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Genetic enhancement of Bacillus cyclodextrin glycosyltransferase production

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Abstract

The bacterial isolates obtained from the botanical garden soil of the National Research Center in Dokki, Giza, Egypt, showed high levels of cyclodextrin glycosyltransferase (CGTase) activity. The CGTase superior *Bacillus* (WT3) was classified as *Bacillus hunanensis* using the 16S-ribosomal RNA sequence and the Basic Local Alignment Search Tool (BLAST). The mutagen ethyl methane sulfonate (EMS) was used to create CGTase mutants of WT3 that produced more. After the testing of 30 mutants chosen in a screening assay, the best mutant for CGTase biosynthesis was WT3-60-8, which produced 84.19 U/mL with a 110.6% relationship to the parental-strain. Protoplast fusions between WT3-60-4 and WT3-60-8 mutants were carried out to increase CGTase activity. Twenty-seven fusants were collected, with fusant (F1-8) showing a 122.4 percent improvement in CGTase activity over its parental strain. Many different DNA banding patterns were observed while fingerprinting with random amplified polymorphic DNA (RAPD). Furthermore, the genetic backgrounds of the two excellent mutants and four superior fusants, as well as the parental-strain, were divided into three clusters, the phylogenetic tree was obtained, and the genetic backgrounds of the two excellent mutants and four superior fusants, as well as the parental-strain, were detected based on genetic distances.

Keywords: *Bacillus*, CGTase, molecular-identification, mutants, fusants, phylogenetic-analysis.

1. Introduction

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) is a key microbial amylolytic activity and a bacterial glycosyl transferase. This enzyme is considered as a type of the glycoside hydrolase family, which is recognized as a-amylase and is the biggest family among glycoside hydrolases **[1]**. CGTase converts starch to cyclodextrin through cyclization activity **[2]**. Cyclodextrin (CD), as the main and most important product of CGTase, is a non-reducing closed-ring malt. CGTases produce α-, β- and γ-cyclodextrins (CDs) which are cyclic forms containing eight, seven and six glucose units respectively, linked with α-1,4-bonds. They also produce larger CDs and oligosaccharides. CGTases are classified as α-, β- and γ- CGTase based on their primary cyclodextrin product. CDs have been known for their modern applications in the agricultural, cosmetic, textile, food, pharmaceutical and chemical industries due to their ability to form inclusion complexes with small hydrophobic molecules **[3]**. Inclusion of organic or inorganic molecules in CDs changes their properties like solubility, reactivity, heat and light susceptibility, etc. **[4]**. Among the three CDs, β-CD is the most useful due to cavity diameter of 7-8A **[5]** and due to the ease of separation of β-CD **[6]**.

The CGTases are known to be produced by various genera of bacterial kingdom such as *Bacillus, Klebsiella, Pseudomonas, Brevibacterium, Thermoanaero bacterium, Corynebacterium, Micrococcus, Clostridium* etc. Several CGTase producers are *Bacillus lehensis* S8 **[7]** *Bacillus* sp. TS1-1, **[8]** *B. megaterium*, , **[9]** *Paenibacillus pabuli* US132 **[10]** and *Klebsiella pneumonia* AS-22 **[11]**. *Bacillus* species are well-known natural producers of CGTase **[12]**. All the organisms producing CGTase that can convert starch predominantly into β-CD, also produce α-, γ-CD and malto-oligosaccharides in varying ratios. However, enzymes that can produce β-CD in higher ratios and small amounts of other CDs and malto-oligosaccharides are important **[13].**

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In a fermentation process, the enhancement of enzyme yields is greatly influenced by physical and biological parameters. The optimal design of the culture medium and the environmental conditions are very critical aspects in the metabolic production and growth of microbial population and development of fermentation processes **[14]**. There are many studies conducted for the purpose of fermentation optimization by several researchers, e.g., **[14, 9, 15, 16, 12].** Screening for the high-yielding enzyme producer microbes considered to be as an important pathway for process development **[17]**. The *Bacillus* genus represents a group of microorganisms that generate several kinds of industrially important enzymes.

