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Molecular Characterization and Batch Fermentation of *Bacillus subtilis* as Biocontrol Agent, II

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Abstract: Fourteen *Bacillus subtilis* isolates (B1 to B14) obtained from different Egyptian sites had different antagonistic and inhibitory effect against six fungal isolates belonging to four different genera, *Rhizoctonia solani*, *Helminthosporium* sp., *Alternaria* sp. and *Fusarium oxysporum*. Random Amplified Polymorphic DNA (RAPD), differential display technique and partial sequencing of 16S rRNA gene were used for characterization of isolates. While RAPD technology, using 4 different random 20-mer primers, revealed different levels of molecular variation among twelve out of the fourteen isolates tested, the remaining two isolates B3 and B5, demonstrated identical profiles. Because of the high degree of homology (93%) between isolates B4 and B7 and the no variation observed between isolates B3 and B5, the differential display technique was used to test the reliability of these results. Isolate B7 was selected for batch cultivation in bioreactor, the biomass achieved was 3.2 g L⁻¹. Inhibitory activity of supernatant was increased near the end of the stationary phase. The activity reached its highest value of 1.7 cm in death phase.

Key words: *Bacillus subtilis*, biocontrol, molecular characterization, 16S rRNA, batch fermentation

INTRODUCTION

The Random Amplified Polymorphic DNA (RAPD) which is a Polymerase Chain Reaction (PCR)-based technique in which amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams *et al.*, 1990; Hilton *et al.*, 1997) is conducted and used as powerful tool for genetic studies involving molecular genetic diversity of microorganisms. A robust RAPD-PCR procedure for large-scale typing of *B. cereus* was developed by Nilsson *et al.* (1998). It is based on a simple DNA preparation, involving only freezing and boiling of cells in water with active carbon. By using a computerized for data collection and processing, an efficient system for handling RAPD patterns for large scale investigation was achieved. The procedure was highly discriminatory for *B. cereus* strains and was found to give reproducible classification of RAPD fingerprints for five reference strains. Istock *et al.*

(2001) analyzed the geographical distribution and structure of *B. subtilis* genomic lineages using RAPD fingerprints. Six major genomic lineages separating at 9-18% similarity were found. One hundred and twenty-six strains of *B. thuringiensis* representing 57 serovars were allocated to 58 genomic types using random amplified polymorphic DNA (RAPD)-PCR patterns (Gaviria and Priest, 2003), also Levy *et al.* (2006) used the RAPD technique to distinguish between seven *Bacillus* species. Differential display is a molecular biology technique that can distinguish between genes that are up- or down regulated. Initially described by Liang and Pardee (1992), differential display encompasses several reverse transcription polymerase chain reaction (RT-PCR) techniques that can produce a large number of cDNA fragments from cellular RNA. First, anchored 3' poly-T primers are used to create cDNAs corresponding to all possible mRNA species. Next, specific gene fragments are amplified by use of random 5' primers.

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Fislage *et al.* (1997) developed a primer set for a prokaryotic differential display of mRNA in the Enterobacteriaceae group. The technique of mRNA differential display was used to identify simultaneously two metabolic genes involved in the degradation of cyclohexanone in a new halotolerant *Brevibacterium* isolate (Brzostowicz *et al.*, 2000). Chia *et al.* (2001) analyzed differential gene expression of *Streptococcus mutans* in response to environmental stress by RNA fingerprinting using arbitrarily primed PCR with a panel of 11 mer primers designed for differential display in Enterobacteriaceae. In a batch process, all substrates are much available in the initial medium and none is supplied extra during the process. The process is stopped after the substrate is converted and the whole batch is harvested together (Sonnleitner, 1998; Wirtz, 2002). A batch growth is a non-steady state process; that is the content of the bioreactor changes with time. The nutrients and the inoculum are added to the bioreactor at the beginning of the batch operation and air is supplied continuously. After a short lag time, *B. thuringiensis* cells begin the exponential growth; the carbon source is oxidized and owing to organic acids accumulation, the pH drops dramatically. The biomass concentration and the cell count increases as the carbon source decreases. The oxygen demand increases during the exponential stage and there are no spores present in the bioreactor (Avignone-Rossa and Mignone, 1995). The production of biocontrol agent of *B. subtilis* R14 was evaluated in a batch bioreactor (Luna *et al.*, 2002). Batch operation has been used for comparison with other modes of growth as a reference. Liu *et al.* (1994) for example compared the results obtained in a batch growth with a fed-batch growth of *B. thuringiensis* subspecies *kurstaki*. The maximum biomass concentration obtained in batch growth was 5.9 g dry weight mL⁻¹ whereas the maximum biomass concentration was 53.7 g dry weight mL⁻¹ in fed-batch growth.

The objective of this study was for molecular characterization of bacterial isolates which have an antagonistic and inhibitory effect against certain plant pathogenic fungi and study the behavior of *B. subtilis* isolate B7 during batch cultivation in bioreactor.

MATERIALS AND METHODS

Bacterial and fungal isolates: Fourteen *B. subtilis* isolates were isolated in 2002-2003 from different locations in Egypt (El-Malaha, El-Amria, El-Nobaria, Abo-Homos and El-Sharkia) and identified according to the morphological, biochemical and physiological tests recommended by Sneath *et al.* (1986) and Collee *et al.*

(1996). Six fungal isolates belonging to four different genera were isolated in 2002-2003 from different hosts and area in Egypt, *R. solani*, *Helminthosporium* sp., *Alternaria* sp. and *Fusarium oxysporum*.

Antagonistic and inhibitory effect of *B. subtilis* isolates (in vitro): Antagonistic effect of fourteen *B. subtilis* isolates and inhibitory effect of supernatant fluid were performed against all fungal isolates according to Toure *et al.* (2004) and Chitarra *et al.* (2003). The data obtained were statistically analyzed using the Statistical Analysis System (SAS).

Genotypic characterization

DNA extraction protocol: Total DNA was extracted from *B. subtilis* isolates according to Istock *et al.* (2001).

