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Cleaved Amplified Polymorphic Sequences (CAPS) Of *Bacillus Cereus* And Mutagenic Effect Of Acridine Orange On Alpha Amylase Production.

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ABSTRACT

The current study aimed to isolate and characterize local isolates from Egyptian soils using 16s rRNA technique and improve α-amylase enzyme production by Acridine orange (AO) as a mutagenic agent. The results of molecular characterization dependent on 16S rRNA using NCBI BLAST search assures that these isolates were closely related to the *Bacillus cereus.* Mutagenesis by (AO) concentration 0, 10, 20, 30, 40, 50, 60 and 70 µg/mL was used for induction a mutation from *Bacillus cereus*. Alpha amylase production was determined in wild type and its mutants. Sixteen mutants gave relatively better amylase production than wild type. These mutants could be used in industries for the efficient conversion of starch to oligosaccharides. Cleaved Amplified Polymorphic Sequence (CAPS) for the analysis of genetic marker of 16S rRNA gene of *Bacillus cereus* and mutants with HaeIII enzyme has done and show the different fragments in mutants comparing with the wild type. These results gave that this technique PCR doubled with restriction enzyme analysis (CAPS) may be used to more quickly analyses the results for detection and identify the bacterial.

Keywords: 16S rRNA, HaeIII, CAPS, AO.

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INTRODUCTION

The members of the genus *Bacillus* cover an excellent diversity of microorganism species and have an omnipresent distribution within the atmosphere as a result of their impedance spores and also the capability of vegetative cells to secrete a large style of enzymes, this genus is very important for the assembly of commercially important biomolecules (insecticides, enzymes, antibiotics, etc.) Kramer and Gilbert (1992).

α-amylase (EC 3.2.1.1), an enzyme degrades α-1,4-glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides consist of maltose, glucose and alpha limit dextrin.

The enzyme is employed particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal and oil drilling (Ajayi and Fagade, 2003). Normal mutation and choice techniques are exploited for the advance of microorganism cultures. Chemicals like nitrous acid are normally used (Prescott and Dunn's, 1987). Further work remains lacked on the optimum of fermentation conditions for α-amylase growth by mutant strain of *B. cereus*. within this study, a bacterial strain of *B. cereus* was refinement for α-amylase production using acridine orange (A0) as a mutagen. Screening and chosen of a hyper producer were performed.

In a recent study, different molecular material is in experimental protocols. The molecular techniques are gauge tools for the analysis of microorganisms from feed and other biological substances. These techniques provide methods to screen for a broad range of agents during one test Field and Wills (1998). Molecular techniques are employed for strain identification. Among them, the RFLP fingerprinting technique is taken into account the foremost sensible method for strain identification. PCR-RFLP (CAPS) can replace AFLP, RAPD within the estimation of genetic diversity Lu *et al.,* (1996). within this study, the genetics of *Bacillus cereus* studied by molecular analysis. restriction endonucleases are the foremost serious tools in gene manipulation techniques. Jang *et al.* 2000 have projected a universal PCR capable of amplifying variety of the 16S rRNA gene of eubacteria, comprising *Staphylococcus aureus.*

The format of the amplified products from multiple forms of bacteria were the same (996 bp), but the restriction shapes of whole PCR maker generated by HaeIII digestion were unlike.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Samples of agricultural soil were collected from Tanta Governorate (Egypt). The soil samples were serially diluted and spread onto nutrient agar medium. The isolated bacterial colonies were purified to homogeneity by quadrant streaking, stored in nutrient agar slants. The bacteria isolated were tested for α-amylase production.

Induction of mutation with acridine orange

Acridine orange (AO) was filter-sterilized and AO concentrations between 10 and 100 µg⁄ mL was dispensed in test tubes containing 2 mL of nutrient broth with one tube kept as control. These tubes were inoculated with 0.5 mL of overnight grown culture, incubated at 37°C for 18 h and centrifuged at 3000 g. Acridine orange was removed by repeated washings with water. Cells were suspended in phosphate buffer (pH 7.5) and absorbance was taken at 550nm to assess the growth and MIC of AO. In order to test the mutagenicity of AO, treated *B. cereus* cells were grown by plating serial dilutions onto nutrient agar and the frequencies of viable cells in the total bacterial population were determined by comparing the number of colonies to the volume of sample plated and dilution used. Data from three such independent experiments were pooled.