In addition, the random amplified polymorphic DNA (RAPD) technique has been used to establish the genetic relationships within various microbial isolates through DNA-fingerprinting of the microbial strains. RAPD assay of genomic polymorphism has been used to compare differences between the various bacterial strains at intraspecific and interspecific levels. **[18, 19**, **20].** This study was therefore initiated to take advantage of the isolation, molecular identification, mutagenesis and protoplast fusion in *Bacillus* to obtain CGTase overproducing strains and molecular typing of the excellent mutants and fusants.

2. Materials and methods

2.1. Isolation of CGTase producing bacteria:

Soil samples from the botanical garden of National Research Center, Dokki, Egypt, were collected in the sterile culture tubes. The tubes were labeled properly and stored at 4ºC until examination. CGTase producing bacteria isolation was performed by suspending 1 gm of soil sample in 9 ml of sterile distilled water. After that, the mixture was vortexed vigorously and serially diluted up to 10^{-3} with sterile distilled water. An aliquot of 0.1 ml from each dilution was taken and then spread evenly over the surface of Horikoshi medium Usharani et al., **[21]**: containing 2% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.02% $MgSO_4$.$7H_2$ O, 0.1% K_2 HPO_4 , 1% Na_2 CO_3 , 0.02% phenolphthalein and 2% agar was utilized. The medium was adjusted to pH 9.5 to isolate alkaliphilic bacteria and incubated at 37^0 C. Isolated colonies around which a pink hollow zone was observed were retested and considered CGTase producers for further study.

2.2 Molecular identification of *Bacillus strain*:

Using the GeneJET Genomic DNA Purification Kit procedure, deoxyribonucleic acid (DNA) was extracted from the *Bacillus* strain (WT3) (Thermo K0721, Thermo-Fisher Scientific, Inc., Waltham, Massachusetts, USA). The 16S ribosomal gene was amplified using Maxima Hot Start PCR Master Mix (Thermo K1051, Thermo-Fisher Scientific, Inc., Waltham, Massachusetts, USA), with the following nucleotide sequences for the 16S primers: 27F primer-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R primer-5' TACGGTTACCTTGTTACGACTT -3'. GeneJET PCR Purification Kit was used to clean up the PCR product (Thermo K0701). The PCR product's DNA was sequenced using forward and reverse primers on an Applied Biosystems (ABI) 3730xl DNA sequencer (GATC Biotech, Constance, Germany).

2.3. Phylogenetic analysis:

Using the Basic Local Alignment Search Tool, the *Bacillus* strain (WT3) 16S ribosomal DNA (rDNA) sequence was compared to sequences in the National Center for Biotechnology Information (NCBI) GenBank database (BLAST). The sequences were compared to those of reference taxa obtained from publicly available databases. The evolutionary distances were calculated using a parameter model, and the phylogenetic tree was generated based on NCBI Neighbor Joining. Under the accession number MN310554.1, the 16S rDNA sequence was submitted to the NCBI-Gene Banklt nucleotide sequence database.

2.4. Enzyme Production:

The enzyme was produced in bulk through submerged fermentation at 37°C on shaker incubator 150 rpm using the modified Horikoshi's medium Usharani et al., **[21]** (containing 2% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.02% $MgSO_4$.$7H_2$ O, 0.1% K_2 HPO_4 , 1% Na_2 CO_3 at pH 9). 2 ml of broth (Horikoshi's medium) inoculated with a loopful of pure culture and allowed to grow at 37°C for 48 hours served as inoculum for submerged fermentation. The broth was harvested on the 4th day and was centrifuged at 10,000 rpm for 5 min to obtain the cell-free extract; the supernatant served as the source of CGTase.