Random Amplified Polymorphic DNA (RAPD): Four 20-mer arbitrary primers were used for RAPD analysis (A1A13, A9B7, A1 and A7) (Abdel Ghany and Zaki, 2003). Sequences of all primers are shown in Table 1. For RAPD analysis, PCR amplification was carried out in a total volume 25 µL containing 2.5 µL 10x buffer, 2 µL 25 mM MgCl₂, 2 µL 2.5 mM dNTPs, 1 µL 10 pmol primer, 1 µL 50 ng of bacterial genomic DNA and 0.2 µL (5 U µL⁻¹) Taq DNA polymerase (Promega, Germany). PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for one cycle at 95°C for 5 min followed by 34 cycles each with 30 sec at 95°C for denaturation, 1 min at 45°C for annealing and 2 min at 72°C for elongation. Reaction mixture was then incubated at 72°C for 10 min for final extension (Istock *et al.*, 2001).

Differential display analysis: Total RNA was extracted from *B. subtilis* isolates using TRI-reagent (Chin *et al.*, 2000). Reverse transcription reactions were performed using oligo dT primer (5'-TTTTTTTTTTTTTTT-3'). Each 25 µL reaction mixture contained 2.5 µL 5x buffer with MgCl₂, 2.5 µL 2.5 mM dNTPs, 1 µL 10 pmol primer, 2.5 µL RNA and 0.2 µL (5 U µL⁻¹) reverse transcriptase (Promega, Germany). PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed at 95°C for 5 min, 42°C for 1 h, 72°C for 10 min and a soak at 4°C (Chin *et al.*, 2000).

Table 1: Sequence of all primers used in this study

Primer	Nucleotide sequence, 5' to 3'
A1A13	CAGGCCCTTCCAGCACCCAC
A9B7	GGTGACGCAGGGGTAACGCC
A1	CGAGGCCCTTCCAGCACCCAC
A7	GAAACGGGTGGTGTATCGCAG
P0	GAAGAGTTTGATCCTGGCTCAG
P1	GCACTGAGG
P2	CGCTGTCCG
P6	CTACGGCTACCTTGTTACGA

Three primers were used in the differential display analysis one of them is specific for 16S rRNA gene (P0) but the other were arbitrary in sequence (P1 and P2). Sequence of primers is shown in Table 1. The reaction mixture for differential display PCR was carried out in a total volume 25 μ L containing 2.5 μ L 10x buffer with $MgCl_2$, 2 μ L 2.5 mM dNTPs, 1 μ L 10 pmol primer, 1.5 μ L cDNA and 0.2 μ L (5 U μ L⁻¹) Taq DNA polymerase (Promega, Germany). PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for one cycle at 95°C for 5 min, then 34 cycles as follows: 30 sec at 95°C for denaturation, 1 min at 45°C for annealing and 2 min at 72°C for elongation. Reaction was then incubated at 72°C for 10 min for final extension (Chin *et al.*, 2000).

Amplification of 16S rRNA gene: The full length (1550 bp) of 16S rRNA gene was amplified for all *B. subtilis* isolates by using two universal primers (P0 and P6) (Sow *et al.*, 2005). Sequence of primers is illustrated in Table 1. PCR amplification was carried out in a total volume 50 μ L containing 5 μ L 10x buffer, 4 μ L 25 mM $MgCl_2$, 4 μ L 2.5 mM dNTPs, 2 μ L 10 pmol forward primer, 2 μ L 10 pmol reverse primer, 2 μ L 50 ng of bacterial genomic DNA and 0.4 μ L (5 U μ L⁻¹) Taq DNA polymerase (Promega, Germany). PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for one cycle at 95°C for 5 min followed by 34 cycles each with 45 sec at 95°C for denaturation, 1 min at 50°C for annealing and 2 min at 72°C for elongation. Reaction mixture was then incubated at 72°C for 10 min for final extension (Sambrook *et al.*, 1989).

Sequencing of 16S rRNA gene and the accession number: QIAquick PCR purification kit (Qiagen, Germany) was used to purify the amplified products of 16S rRNA gene.

The amplified product (1550 bp) of 16S rRNA was sequenced by the use of a Big Dye terminator cycle sequencing kit. Sequencing products were purified using Centri-Sep spin columns and were resolved on an ABI PRISM model 310 automated DNA sequencer at the Agriculture Genetic Engineering Research Institute (AGERI) in Egypt.

Fermentation experiments

Bioreactor: Batch and fed-batch cultivations were performed in a 3 L bench-top bioreactor (Bioflow III, New Brunswick, N J, USA) equipped with two 6-bladed disc-turbine impeller and four baffles and connected to a digital control unit. The process was automated through a computer aided data bioprocessing system AFS-BioCommand multi-process management program.

The set points for temperature and pH value were 30°C and 7, respectively and pH was controlled by automatic feeding of 2N NaOH. Compressed air was supplied initially at 0.5-1.0 VVM (air volume per broth volume per minute) through sterile filter. It was manually controlled in parallel with agitation speed (150-900 rpm) to maintain the dissolved oxygen level above 20%. Dissolved oxygen and pH values were determined on-line using METTLER TOLEDO electrodes. Antifoam A (Sigma) was used for eliminating foaming.

Batch fermentation: Batch cultivation was initiated in a bioreactor using a volume of 1900 mL of fresh number 3 medium of which each 1 L contained 10 g peptone, 10 g glucose, 1 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and a pH of 6.8 (Asaka and Shoda, 1996) which was then inoculated with 100 mL of shake flask pre-cultured seeds which had previously been prepared as follows: a single colony of *B. subtilis* B7 (Abo-Homos) which was selected from fourteen isolates had antagonistic effect against all tested fungi, but showed more antagonistic effect against *Alternaria* sp. (Matar *et al.*, 2008(I)) was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of No. 3 medium and cultured overnight at 30°C with constant shaking at 200 rpm. During the time of cultivation, several samples of culture were taken and cell number was determined by measuring of optical density (OD) at 550 nm. Glucose level was estimated by enzymatic (GOD-PAD) colorimetric kit (Diamond Diagnostic, Egypt) and the inhibitory activity assay of supernatant fluid was performed against *Alternaria* sp. according to Chitarra method (Chitarra *et al.*, 2003). Also, 10 mL of cultivated broth was separated overtime to apply the dry test method.

