNA sequence tool used to find large, non-overlapping, open-reading frames and works for all restriction enzymes. It provides a website, which allows users to check nucleotide sequences for restriction enzyme sites. The sequences were submitted and *Alu*I enzyme was chosen for digestion. Finally gel photograph using 2% agarose was viewed (Ash et al., 1991).

### 2.5. Bacterial strains and growth conditions

*Bacillus* strains used in this work were cultivated in Luria-Bertani (LB) broth or agar at 37 °C. Defined medium was used for cellular production of levansucrase (Yanase et al., 1992). It had the following composition (g/L): yeast extract, 2.5; commercial sucrose, 80; $MgSO_4$, 0.2 and $K_2HPO_4$, 5.5. The medium was completed by the addition of 1 L distilled water and the pH was adjusted to 7.0 before autoclaving. The parameters included initial incubation temperature (25–45 °C); different concentrations of sucrose (80–160 g); incubation time (16–48 h); (50–150) rpm; pH (5-9) and NaCl (1–4% w/v) were studied. The sucrose was substituted with fructose, glucose, lactose (80 g/L) and beet molasses (equivalent to 80 g sucrose) to study their effects on enzyme production.

### 2.6. Cellular production

Cultivation was carried out in 250 mL Erlenmeyer flasks. Each flask contained 50 mL production medium and was autoclaved for 15 min. The flasks were then inoculated with 2.0 ml inoculum and incubated for 24 h at 30 °C. The culture broth was then centrifuged in a cooling centrifuge (K70; Janektzki, Germany) at 10,397 × *g* to separate the bacterial cells from the supernatant.

### 2.7. Assay of levansucrase

Levansucrase assay was performed according to the method of Yanase et al. (1992) with some modification. 0.5 ml of culture filtrate was incubated with 1 ml 20% (w/v) sucrose and 0.5 mL 0.1 M acetate buffer at pH 5.2 and incubated at 37 °C for min. The decreasing amounts of sugars produced were measured by glucose oxidase kits.

One unit of enzyme activity was defined as the amount of enzyme that produced decreasing sugars equivalent to 1 μmol of glucose/min.

### 2.8. Separation of levan polymer

The levan producing organisms were cultivated on a defined medium as described above. After growth, the culture was centrifuged to remove bacterial cells; the levan was precipitated with two volumes of absolute ethanol. The precipitate was collected and dried under vacuum.

### 2.9. Chromatography

Paper chromatography was performed according to Block, Durrum, and Zweig (1995). Hydrolytic products of levan were analyzed by either paper chromatography on whatman No. 1. The reaction mixtures at the end of incubation time were boiled for 3 min to stop the reaction. Chromatographic development was carried out with a solvent system of n-butanol:acetone:water (4:5:1) and detected by spraying with aniline hydrogen phthalate. The acid hydrolysate of the polysaccharide produced by the six isolates

was analyzed using high-pressure liquid chromatography (HPLC). A 7.8 mm × 300 mm PL-HI-PLEXPB column was linked to a differential refractometer. The column temperature was maintained at 80 °C. The aqueous mobile phase was delivered at a flow rate of 0.6 ml/min.

### 2.10. Determination of molecular weight

Different concentrations of levan and oligosaccharide were prepared and the flow time of equal volume for each concentration at 30 °C was determined in a U-shaped Ostwald viscometer. Flow time of the same volume of distilled water was also determined as control. Thus, specific viscosity/C (gsp) was estimated.

A plot of levan and oligosaccharide concentration (C) against intrinsic viscosity (C) (gsp/C) therefore yielded a straight line.

### 2.11. Antivirus detection

Two types of viruses were used, highly pathogenic avian influenza H5N1 virus Egyptian isolate, was used at titre of $10^6$ EID50/mL (embryo infective dose per mL) and adenovirus type 40 with different doses $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ infectious particles/mL obtained from the Holding Company for Biological Products & Vaccines (VACSERA).