2.5. Enzyme Assay:

CGTase activity was measured as β-CD forming activity according to Goel and Nene **[22]** with some modification as the follow: Aliquot (750 µL) of 40 mg starch solution prepared in 100 mL (50 mM sodium phosphate buffer pH7), was pre-incubated at 50°C for 5 min. Then, 100 µL of enzyme sample was added and after incubating for 20 min at 50ºC, the reaction was quenched by adding 250 µL of Na_2 CO_3 (1M) in water. Subsequently, 200 µL of 0.012gm phenolphthalein prepared in 100ml of (95% Ethanol) was added and add 700µL of distilled Water after standing at room temperature for 15 min, the color intensity was measured at 550 nm, as a standard, the

soluble starch and enzyme were replaced by 0.5 mg of β-CD and 0.1 ml of water, respectively. A calibration curve was made using β-CD (Sigma- Aldrich, St. Louis, MO, USA) in 100 mM sodium phosphate buffer at pH 6.0. One unit of enzyme activity was defined as the amount of enzyme that formed 1µmol β-CD per min under the conditions defined above.

2.6. EMS Mutagenesis:

The ability of bacteria to generate random mutations was assessed using the EMS method (Ethyl methane sulfonate (EMS, C3H8O3S) is a mutagenic and carcinogenic volatile organic solvent. It produces nucleotide substitution, specifically guanine alkylation, causes random mutations in DNA. Five ml of 24 h old *Bacillus* strain is centrifuged (5.600 x g, 4ºC for 3 min) separately grown in Horikoshi's medium. EMS was applied to a final concentration of 200 mM after the cells were resuspended in 5 ml sodium phosphate buffer (pH 7.0, 50 mM) according to Bazaraa et al., **[23]**. The tube was shacked for 20, 40, and 60 minutes at 30ºC. To neutralize EMS, 500 µl sodium thiosulphate (0.4 M) was added after the required time. The cells were harvested by centrifugation at (5.600 x g, 10 min, at 4ºC) and washed twice with the same buffer. The formed pellet was reconstituted in phosphate buffer (pH 7.0, 50 mM), and sufficient dilutions were plated on the Horikoshi medium surface and incubated for 2 days at 37ºC. After calculating the number of colonies mutagen after growth, the colonies with the highly pink zone were chosen for further study.

2.7. Protoplast Fusion:

2.7.1. Antibiotic resistance tests:

On mutant strains, an initial antibiotic sensitivity test was carried out with different antibiotic discs containing (µg/disc): ampicillin (AM), 10; amoxicillin/clavulanic acid (AMC), 30; amoxicillin (AML), 25; chloramphenicol (C), 10; gentamycin (GM), 10; colistin (CT), 25; doxycycline (DO), 30; rifampicin SV (RF), 5; ampicillin/sulbactam (SAM), 20 and streptomycin (S), 10. For the collection of resistant mutants, the most appropriate antibiotic disc was used. The antibiotic resistant mutants were collected from the nearest area around the discs (inhibition zone) and transferred to Horikoshi's plates for further studies.

2.7.2. Protoplast formation buffer (P)

The used protoplast formation buffer **[24]** contained (g/L): NH_4Cl_2, 1.25; sucrose, 171.15; $MgCl_2$ $6H_2O$, 5.33; Na_2SO_4 $10H_2O$, 0.373; NaCl, 0.075; KCl, 0.045 and tris base, 15.

2.7.3. Protoplast regeneration medium (DM3)

The medium described by Jensen and Hulett **[25]** was utilized, and it contained (g/L): glucose, 10; $MgCl_2$ $6H_2O$, 4.1; yeast extract, 10; casamino acid, 10; K_2HPO_4, 3.5; KH_2PO_4, 1.5; sodium succinate, 13.5; NaCl, 10 and bovine serum albumin (BSA), 0.5. The pH was adjusted to 7.3. To prepare such medium, all components (without BSA) with constant heating (50 °C in water bath) and stirring. Then 2% agar was added, and this solution was sterilized at 121 ºC for 15 min. BSA was dissolved in the remained DM3 (without BSA), sterilized by filtration (0.45 µ Millipore filter) and directly added to the medium exactly before pouring at 50 ºC.