RESULTS

Data of antagonistic and inhibitory effect of *B. subtilis* isolates (*in vitro*) was explained by Matar *et al.* (2008(I)).

When Random Amplified Polymorphic DNA (RAPD) technique was used to molecularly differentiate among the 14 *B. subtilis* isolates, using four 20-mer arbitrary primers (Table 1), clear differences among twelve isolates on the bases of amplified product band patterns observed with each primer (Fig. 1, 2). Notably, high similarity (93%) was observed between B4 (El-Nobaria) and B7 (Abo-Homos) and also between B8 (El-Malaha) and B9 (El-Malaha). Exceptionally, isolates B3 (El-Malaha) and B5 (El-Amria) gave identical band patterns with all the four primers used. Results indicated that 256 amplicons were produced by all primers. The number of amplified

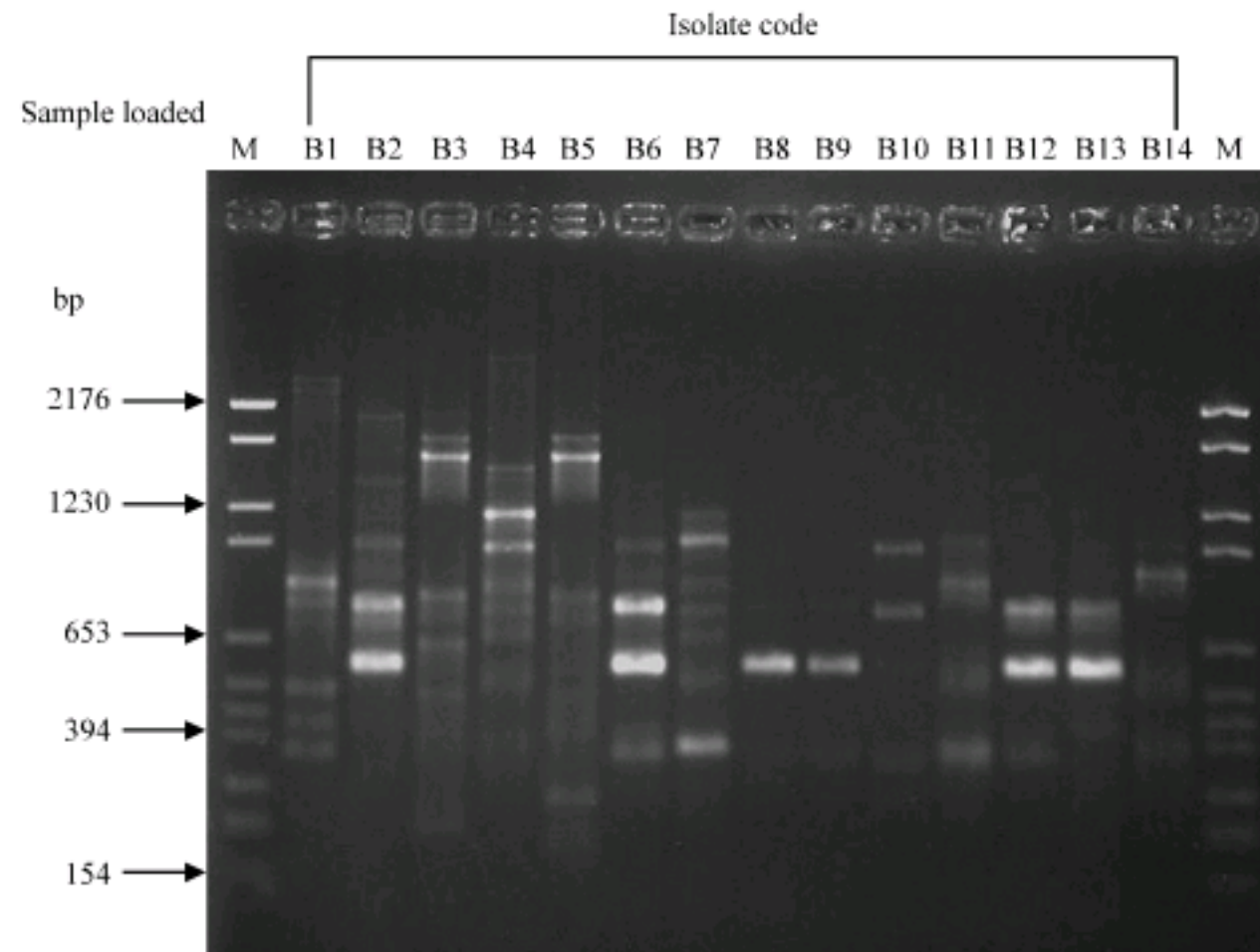


Fig. 1: RAPD-PCR patterns of *Bacillus subtilis* isolates with primer A9B7. Lanes on both sides of the gel labeled M are loaded with PBR 328 Mix DNA marker. B1 to B14 are the fourteen *Bacillus subtilis* isolates investigated. Isolates B1, B3, B8 and B9 are those of El-Malaha; B5 and B6 are of El-Amria; B4, B10, B11 and B12 are of El-Nobaria; B7, B13 and B14 are from Abo-Homos; B2 is that isolated from El-Sharkia