### 2.12. Specific pathogen free (SPF) eggs

SPF embryonated chicken eggs were used at nine days old and inoculated *via* the allantoic sac route. SPF eggs (Brown et al., 2007) were obtained from Nile SPF Eggs, Koomoshiem, Fayoum, Egypt.

### 2.13. Cytotoxicity test

It was done according to Simões, Amoros, and Girre (1999) and Walum, Strenberg, and Jenssen (1990). Briefly, All samples (100 mg) were dissolved in 500 μL of water or ethanol. Samples A, E, M, and K were dissolved in ethanol while samples C and G were dissolved in water.

Decontamination of samples was done by adding 12 μL of 100× of antibiotic–antimycotic mixture to 500 μL of each sample. Then, bi-fold dilutions were done to 100 μL of original dissolved samples and 100 μL of each dilutions were inoculated in Hep-2 cell line (obtained from the Holding Company for Biological Products & Vaccines VACSERA, Egypt) previously cultured in 96 multi well plates (Greiner-Bio one, Germany) to estimate the non toxic dose of the tested samples. Cytotoxicity assay was done using cell morphology evaluation by inverted light microscope and cell viability test applying trypan blue dye exclusion method.

### 2.14. Cell morphology evaluation by inverted light microscopy

Hep-2 cell cultures ($2 \times 10^5$ cells/mL) were prepared in 96-well tissue culture plates (Greiner-Bio one, Germany). After 24 h incubation at 37 °C in a humidified 5% (v/v) $CO_2$ atmosphere cell monolayers were confluent, the medium was removed from each well and replenished with 100 μL of bi-fold dilutions of different samples tested prepared in DMEM (GIBCO BRL). For cell controls 100 μL of DMEM without samples was added. All cultures were incubated at 37 °C in a humidified 5% (v/v) $CO_2$ atmosphere for 72 h. Cell morphology was observed daily for microscopically detectable morphological alterations, such as loss of confluence, cell rounding and shrinking, and cytoplasm granulation and vacuolization. Morphological changes were scored (Simões et al., 1999).

### 2.15. Cell viability assay

It was done by trypan blue dye exclusion method (Walum et al., 1990). HEp-2 cell cultures ($2 \times 10^5$ cells/mL) were grown in 12-well tissue culture plates (Greiner-Bio one, Germany). After 24 h incubation, the same assay described above for tested samples cytotoxicity was followed by applying 100 μL of tested samples dilutions (bifold dilutions) per well. After 72 h the medium was removed, cells were trypsinized and an equal volume of 0.4% (w/v). Trypan blue dye aqueous solution was added to cell suspension. Viable cells were counted under the phase contrast microscope.

### 2.16. Haemagglutinating activity assay

This was applied for the allantoic fluids of the inoculated eggs and measured by micro technique of haemagglutination (HA) test (Takatsy, 1955).

### 2.17. Evaluation for antiviral activity

Three experiments were conducted.

#### 2.17.1. Experiment 1

One hundred and five emberyonated chicken eggs (ECEs) were examined, equal volumes of HPAI H5N1 virus and original extracts were separately used at three levels:

Level 1: Equal volumes of HPAI H5N1 virus and the original undiluted samples were mixed and incubated at room temperature for 1 h then inoculated into the allantoic sac of five ECEs for each product sample at dose 0.2 mL/ECE.
Level 2: Equal volumes of HPAI H5N1 virus and the 1/5 dilution of each sample were mixed and preceded as level 1.
Level 3: Equal volumes of the virus and the 1/10 dilution of each sample were mixed and preceded as level 1. In addition, five ECEs were inoculated with the virus that mixed with equal volume of saline at a dose of 0.2 mL/ECE of saline alone (negative control). The ECEs are inoculated at 37 °C and candled every 2 h till all the positive control ECEs are died.