Production Medium

Soluble starch 10g, Peptone 5g, KH_2PO_4 1g, Yeast extract 5g, NaCl 5g, $MgSO_4$ 0.2g, $CaCO_2$ 0.2g, pH 7.0 (Haq *et al.,* 2010).

Luria broth Medium(LB)

Luria- Bertani broth (LB) used to propagate bacterial strains at 37^0C with vigorous aeration. For solidification the medium, 1.5 % of agar was added (**Davis *et al.,* 1980).**

Minimal medium (Faires*et al.,* 1999)

K_2HPO_4 70mM, KH_2PO_4 30 mM, $(NH_4)_2SO_4$ 25 mM, $MgSO_4$ 0.5 mM, $MnSO_4$ 10 mM, Ferric ammonium citrate 22 mg**,** Glucose 1g, Agar was added as solidified agent 2% (w/v).

Selection and characterization of mutants

The bacterial isolates were grown in nutrient broth media for 7–8 h at 37°C. Two milliliters of AO solution (50 µg/mL) were added to an equal volume of half-grown cultures. These cultures were incubated overnight at 37°C and bacterial cells were harvested by centrifugation. Treated cells that were presumed to be mutants and untreated ones (control) were inoculated into nutrient broth and incubated at 37°C for 18 h. Overnight grown cultures were serially diluted in nutrient broth and were grown on nutrient agar containing 1% soluble starch. The colonies were replica plated after overnight growth and were stained with iodine reagent (0.1 M iodine in 0.4 mol. KI)

Enzyme assay

α- amylase was estimated according to the method of Rick and Stegbauer (1974). One milliliter of enzyme extract was added to a test tube containing 1.0 mLof 1.0 % soluble starch solution, pH 7.0. The mixture was incubated at 40ºC for 10 min. After the incubation, 3.0 mL of DNS reagent was added to each of the tubes. The tubes were placed in boiling water for 5 min and cooled to room temperature. The contents of tubes were diluted up to 10 mL with distilled water. The optical density (OD) of reaction mixture was determined at 570 nm using a spectrophotometer. One unit of enzyme activity is equivalent to that amount of enzyme, which in 1 min librates1mg reducing sugars from 1% soluble starch.

DNA Extraction

DNA extraction and purification were carried out according DNeasy Blood & Tissue Kits (QIAGEN-Germany).

DNA Preparation and PCR Amplification

A 1600bp fragment was amplified from the 16S rRNA gene using universal primers 8F (5-AGTTGATCCTGGCTCAG-3), and reverse primer 1492R (5-ACCTTGTTACGACTT-3) were used to amplify the 16S rRNA gene with the program a denaturation at 94°C for 5 min and subsequent 35 cycles of denaturation at 94°C for 30 sec. annealing at 55°C for 30 sec. and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl the reaction product in a 1% agarose gel.

16S rRNA Sequencing and Data Analysis

Sequencing analysis was performed on a 1600 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rRNA sequences were aligned and compared with other 16S rRNA in the GenBank by using the NCBI Basic Local alignment search tools BLAST-n program (http://www.ncbi.nlm.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000.

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PCR–RFLP (Cleaved Amplified Polymorphic Sequences (CAPS)

Further reaction mixture for restriction digestion was prepared by mixing 8.5 µL of purified PCR products, 5 U of restriction endonuclease, HaeIII (Fermentas) and 1.0 µL of 10X recommendation buffer. Reaction mixture was incubated overnight in water bath at 37°C. Restriction digested DNA was analyzed by horizontal electrophoresis in 2% agarose gels with 100 bp DNA marker. The gels were visualized on a gel documentation system (Alpha Innotech). Photograph of gel has been shown in Figure 2.

Data analysis

Phylogenetic analysis was performed using Past3- Software. For phylogenetic study of this data matrix consisting of 16s PCR–RFLP profile data were transformed to estimate distances (Nei and Li, 1979). The unweighted pair group method using arithmetic averages (UPGMA) was used for cluster analysis (Sokal and Michener 1958).