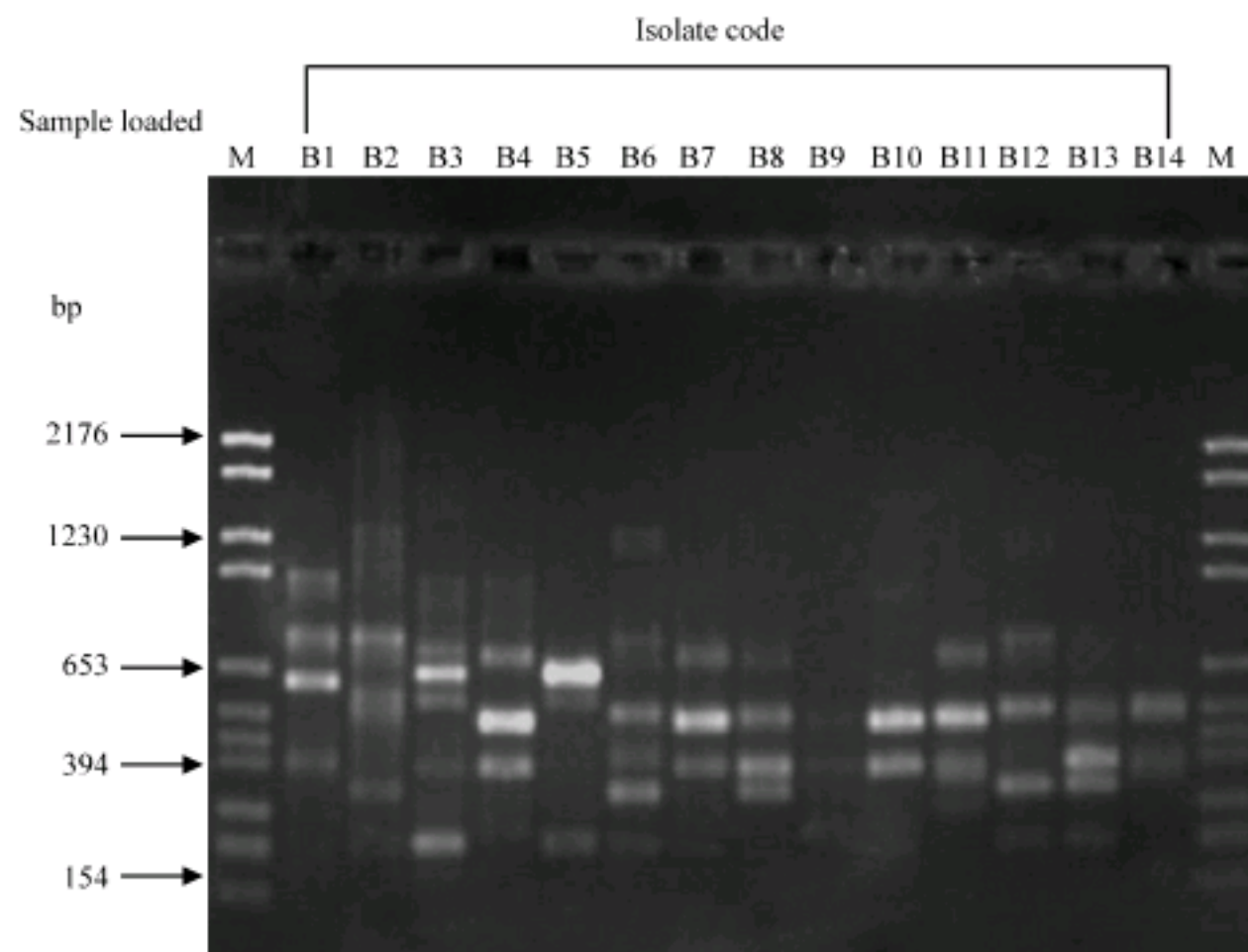


Fig. 2: RAPD-PCR patterns of *Bacillus subtilis* isolates with primer A1. Lanes on both sides of the gel labeled M are loaded with PBR 328 Mix DNA marker. B1 to B14 are the fourteen *Bacillus subtilis* isolates investigated. Isolates B1, B3, B8 and B9 are those of El-Malaha; B5 and B6 are of El-Amria; B4, B10, B11 and B12 are of El-Nobaria; B7, B13 and B14 are from Abo-Homos; B2 is that isolated from El-Sharkia

products produced by each primer varied from 46 with primer A1A13 to 75 with primer A9B7. However, primer A1A13 and A7 failed to show any amplified products with isolate B9 (El-Malaha).

The RAPD band patterns resulting from the use of the four primers were analyzed using UPGMA method to construct a similarity matrix and to generate a dendrogram indicating the relationship between the fourteen tested

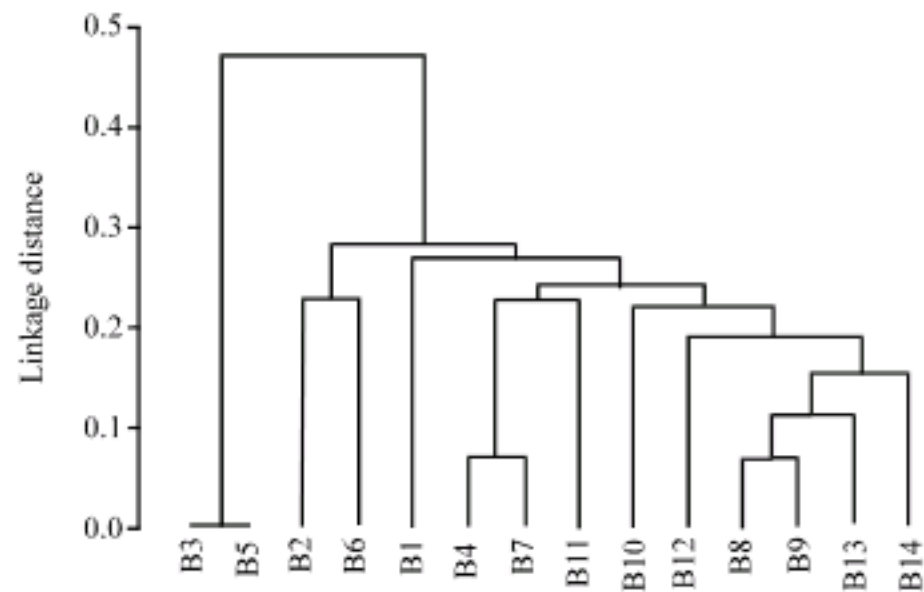


Fig. 3: Dendrogram obtained by clustering (UPGMA method) based on the band pattern obtained by the RAPD-PCR analysis of fourteen *Bacillus subtilis* isolates from different location according to Jaccard similarity