#### 2.17.2. Experiment 2

One hundred and five SPF ECEs were used in this experiment; equal volumes of HPAI H5N1 virus and the original samples were mixed with equal volume of the original samples and inoculated directly into the allontoic sac of five ECEs for each product sample at a dose of 0.20 mL/ECE for each product sample at a dose of 0.2 mL/ECE. Five ECEs were inoculated with equal volume of the HPAI H5N1 virus and saline at dose of 0.2 mL/ECE (positive control). Another five ECEs were inoculated with 0.20 mL/ECE of saline alone (negative control). All the ECEs were incubated at 37 °C and controlled every 2 h till the ECEs of the positive control are died.

### 2.18. Experiments 3

One hundred and five SPF ECEs of nine days old were used in this experiment. 0.10 mL of the HPAI H5N1 virus was inoculated *via* the allontoic sac of each ECE into 100 ECEs and then the inoculated ECEs were incubated for 1 h at 37 °C. The original samples were inoculated into five ECEs, which previously inoculated with the virus at a dose of 0.1 ml. Another five ECEs were inoculated with 0.2 ml/ECE of the mixed virus and saline. Other five ECEs were inoculated with 0.2 ml/ECE of saline alone. The ECEs were inoculated at 37 °C and candled every 2 h till ECEs of the positive control are died.

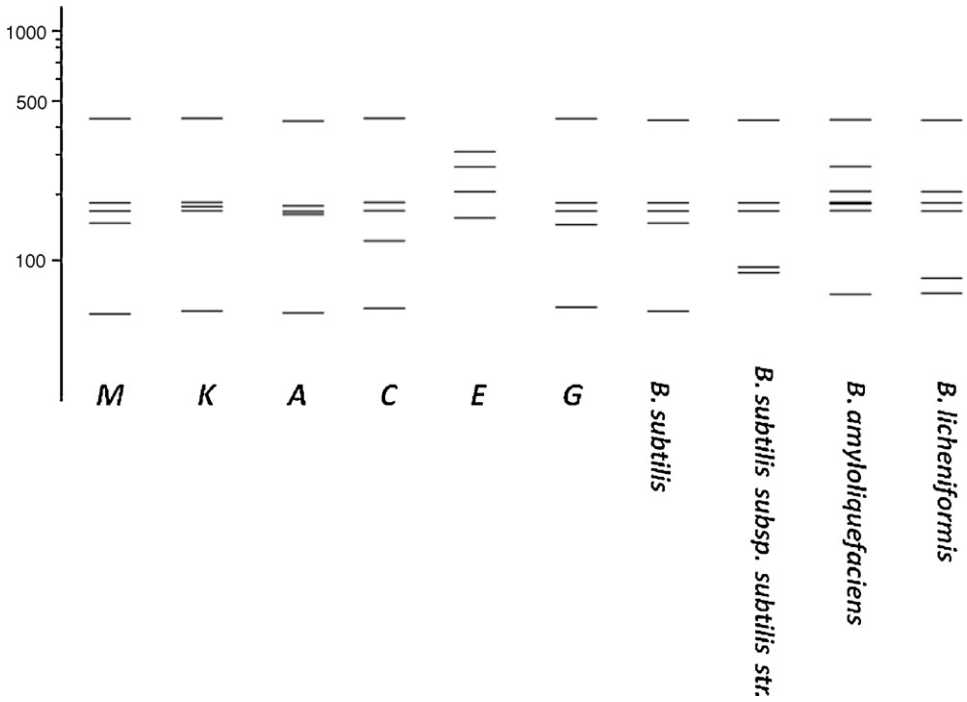


**Fig. 1.** The analogical electrophoresis of *Bacillus* isolates compared to *Bacillus* strains (from the gene bank) by "AluI",using NEBcutter 2.0. The accession no. of the control *Bacillus* strains: *B. subtilis* gi|269313996|; *B. subtilis* subsp. *subtilis str.* AL009126.3; *B. amyloliquefaciens* gi|229484923|; and *B. licheniformis* gi|270356913|.