RESULTS AND DISCUSSION

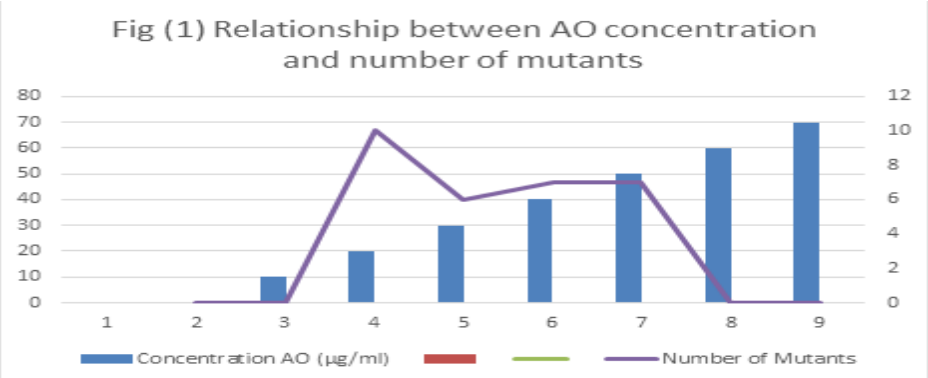
Effect of AO on growth and survival

Bacterial development was gradually suppressed by increasing the concentration of AO from zero to 70 µg/mL (Table 1 and Fig.1). The results in Table 1 and Fig.1 showed that a little bit or negligible growth was observed at 50 and 60 µg/ mL of AO and no growth was found out with AO concentrations higher than (70 µg/ mL). *Bacillus cereus* survival percent mean for the seven concentrations were found to be; 100, 67.66, 45.93, 30.48, 21.69,15.37, 12.33and 0.0 respectively. Negative relation was found between AO concentrations and survival percentage mean.

Table 1: *Bacillus cereus* cell survived different AO concentrations and mutant number.

Concentration AO (µg/mL)	No. of cell survived/mL (10^5)	Survival (%)	Number of Mutants
0.0	288.33	100	0.0
10	195.11	67.66	0.0
20	132.44	45.93	10
30	87.88	30.48	6
40	62.55	21.69	7
50	44.33	15.37	7
60	12.33	4.27	0.0
70	0.0	0.0	0.0

The data showed in Table (1) and Fig (1) revealed that the effect of AO on bacteria survival was severe at all concentrations and the complete lethality was obtained at 70µg/mL LMM. 30 mutants from all concentrations were selected on complete and minimal media at 30 ºC. These results were agreements with (Francis, 2011) on *Xanthomonas campestris*.



Evaluation of alpha amylase production of *Bacillus cereus* and their mutants as follow

Table (2) showed 30 mutants of the parental strain *Bacillus cereus.* (AO) were screened for enhanced α-amylase production, the 14 mutants compared to the parental strain; showed low enzyme productivity however, the rest mutants (16) gave relatively better amylase production. Employees have employed random mutagenesis for α-amylase production by exposure the organism to chemically like EMS (Nadeem et al., 2010 and Haq *et al.,* 2012) in genus *Bacillus*. This increase happened due to increase in gene transcribe number and amplification of the DNA area (Cherry, *et al*.; 2009) in *Aspergillus niger*.

Table 2: Alpha amylase production of *Bacillus cereus* and the 30 mutants growing on nutrient starch agar medium for 3 days on 30°C.

Mutant code	AO (µg/mL)	Enzyme activity U /mL /min
Wild type	0.0	107
M1	20	117
M2	20	120
M3	20	119
M4	20	119
M5	20	123
M6	20	128
M7	20	125
M8	20	165
M9	20	127
M10	20	121
M11	30	119
M12	30	120
M13	30	129
M14	30	130
M15	30	131
M16	30	120
M17	40	101
M18	40	110
M19	40	310
M20	40	107
M21	40	106
M22	40	100
M23	40	110
M24	50	100
M25	50	104
M26	50	105
M27	50	90
M28	50	80
M29	50	88
M30	50	95

16S rRNA PCR

DNA isolated from wild Type and some of mutants were tested by the universal 16S rRNA PCR. Each the DNA pattern generated a PCR output of the expected size 1500 bp (Fig.2).