isolates of *B. subtilis*. The presence or absence of any particular DNA bands was the only parameter considered in the computer analysis. The dendrogram generated to show linkage distance (Fig. 3) indicated that the fourteen *B. subtilis* isolates were classified into two main Clusters (Cluster I and Cluster II). Cluster I represented only one group which included the two *B. subtilis* isolates B3 (El-Malaha) and B5 (El-Amria). Cluster II split into two Sub-clusters: Sub-cluster 1 that divided further into two Groups: Group 1 which included isolate B2 (El-Sharkia) and Group 2 which included B6 (El-Amria), whereas Sub-cluster 2 divided into two Groups: Group 1 included B1 (El-Malaha) and Group 2 divided into two Sub-groups: Sub-group 1 contained B11 (El-Nobaria), B4 (El-Nobaria) and B7 (Abo-Homos) and Sub-group 2 contained B10 (El-Nobaria), B12 (El-Nobaria), B14 (Abo-Homos), B13 (Abo-Homos), B8 (El-Malaha) and B9 (El-Malaha), respectively.

The results obtained when the differential display technique was applied to *B. subtilis* isolates B3 (El-Malaha) and B5 (El-Amria), which could not be differentiated when analyzed by the RAPD-PCR, using the primer P0 of the 16S rRNA gene revealed clear differences. While, three fragments differing in molecular size, 637, 1083 and 1767 bp were shown with isolate B3 (El-Malaha) but not shown with B5 (El-Amria). Two fragments of a size of 763 and 860 bp were appeared with B5 (El-Amria) but not with B3 (El-Malaha). Similarity results were obtained (Fig. 4) where isolate B4 (El-Nobaria) and B7 (Abo-Homos), which showed high percentage of similarity when exposed to RAPD analysis, were tested with the same primer (P0). While only one fragment of 860 bp was observed with isolate B4 (El-Nobaria) but not with B7 (Abo-Homos), four fragments differing in molecular size, 2361, 2457, 3398 and 3910 bp were detected with B7 but not with B4.

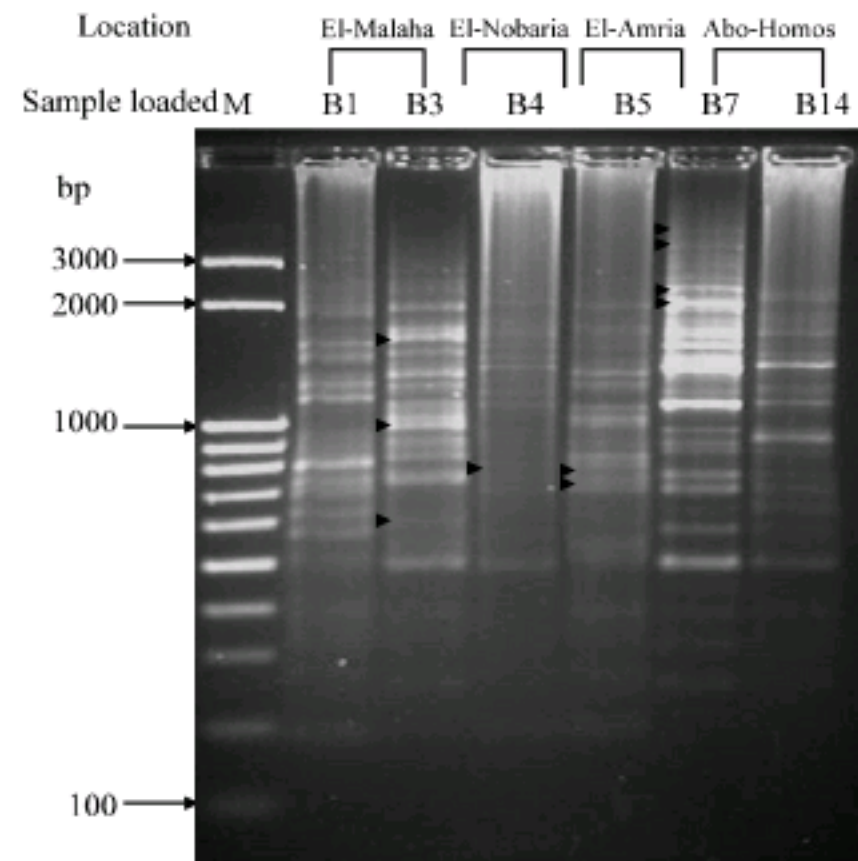


Fig. 4: Differential display RT-PCR for six *Bacillus subtilis* isolates using primer P0. The lane labeled M at the left-hand side of the gel was loaded with M25-3 k bp ladder; the other lanes were loaded with B1 and B3 (El-Malaha), B4 (El-Nobaria), B5 (El-Amria), B7 and B14 (Abo-Homos). Arrows within the gel point out polymorphic bands