### 2.19. Antiviral effect of tested samples on adenovirus type 40

Seventy five microliters of non toxic dilutions were mixed with 75 μL of different doses $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ infectious viral particles/mL of adenovirus type 40 provided by American Type Culture Collection (ATCC). Then the mixture was incubated overnight at 4 °C. Inoculation of 100 μL of 10 fold dilutions of treated and untreated adenovirus was done into Hep-2 cell line in 12 multi well-plates. After 1 h incubation for adsorption at 37 °C, 1 mL medium (DMEM) was added to each well. The cell line was observed daily for one week then, three times freezing and thawing for tested plates were done. Nested PCR was done for confirmation of adenovirus (presence/absence) in each well (Puig et al., 1994).

## 3. Results

### 3.1. Molecular identification of the levansucrase producers strains

#### 3.1.1. 16S rRNA sequences and their analogical electrophoresis

The six selected isolates with remarkable levansucrase activity were previously subjected to molecular identification based on 16S rRNA gene sequencing method. The DNA of the isolates was extracted as described in Section 2 and the 1.5 kb 16S rRNA gene was amplified from each DNA by PCR using primers bac-F and bac-R. The PCR amplification, purification and sequencing were performed as described previously. The 1.5 kb obtained sequences were aligned and clustered with sequences from the NCBI database. 16S rRNA gene sequence analysis indicated that the six isolates (K, M, A, C, E, and G) were *Bacillus* spp. with 99% identical to any of these three species *B. subtilis*, or *B. licheniformis*, or *B. amyloliquefaciens* and they clustered into a monophyletic line in a phylogenetic tree (data not published yet).

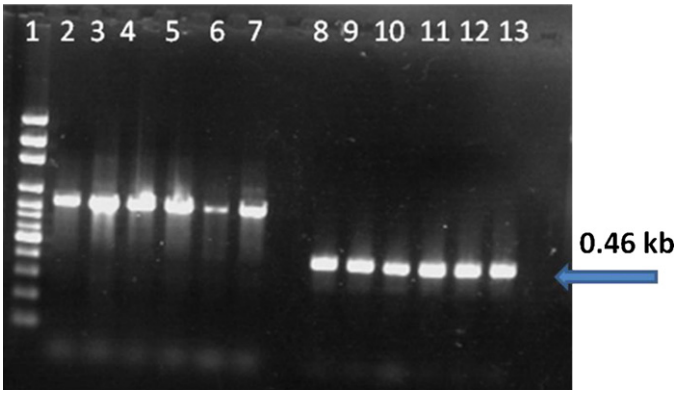


**Fig. 2.** PCR using the chromosomal DNA of the 6 Bacillus isolates. Lane 1, 100 bp ladder; lane 2–7, using primers recR × recF; lane 8–13, using *B. subtilis* specific primers ytcF × ytcR.

To distinguish and clear identification of these strains on the species level the analogical electrophoresis, using NEB cutter was applied to identify the 16S rRNA results, which have been sequenced, as the strains of the same species expected to have

**Table 1**
Primers used for gene amplification.

| Target | Primer name | Oligonucleotide | | Reference |
|---|---|---|---|---|
| 16–23S intergenic region | L516SFL523SR | 5′-TCGCTAGTAATCGCGGATCGGC-3′ | 5′-GCATATCGGTGTTAGTCCCGTCC-3′ | Yoon et al. (2001) |
| *rec*A gene | recA-F recA-R | 5′-TGAGTGATCGTCAGGCAGCCTTAG-3′ | 5′-CYTBRGATAAGARTACCAWGMACCGC-3′ | Kwon et al. (2009) |
| Hypotheticl gene | ytcP-F ytcP-R′ | 5′-GCTTACGGGTTATCCCGC-3′ | 5′CCGACCCCATTTCAGACATATC-3′ | Kwon et al. (2009) |