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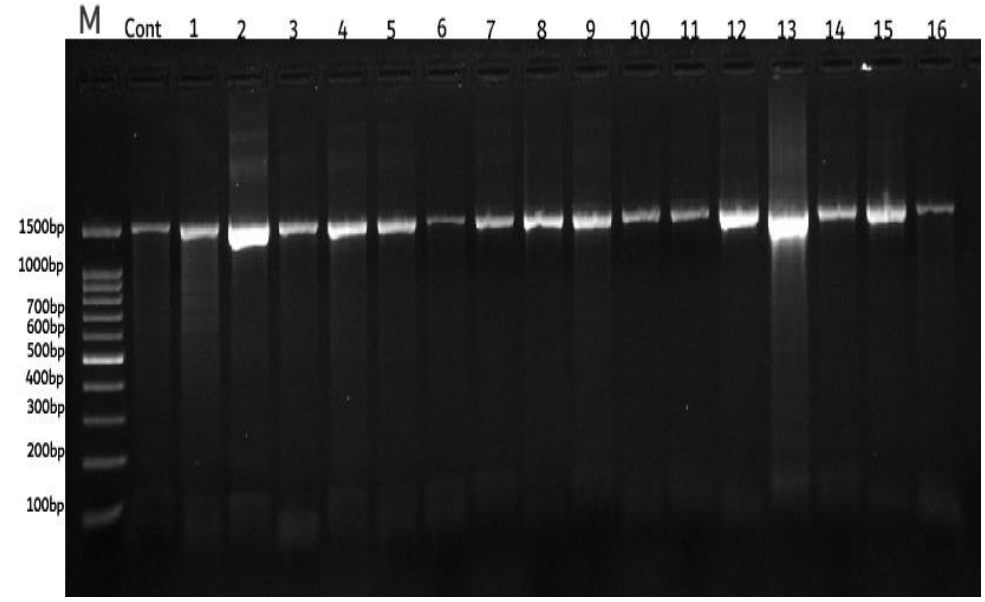

Figure 2: Electrophoresis on 1% agarose gel of universal PCR products: Pattern in various lanes, that are the same, were PCR outputs from wild type, (lane 1), mutants (lane 2 to lane 17) and M: molecular weight marker, DNA ladder.

PCR-RFLP markers: **Cleaved Amplified Polymorphic Sequences (CAPS)**.

16S rRNA - PCR–RFLP analysis

To discover the genetic relationship between each mutants and wild type Including 16S rRNA PCR–RFLP was executed. The position of 16S rRNA genes was amplified and assimilation with HaeIII (Fig.3). The data showed that 3–4 DNA fragments of 120–600 bp was spotted in each mutant and wild type (Fig.3). Depends on the number of fragments generated in all mutant different pattern were observed. In control; *Bacillus cereus;* four patterns were observed and showed similar banding pattern to mutant no 2,7,8,9,11,12,13,15 and 16. The mutant of *Bacillus cereus* handed different banding patterns with control were 3 to 6, 10 and 14.

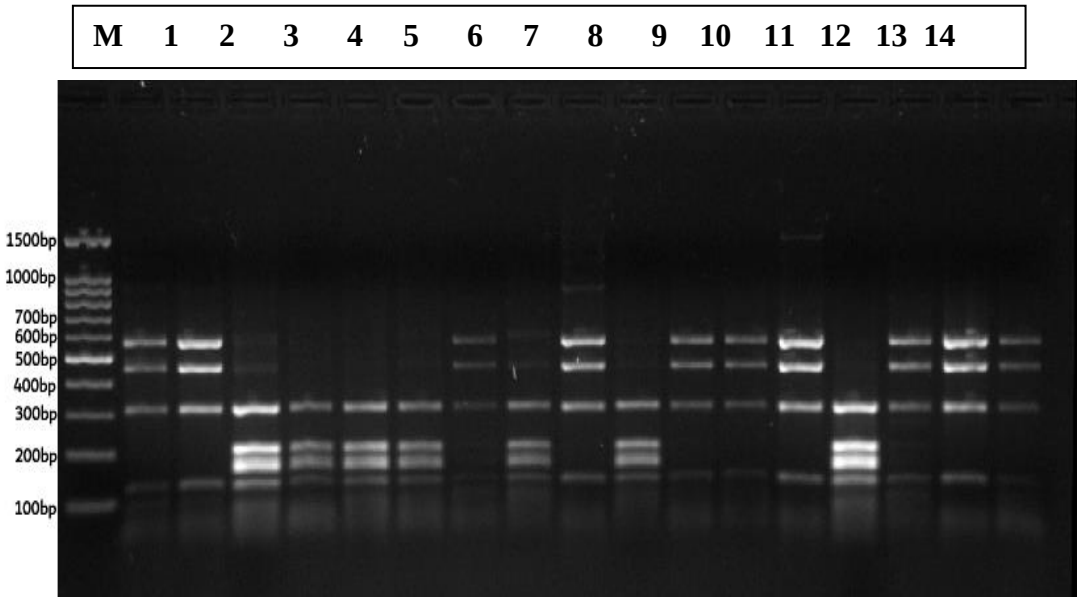

Figure 3: HaeIII digestion shapes of universal PCR products. Samples in various lanes were HaeIII-digested PCR output from the following bacteria: lane 1 wild type; lane 2 to lane 17 mutants, M; molecular weight marker, DNA ladder.