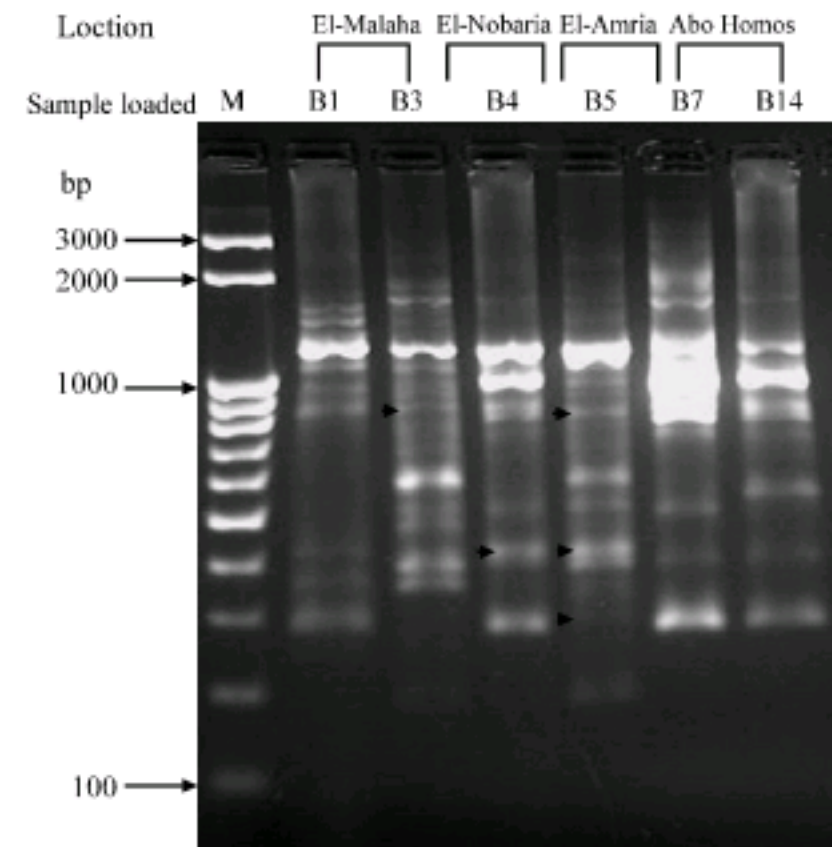


Fig. 5: Differential display RT-PCR for six *Bacillus subtilis* isolates using primer P1. The lane labeled M at the left-hand side of the gel was loaded with M25-3 k bp ladder; the other lanes were loaded with B1 and B3 (El-Malaha), B4 (El-Nobaria), B5 (El-Amria), B7 and B14 (Abo-Homos). Arrows within the gel point out polymorphic bands

Supportive results of polymorphism were observed (Fig. 5) when the arbitrary primer (P1) was used as, one fragment of a size of 980 bp was shown with isolate

Table 2: The accession No. and isolate code of 16S rRNA gene of *Bacillus subtilis* isolates in the GenBank

Isolate code	Location	Accession No.
zag1	El-Malaha	EF150883
zag4	El-Nobaria	EF150884
G-GANA7	Abo-Homos	EF583053
G-GANA8	El-Malaha	EF583054
G-FAT10	El-Nobaria	EF583055
G-HEB11	El-Nobaria	EF583056
zag14	Abo-Homos	EF150885

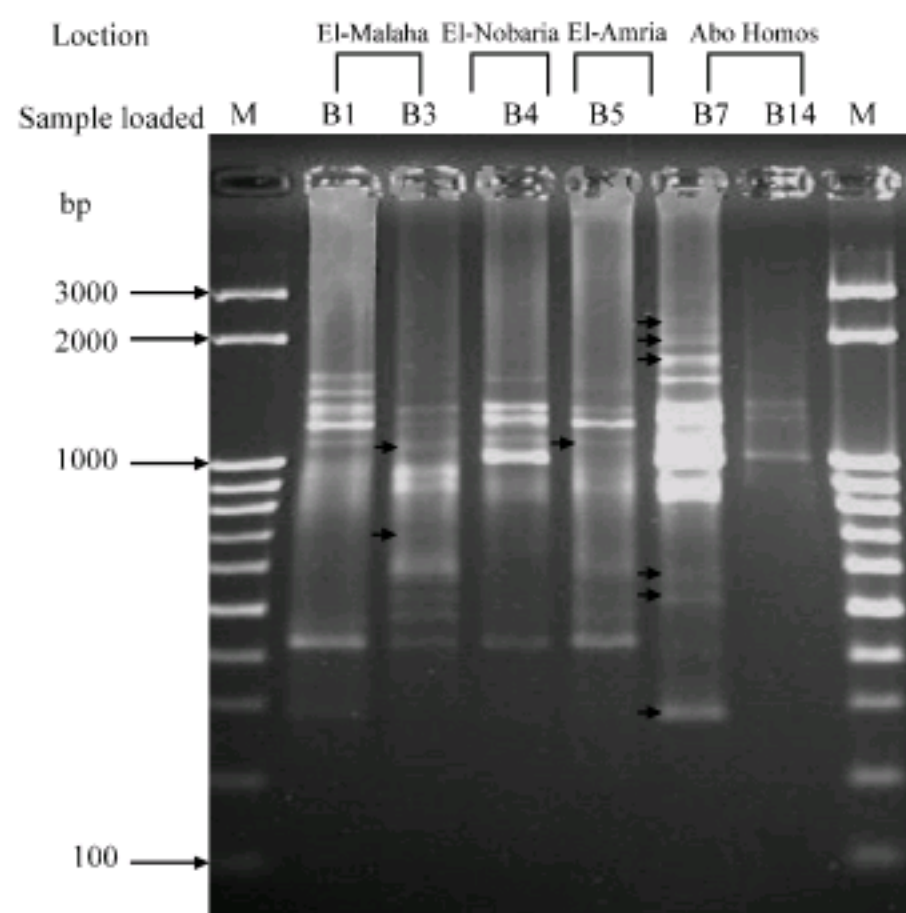


Fig. 6: Differential display RT-PCR for six *Bacillus subtilis* isolates using the arbitrary primer P2. The Lanes labelled M at both sides of the gel were loaded with M25-3 k bp ladder; the other lanes were loaded with B1 and B3 (El-Malaha), B4 (El-Nobaria), B5 (El-Amria), B7 and B14 (Abo-Homos). Arrows within the gel point out polymorphic bands

B3 (El-Malaha) but not with B5 (El-Amria). Three fragments of 295, 435 and 948 bp were appeared with B5 but not with B3. One fragment of 430 bp was evident with B4 (El-Nobaria) but not with B7 (Abo-Homos).