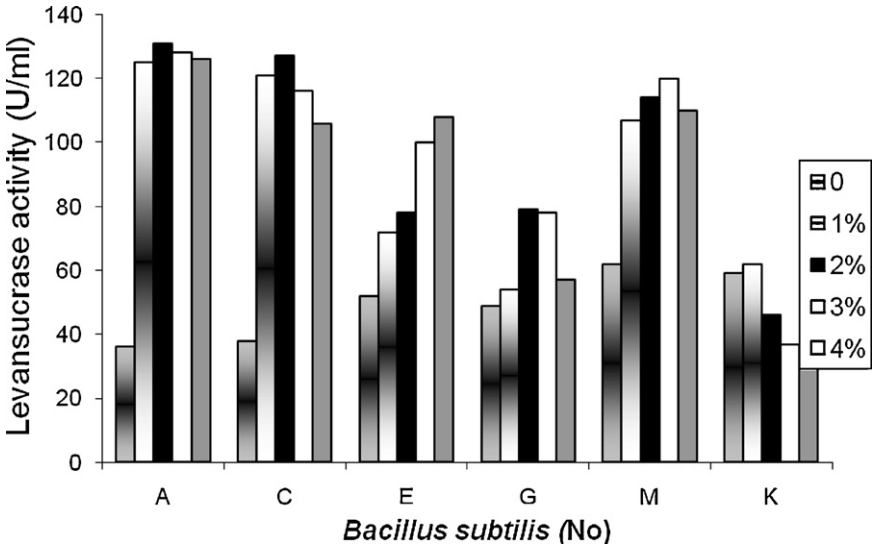


**Fig. 3.** The effect absence and presence of different concentrations of NaCl on levansucrase production from honey isolate. *Note*: NaCl conc (0–4%) represented from left to right.

almost the same sites when digested with *Alu*I. Fig. 1 showed that the isolates M and G have the same size fragments as *B. subtilis* gi 269313996 while the other isolates A, C, E, K showed different *Alu*I fragments which differ to the *Alu*I fragments generated from 16S rRNA sequence of *B. subtilis*, or *B. licheniformis* or *B. amyloliquefaciens*.

It was clear that 16S rRNA gene alone could not distinguish these three closely related *Bacillus* species.

### 3.2. Identification by 16–23S intergenic region

For distinguish between these three closely related strains the 16–23S intergenic region was amplified by primers L516SF × L523SR, then the sequence was determined for the six isolates. The homology results for the 16–23S intergenic region showed that: K and M strains showed 100% to *B. subtilis* but strains A, C, E, G might be either *B. subtilis* (99%) or *B. amyloliquefaciens* (85%) (Table 1).

### 3.3. Identification by recA sequence

Hence the *rec*A gene has been used as a molecular chronometer in addition to rRNA genes The 1.2 kb band was gel isolated and subjected to sequencing results and showed that the four strains A, C, E, and G were *B. subtilis* rather than *B. amyloliquefaciens*. Still these results need more confirmation.

### 3.4. Identification by specific-PCR for B. subtilis

To solve this problem, identification using specific-PCR for *B. subtilis* was described. Based on *ytc*P gene encoding a hypothetical protein, a PCR primer pair ytcpF and ytcpR were designed for *B. subtilis* species for specific amplification purpose. Using this primer