2.7.4. Selection of the proposed cross

Antibiotic susceptibility testing was first performed on mutant strains, which obtained after EMS-mutagenesis of WT3 wild type strain, using the above antibiotic discs to perform protoplast fusion. The appropriate protoplast crossing was chosen based on disc resistance and sensitivity.

2.7.5. Protoplast fusion process

According to Jensen and Hulett **[25]**, protoplast fusion was performed. Each parental strain was inoculated into ten ml of Horikoshi's broth medium and incubated overnight at 37 ºC. Cells were then collected, washed by protoplast formation buffer (P) and resuspended in 2 ml of the same buffer containing 1mg of lysozyme. The cells were incubated at 37 ºC with gentle agitation (100 rpm). The formation of protoplasts was confirmed microscopically using a phase contrast microscope. Within one hour of incubation, cell wall removal and protoplast formation were observed. Protoplasts were harvested and resuspended in P after centrifugation at 4000 x g for 10 minutes at 4 ºC. Two protoplast suspensions from different parents were mixed in 1:1 ratio, and then harvested by centrifugation at 4000 x g for 10 min at 4 ºC. The pellet was suspended in 1 ml P containing 100 l polyethylene glycol (PEG) 4000 (40 percent) for 10 minutes after the supernatant was discarded. The pellet was resuspended in 3 ml of PB after centrifugation of the suspension. Five millilitres of soft DM3 (0.4 percent agar) medium were combined with 0.5 mL of protoplast suspension at 45ºC then plated onto the surface of solid DM3 agar plates and incubated at 37 ºC. The plates were incubated at 37 ºC for 2 days after inserting the antibiotic discs, and the resistant fusants were picked up, sub-cultured, screened for antimicrobial activity, and deposited at 4 ºC.

2.8. Random amplified polymorphic DNA (RAPD PCR):

The mutant's molecular analysis was carried out using 2x PCR Master Mix Solution (i-StarTaq), Hot-Start

(iNtRON Biotechnology Inc., South Korea Product Catalog No: 25166). Except for primer and DNA template, the 1x PCR Master Mix vials contain all the reagents required for in vitro DNA amplification. Operon Technologies Inc, Venlo, Netherlands, provided three separate primers for this analysis. The sequences for the three primers are as: first primer (RP1) 5′-GGG GTT TGC CAC TGG-3', second primer (RP3) 5'-GTG TTG TGG TCC ACT-3' and third primer (RP5) 5′-TGA GTG GTC TAC GTG-3'. To each PCR bead, a random primer (12 ng) and purified DNA sample (40 ng) were added. Using sterile distilled water, the total volume of the amplification cocktail was made to 25 µl. The PCR protocol was initial denaturation, 94°C for 4min and 35 cycles of subsequent denaturation, 94°C for 45 sec; annealing temperature, 55°C for 30 sec; extension temperature, 72°C for 1 min and final extension, 72°C for 10 min PCR products were analyzed on 10 x 14 cm 1% agarose gel electrophoresis with DNA ladder standard 100 bp ((iNtRON Biotechnology Inc., South Korea). for 30 min using Tris-borate- EDTA Buffer and visualized by ultraviolet illumination after staining with 0.5 µg/ml ethidium bromide.

3. Results and Discussion

3.1. Screening for CGTase producing isolates:

Initially, several isolates which have a pink zone were isolated from the botanical garden soil at the National Research Center, Dokki, Giza, Egypt. The pink zone of these isolates was evaluated on the Horikoshi medium and selected the highly pink zone of them as shown as in (Fig. 1). Furthermore, the most active strain, WT3, was chosen, and a breeding program was carried out to increase its efficiency, in addition to identifying it at the molecular level using 16S-rRNA. The isolated *Bacillus* colonies with highly pink zone are candidates for production of CD according to More et al., **[26]** and Upadhyay et al., **[27]**.