When differential display analysis with the arbitrary primer P2 was conducted (Fig. 6), two fragments of a size of 741 and 1186 bp were observed with B3 (El-Malaha), but not with B5 (El-Amria) and only one fragment of 1199 bp was detected with B5 with no corresponding band with B3. Likewise, the same primer gave six fragments (286, 522, 596, 1856, 2094 and 2335 bp) with B7 but these fragments were not generated with B4.

Approximately 1550 bp of the 16S rRNA gene was amplified for all the fourteen *B. subtilis* isolates using universal primers (Table 2). Consequently, the obtained amplicon was later subjected to sequencing analysis.

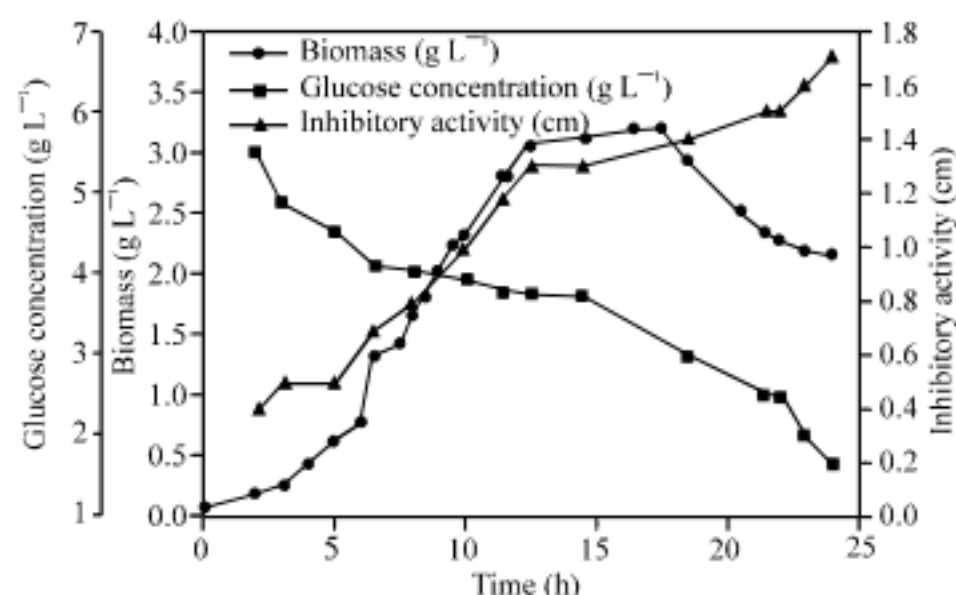


Fig. 7: Biomass, glucose concentration and inhibitory activity of culture supernatant as a function of time for batch cultivation of *Bacillus subtilis* isolate B7

When the purified product (1550 bp) of the 16S rRNA gene was subjected to sequencing using ABI PRISM model 310 automated DNA sequencer, the following partial sequence was obtained for *B. subtilis* isolates B1 (El-Malaha), B4 (El-Nobaria), B7 (Abo-Homos), B8 (El-Malaha), B10 (El-Nobaria), B11 (El-Nobaria) and B14 (Abo-Homos) (Table 2).

Search in databases to identify the bacteria was achieved in BLAST search at the National Center for Biotechnology Information site (<http://www.ncbi.nlm.nih.gov>). The search revealed that these sequences were identical to that of *B. subtilis*.

Bacterial isolates (B1, B4, B7, B8, B10, B11 and B14) were identified according to partial sequence of 16S rRNA gene as *B. subtilis* and scored in GenBank with accession No. (EF150883, EF150884, EF583053, EF583054, EF583055, EF583056 and EF150885) (Table 2).

Batch cultivation of *B. subtilis* isolate B7 was carried out in 3 L bench top bioreactor (Bioflow III, New Brunswick, NJ, USA) at a constant temperature of 30°C. Figure 7 shows biomass, concentration of consumed glucose and inhibitory activity of supernatant of isolate B7 plotted against the time. Cells were grown directly after the lag phase which lasted for about 2 h. Cell mass was then increased exponentially over time with a constant specific growth rate, $\mu = 0.3 \text{ h}^{-1}$ within the exponential phase. The behavior in this phase allows to calculate the yield coefficient $Y_{x/s}$ (0.45 g cells g glucose). During this phase, the specific growth rate of 0.3 h^{-1} was determined from the logarithmic relationship shown in Fig. 8. In the Fig. 8, the values of the $\ln X$ (values of X was obtained by using the correlation coefficient δ , 0.42 calculated from the linear relationship between dry mass weight and optical density) was presented by a straight line on a semi-logarithm plot versus time. The biomass achieved was 3.2 g L^{-1} . Inhibitory activity of supernatant was increased near the end of the stationary phase. The activity reached its highest value of 1.7 cm in death phase.

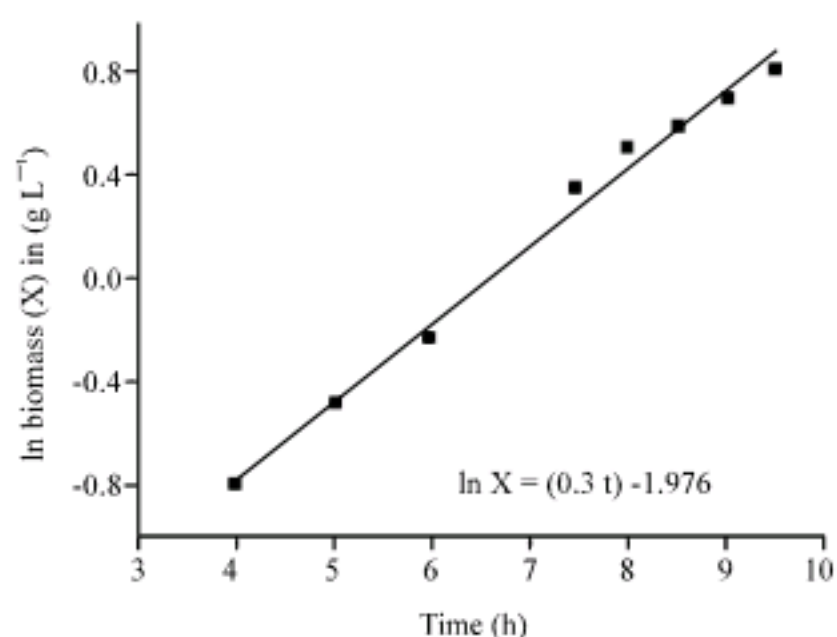


Fig. 8: ln biomass (X) in g L⁻¹ as a function of time in the exponential growth phase of batch cultivation of *Bacillus subtilis* isolate B7