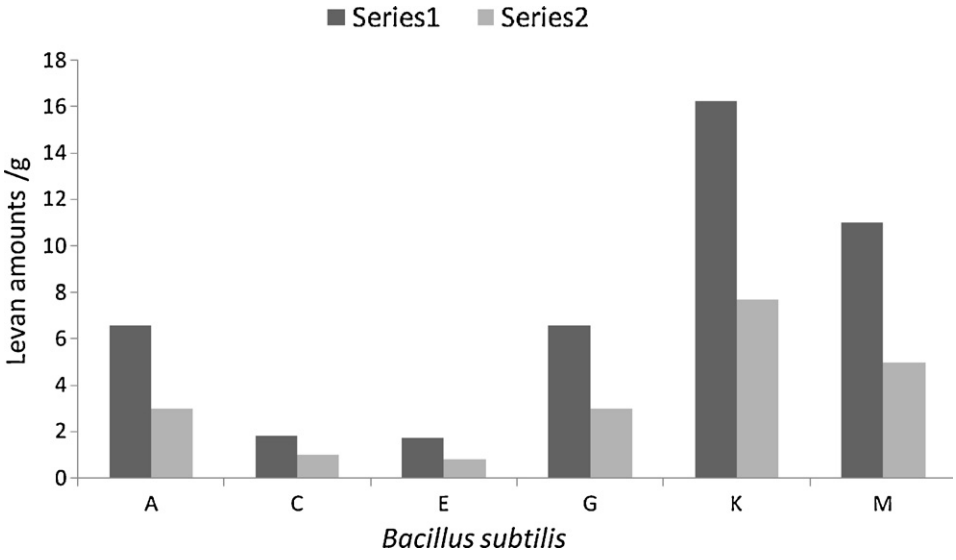


**Fig. 4.** The difference between amounts of levan produced in sucrose medium (series 1) and in molasses medium (series 2).

pair, a 0.46 kb fragment was amplified only from *B. subtilis* strains, whereas no similar band was detected from *B. licheniformis* or *B. amyloliquefaciens* strains. These primers were subjected to the six isolates and PCR resulted in the 0.46 kb band as shown in Fig. 2 and this confirmed that all isolates A, C, E, G, K, M surely belong to *B. subtilis* not *B. amyloliquefaciens.*

### 3.5. Levan and levansucrase production

The six honey isolates were tested for production of levansucrase, the optimized conditions for the isolates ranged from 8 to 12% (w/v) commercial sucrose, 37–40 °C, 24–28 h, 50–100 rpm and pH 6–7.0 (data not shown). Among all the tested isolates, M and K isolates showed the highest levansucrase activities (62 and 59 U/mL). The presence of NaCl (1–4%, w/v) showed great influence in enzyme activity, the enzyme production increased from 2 to 3 folds according to the strain (Fig. 3). Paper chromatography of the product hydrolysate revealed the presence of only fructose and tiny traces of glucose, pointing to the levan nature of the product. Furthermore, the acid hydrolysate of the polysaccharide produced by the isolates was exclusively fructose, as revealed by HPLC. Levan was harvested by precipitation from the culture broth by addition of ethanol. The yield and consistency of the product varied according to the isolate. The final products were a brownish-white gummy material, which could be freeze-dried or vacuum-dried. The highest amount of levan was produced on the medium containing commercial sucrose, followed by beet molasses (Fig. 4). While a small amount of microbial polysaccharide (alcohol precipitate) was also produced when the organism was grown on lactose and glucose, it was worthy to record that no polysaccharides were produced on fructose. The amounts of levan decreased 40–50% in the presence of NaCl. Isolates M, K, A, C, E, and G produced 11, 16.25, 6.60, 1.81, 1.74, 6.6 g/L levan, respectively under the optimized conditions. The levan products consisted of one fraction characterized by high and low molecular masses (40.938, 71.887, 43.487, 154.638, 77.753 and 14.200 kDa for isolates K, M, A, C, E, and G, respectively) (Fig. 5).

### 3.6. Cytotoxicity test

The non toxic doses for samples C and G were 0.5 mg/mL, which both of them were dissolved in water. On the other hand, the non toxic doses for samples A, E, and M were 0.2 mg/mL while the non-toxic dose of sample K was 0.1 mg/mL. All of them were dissolved in ethanol.

### 3.7. Anti-adenovirus type 40 assay

Some samples (C, E, G and K) showed weak effect on adenovirus 40 which did not exceed 10%. The two samples A and M showed antiviral effect on adenovirus type 40 ranged from 50 to 60% as shown in Table 2.