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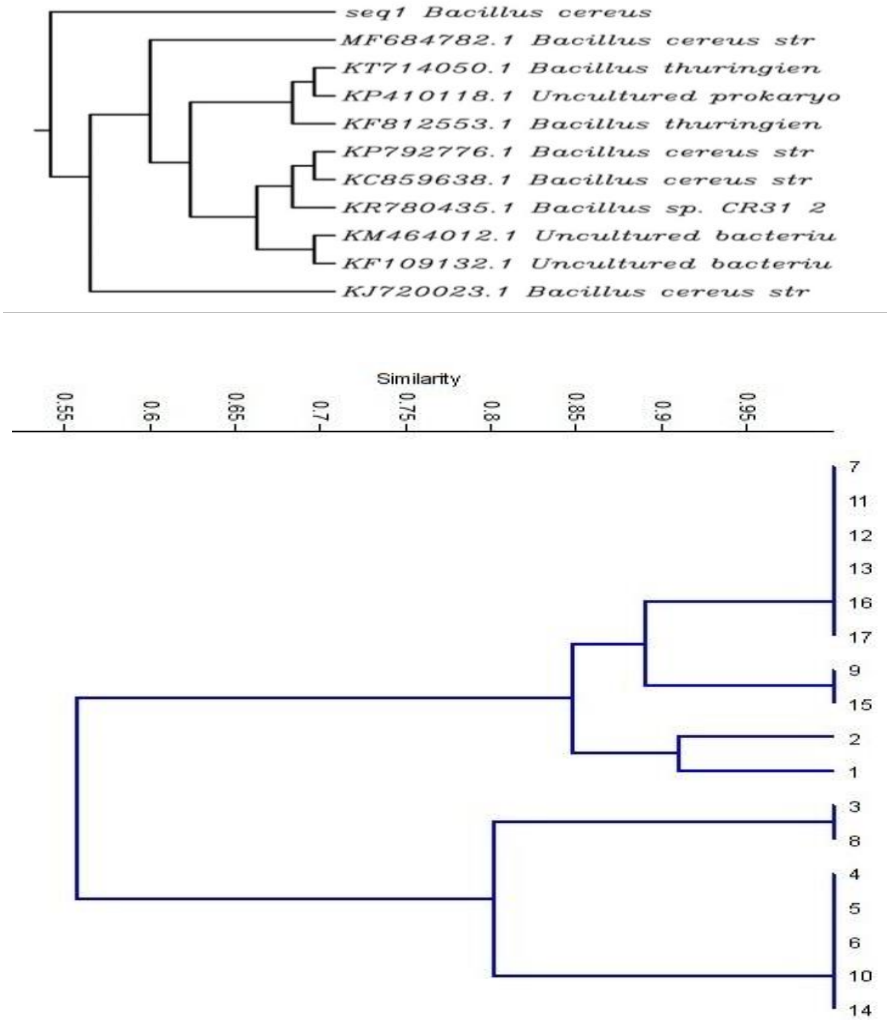
Dendrogram derived nixing numerical test of 16s PCR–RFLP data collected the wild type and mutants in two main clusters (Fig. 4). The restriction assimilates of amplified 16S rRNA gene by HaeIII enzyme have given a fragment of concerning 300 bp length in every mutant and wild type. Sequence datum of this fragment (from NCBI) were used to find certainly length of the fragment (300 bp) and to evolution specific primers for amplification of this line. Further sequence input and multiple alignment of 300 bp long sequences of various species of *Bacillus* has revealed that this is an easy material for classification of the members of *Bacillus* and related genera. New interest information provided by our study is that almost each species of Bacillus and belonged genera have restriction enzyme position for HaeIII enzyme which give a product of 300 bp. These results agree with Rahmani*, et al.* (2006) on *Pseudomonas aeruginosa*

Figure 4: Phylogenetic tree depends on partial 16s PCR–RFLP sequences, showing the relations among wild type *Bacillus cereus* and mutants. The tree was constructed utilization the CLUSTAL-X and neighbor-joining method.



CONCLUSION

These studies suggest that the universal PCR method accompanied with RFLP is a very valuable and rapid method, for disclosure and identification of bacteria.

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