Modeling of batch fermentation: The most common culture system is the batch culture, due to its simplicity and low cost. This is a closed system in which there is no input or output of materials. The microbial population cell density increases constantly until exhaustion of some limiting factor, while other nutrient components of the culture medium decrease over time. Any products produced by the cells during growth also increase in concentration in the culture medium.

In batch culture, it was important to calculate growth yield for *B. subtilis* during cultivation on glucose. Yield coefficient was calculated based on the amount of glucose consumed (Shuler and Kargi, 1992). The following equation was used to calculate growth yield (Y_{XS}).

$$Y_{XS} = \frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S} \quad (1)$$

where, X and X₀ are biomass concentrations (g L⁻¹) at measuring time t and initial time t₀, respectively. S and S₀ are the consumed amounts of glucose (g L⁻¹) at the same times mentioned previously.

In logarithmic growth phase, cell mass density increases exponentially with time t and specific growth rate μ (h⁻¹) is independent of nutrient concentration. According to Shuler and Kargi (1992), the exponential growth rate is first order.

$$dX/dt = \mu X, X = X_0 \quad \text{at } t = 0 \quad (2)$$

by integration of Eq. 2 yields

$$\ln (X/X_0) = \mu t, \text{ or } X = X_0 e^{\mu t} \quad (3)$$

The exponential growth is characterized by a straight line on a semi logarithm plot of ln X versus time.

DISCUSSION

Molecular techniques, such as RAPD-PCR, are powerful tools for obtaining data related to the genomic diversity that exists among the strains belonging to a bacterial species (genetic diversity). Istock *et al.* (2001) used RAPD-PCR for studying geographical diversity of genomic lineages in *B. subtilis* and Drake *et al.* (1996) used single primer G1, could distinguish 16 commercial strains of *Lactobacillus helveticus* by RAPD-PCR profiling. One hundred and twenty-six strains of *B. thuringiensis* representing 57 serovars were allocated to 58 genomic types using random amplified polymorphic DNA (RAPD)-PCR patterns (Gaviria and Priest, 2003). The fourteen *B. subtilis* isolates obtained in this study from different areas in Egypt could be differentiated using random amplified polymorphic DNA technique and it was possible to categorize them in two main Clusters, Cluster I which included one group with two *B. subtilis* isolates, B3 and B5 (similarity 100%) and Cluster II which encompassed four groups containing twelve *B. subtilis* isolates.

Differential display is a molecular biology technique that can distinguish between genes that are up- or down regulated. The failure of RAPD-PCR to show any variation between *B. subtilis* isolates B3 (El-Malaha) and B5 (El-Amria), accside the high similarity percentage between B4 (El-Nobaria) and B7 (Abo-Homos) it demonstrated, differential display technique was performed for obtaining more confirmative results and to possibly differentiate between a members of each pair (B3, B5 and B4, B7). Differentiation was possible using a specific primer to the housekeeping gene, 16S rRNA. When differential display analysis with the arbitrary primer P2 was conducted. This was evident as the two fragment of a size of 741 and 1186 bp which were observed with B3 (El-Malaha) were not found with B5 (El-Amria) and the only one fragment of 1199 bp which was shown with B5 was not detectable with B3. Polymorphism reached its maximum when the same primer (P2) was used to analyze the pair of isolates B4 and B7. This was evident when the six fragments seizing 286, 522, 596, 1856, 2094 and 2335 bp which were obtained with B7 were not present with B4.

The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level (Sacchi *et al.*, 2002). The sequencing of the 16S rRNA and the alignment with sequences present in the GenBank database resulted in the final identification of the studied isolates. The sequencing of 16S rRNA has also successfully been used

for identification of *B. subtilis*, *B. anthracis* and *B. cereus* in other studies (Ash *et al.*, 1991; Ash and Collins, 1992; Green *et al.*, 1999; Siefert *et al.*, 2000).

In batch culture, the lag phase is characterized by a negligible increase in the biomass concentration which lasted for a bout 2 h. The exponential phase is characterized by a sharp exponential increase in the biomass concentration, cell mass was increased exponentially over time with a constant specific growth rate 0.3 h^{-1} , the overall biomass yield was 0.45 g cell/g glucose. At the stationary phase, the growth rate was zero (no cell division), these due to the depletion of substrate, oxygen insufficiency, accumulation of inhibitory metabolites such as acetic acid and also due to the beginning of the sporulation process by *B. subtilis*. Finally during the death phase the cell growth had a negative growth. The growth process is divided into two main stages: vegetative stage and sporulative stage. A *B. subtilis* cell is in its vegetative stage if it is capable of dividing. On the other hand, a cell is in sporulative stage if the cell stops its division and uses the available energy to form a spore.

Unlike the growth phases, the metabolic stages cannot be identified by a change in the biomass concentration with time. The metabolic stages might, however, be identified by the cell morphology, the cell oxygen consumption and the change in the pH of the medium. Regarding the morphology of a vegetative *B. subtilis* cell it can be characterized on the basis of cell structure and the absence of a spore. In this respect, during the vegetative stage, the cells consume the substrates and require high amounts of oxygen and several acids such as acetic acid are produced. As a result, the pH decreases in the liquid medium.

Concerning sporulation, the shift from vegetative to sporulation stage occurs when the nutrients are depleted. In this case, the acids produced during the vegetative stage are already oxidized and the cells oxygen demand is very low. Therefore, the pH remains constant.

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