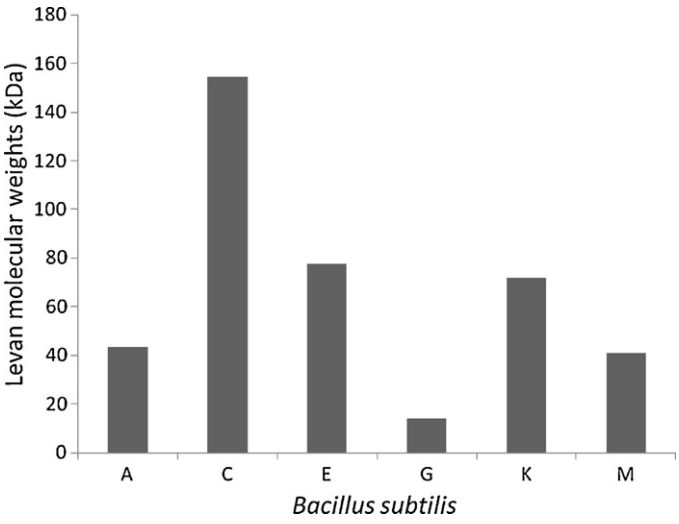


**Fig. 5.** The levan molecular weights from *Bacillus subtilis* isolates.

**Table 2**
Effect of levan A and levan M on the infectivity of enteric adenovirus type 40 (DNA virus).

| Sample | Initial viral doses | Final viral doses | Reduction (%) |
|---|---|---|---|
| A | $1 \times 10^4$ | $4 \times 10^3$ | 60 |
| | $1 \times 10^5$ | $5 \times 10^4$ | 50 |
| | $1 \times 10^6$ | $5 \times 10^5$ | 50 |
| M | $1 \times 10^4$ | $4 \times 10^3$ | 60 |
| | $1 \times 10^5$ | $4 \times 10^4$ | 60 |
| | $1 \times 10^6$ | $5 \times 10^5$ | 50 |

### 3.8. Anti-H5N1 virus assay

All the embryos of the positive controls were died and the allantoic fluid of each was positive for haemagglutination assay (HA), while all the embryo of negative control were not died and the *allantoic* fluid of each was negative for HA. Three levans of code K, M, E showed antiviral against HPAI H5N1. Each of these samples showed antiviral effect when inoculated with H5N1 virus 1 h before inoculation into nine days old ECEs, while they had no effect on the virus when inoculated simultaneously with the virus just after mixing or after the virus inoculation for 1 h (Table 3).

These results also revealed that simultaneous inoculation of the levans product or even after infection was of no value.

## 4. Discussion

*B. subtilis* isolates are biologically and commercially important as producers of a great variety of secondary metabolites such as antibiotics, and enzymes (Desai & Banat, 1997; Roberts, Nakamura,

**Table 3**
Antiviral of levans of code K, M, E against HPAI H5N1.

| Code of sample | Experiment 1 | | | | | | Experiment 2 | | Experiment 3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Level 1 | | Level 2 | | Level 3 | | NDE | +HA | NDE | +HA |
| | NDE[a] | +HA[b] | NDE | +HA | NDE | +HA | | | | |
| Control | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 |
| M | 0/5 | 0.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 |
| C | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 |
| E | 0/5 | 0.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 |
| K | 0/5 | 0.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 | 5/5 | 5.0 |

[a] Number of died.
[b] Haemagglutination assay.

& Cohan, 1996). Within this context, six mobile spore-forming, and Gram-positive facultative aerobic bacilli were isolated from different honey samples and identified as *Bacillus* sp. On the base of morphological, and molecular identification, using 16S rRNA sequence method. This method could not identify the *Bacillus* isolates at the species level where revealed identical to any of three strains *B. subtilis* or *B. licheniformis* or *B. amyloliquefaciens*.

In fact DNA-based identification methods such as 16S rRNA gene sequencing and 16S–23S intergenic region sequencing have been used widely for the purpose of identification and typing of microorganisms isolated from natural environments including fermented foods (Hansen, Leser, & Hendriksen, 2001; Levine, Tang, & Pei, 2005). But identification based on rRNA gene sequences fails to distinguish one species from the other if they share highly similar rRNA genes. This is true for some *Bacillus* species. It is difficult to distinguish *B. subtilis* from closely related *B. licheniformis* or *B. amyloliquefaciens* by rRNA gene sequences because of no significant differences in their rRNA sequences (Nakamura, 1989; Nakamura, Roberts, & Cohan, 1999). Other genes such as *rec*A (Rodriguez et al., 2007) and *dna*J (Shah et al., 2007) have been employed instead of rRNA genes. It is necessary to compare results from different identification methods as a whole before to reach a conclusion (Bourque, Valero, Lavoie, & Levesque, 1995).