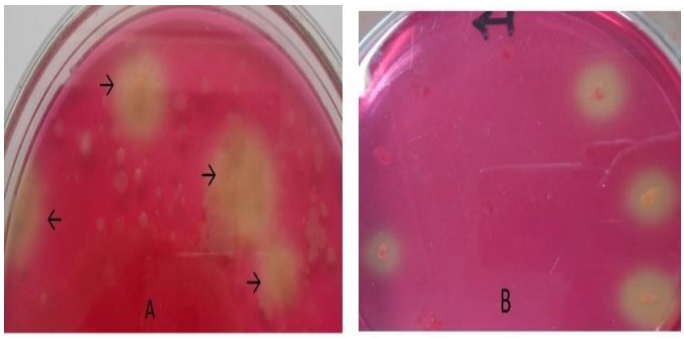

Fig. 1. Detection of CGTase producing isolates using Horikoshi medium, from soil (A) and screening assay (B).

3.2. Molecular identification of *Bacillus* strain (WT3):

The 16S-rRNA gene was used to determine the precise name of the isolated strain (WT3). The 16S-ribosomal ribonucleic acid (rRNA) gene was amplified from the total DNA of the *Bacillus* strain using 16S unique primers. After the amplification by PCR, a product of nearly 1500 bp was detected (Figure 2). The BLAST of the 16S-rRNA gene sequence revealed 93.78% resemblance to the partial 16S-rRNA gene of *Bacillus hunanensis* (NR_108984.1) strain. Based on this resemblance, the *Bacillus* strain (WT3) was identified as of *Bacillus hunanensis* (Figure 3) and it registered in the genebank under an accession number (MN310554.1).

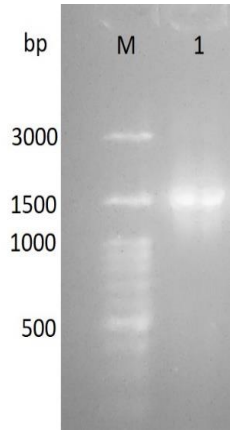

Fig. 2. 16S-DNA amplified band Photograph for *Bacillus* strain (WT3) (lane 1) against 100 bp ladder H3 RTU (Cat. No.DM003-R500 Genedirex, Taiwan).

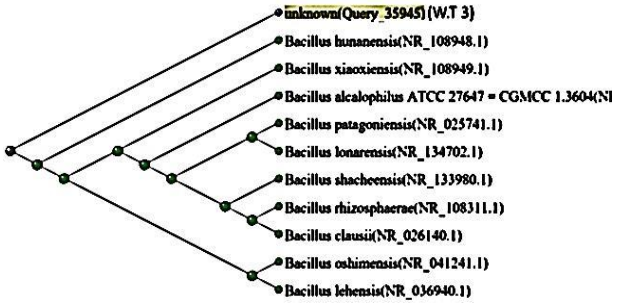

Fig. 3. The NCBI phylogenetic tree based on the 16S-rRNA gene sequences of the potent CGTase-producing *Bacillus* (WT3) strain and the most closely related bacterial strain (*Bacillus hunanensis*).

3.3. EMS-mutagenesis and their effect on *Bacillus hunanensis* (WT3):

Bacillus hunanensis (WT3) spore suspensions were exposed to 200mM EMS-mutagen for different times (20, 40, and 60 minutes), then diluted and overlaid on the Horikoshi medium. It was discovered that as the EMS-exposure period increased, the survival percentages decreased dramatically, from 43.16 percent for 20 minutes to 14.21 and 5.13 percent after 40 and 60 minutes of EMS-mutagenesis, respectively (Table 1). This decline in survival rate may be due to EMS-mutagen-induced damage to nucleic acid and/or defects in other cell components. The findings of Meraj et al. **[28]**; Thakur et al. **[29]** were all in agreement.

Table 1. Survival of *Bacillus hunanensis* (WT3) spores after exposure to the EMS mutagen.