To solve the ambiguity in differentiating them based solely on the 16S rRNA gene, it was turned to 16–23S intergenic region, *rec*A gene, and ended with *B. subtilis* specific primers. It is specifically useful to distinguish organisms with highly similar rRNA genes *rec*A gene was amplified from the chromosomal DNA of bacilli isolates by PCR, using primers (recF and recR) (Payne et al., 2005). When rRNA and *rec*A gene sequences were considered together, it was possible to conclude that the G, M isolates are belonging to *B. subtilis* but the other isolates A, C, E needed further identification. For the accurate distinguish between the 6 isolates, the specific-PCR for *B. subtilis* based on *ytc*P gene was used and the results revealed that the six strains are belonging to *B. subtilis*. The present results proved high phenotypic and genotypic variability among *B. subtilis* isolates, where they showed different morphological and biological properties suggesting them as new different species of *B. subtilis* with valuable impact in the industry. Many authors reported in the production of levansucrase from *B. subtilis* (Euzenat, Guibert, & Combes, 1997; Le Gorrec, Connes, & Guibert, 2002). The various sugars, initial pH, fermentation temperature, and agitation speed affected the levansucrase production by *B. subtilis* (Abdel-Fattah et al., 2005; Shih et al., 2005). The result ensure the halophilc feature of *B. subtilis* levansucrases, this comes from its osmophilic character. Enhancements of levansucrase production in the presence of NaCl were reported (Euzenat, Guibert, & Combes, 2006; Poli et al., 2009). As far as we are aware no studies were reported on the effect of NaCl on the enzyme production. It seemed that levan production by the isolates was dependent mainly on commercial sucrose media, where the use of beet molasses, glucose, and lactose led to noticeable reduction in levan synthesis. Beet molasses was used as low-cost substitutes for sucrose in commercial levan yield (Han & Watson, 1992). The decrease in levan yield in the molasses medium (2.533 g/L) when compared to the commercial sucrose (21.685 g/L) was also reported (De Oliveira et al., 2007). Although higher *Halomonas* sp. AAD6 biomass concentrations were observed when glucose, maltose, fructose and galactose were used as carbon sources, levan levels were very low comparing with sucrose (Poli et al., 2009). The six isolates produced different levan weights, with wide range of molecular mass. On the other hand, it was reported that halophilic *Halomonas* sp. AAD6 cells grown in the presence of sucrose afforded the highest levan production levels (1.073 g/L) (Poli et al., 2009). Also, *B. polymyxa* produced about 40 g/L extracellular polysaccharide per liter in sucrose medium, which was about three times that produced by familiar levan producers (Han, 1989). Levan antitumor activity was reported by many authors (Yoon, Yoo, Cha, & Lee, 2004) but as far as we are aware nothing was reported on antiviral activity of this fructose polymer. The present findings showed antiviral effects of K, M, and E levan products on H5N1 virus, While, A and M levan products showed antiviral effects on adenovirus type-40. It was obvious that the product M was entirely effective against both respiratory RNA virus (H5N1) and enteric adenovirus type 40 (DNA virus). It was apparent that each of effective levan showed antiviral effect when inoculated with H5N1 virus 1 h before inoculation into nine days old ECEs, while they had no effect on the virus when inoculated simultaneously with the virus just after mixing or after 1 h of inoculation.

## 5. Conclusion

The outcome of this study is the probable suitability of the produced levan as a safe and cheap natural product adequate for effective antiviral treatments with applying the known roles concerning the use of these compounds. In this respect and as far as we are aware nothing was reported on levan as antivirus. In addition, this article affords honey micro flora as a new and important sources of levansucrase enzymes, also it could have biotechnological applications in pharmaceutical industries.

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