Exposure time	Counted colonies	Survival %
0	1520	100
20	656	43.16
40	216	14.21
60	78	5.13

3.4. CGTase-production after EMS-mutagenesis:

Mutations caused by mutagenic agents induce variations in the nucleotide sequence of DNA. Natural mutations are thought to be uncommon compared to induced mutations, but when chemical and physical mutagenic agents are used, significant changes in the DNA sequence (base pair replacement, addition, and deletion) occur at random in microbial DNA. To improve the genome of bacterial strains, traditional mutagenic agents such as ultraviolet light (UV) and chemical mutagenic agents (EMS and N-methyl-N′-nitro-N-nitrosoguanidine, {NTG}) are used, which typically generate different bacterial DNA mutations. Induction of mutant strains is often used on a commercial scale to facilitate large-scale enzymes productivity. After 20 minutes of EMS-treatment of *B. hunanensis* (WT3, parent strain) with EMS-mutagen (Table 2), no mutant generated CGTase lower than the parent strain after evaluated of ten mutants selected through screening assay (Figure, 4A). After 20 minutes of EMS, the best isolate was WT3-20-5, which produced 81.24 U/mL of CGTase, or 106.8 percent of the parent production, and was designated the greatest CGTase producer. The following greatest CGTase producer after 20 min of EMS-mutagenesis was WT3-20-4 which produced 81.19 U/mL of CGTase, or 106.6 percent of the parent.

Furthermore, after 40 minutes of EMS-treatment of *B. hunanensis* (WT3, parent strain) with EMS-mutagen (Table 3), after evaluating ten mutants chosen by a screening experiment (Figure, 4B), no mutant produced CGTase at a lower level than the original strain (Figure, 4B). WT3-40-4, the highest mutant, produced 83.61 U/mL of CGTase, or 109.8% of the parent production, and was named the highest CGTase producer. WT3-40-3 and WT3-40-8 were the next highest CGTase producers after 40 minutes of EMS-mutagenesis. These mutants produced 81.63 U/mL of CGTase, or 107.2 percent of the parent.

Meanwhile, no mutant generated CGTase lower than the parent strain following 60 minutes of EMS-treatment of *B. hunanensis* (WT3, parent strain) with EMS-mutagen (Table 4), after evaluation of 10 mutants identified using screening assay (Figure, 4C). WT3-60-8 was chosen as the highest CGTase because it produced 84.19 U/mL of CGTase, or 110.6 percent of the parent production. The following greatest CGTase producer after 60 min of EMS-mutagenesis was WT3-60-4 which produced 83.87 U/mL of CGTase, or 110 percent of the parent.

Mutagenesis of *Bacillus* strains is commonly used to increase enzymes production utilizing a variety of mutagens especially EMS **[30, 31, 32, 33, 34, 35, 36, 37].** There is no research on the genetic enhancement of CGTase, especially employing mutagens, whether physical or chemical, thorough the careful searches on the Internet. However, there has been a lot of research on using site-directed mutagenesis method to increase the CGTase productivity and/or efficiency of microbial cells **[38, 39, 40, 4, 41, 42, 43]**. Therefore, this study is considered the first of its kind to improve CGTase productivity by using EMS-mutagenesis to obtain distinct mutations in CGTase production by *B. hunanensis* (WT3).

3.5. Protoplast Fusion:

The best hyper-producing mutants WT3-40-4, WT3-60-4 and WT3-60-8 were selected for protoplast fusion. First, the mutants were assessed for their

antibiotic susceptibility responses and data indicated that, WT3-40-4 was resistant to amoxycillin and ampicillin/sulbactam **(Table 5).** Additionally, WT3-60-4 was resistant to ampicillin, amoxycillin, gentamycin and ampicillin/sulbactam. Mutant WT3-60-8 was resistant to ampicillin, amoxycillin, amoxycillin/clavulanic acid, chloramphenicol, colistin and ampicillin/sulbactam. According to the antibiotic response patterns, three crosses between WT3-60-4 and WT3-60-8 were suggested to carry out the intraspecific protoplast fusion.

Table 2. CGTase production of selected mutants after treatment of *B. hunanensis* (WT3) with EMS-mutagen for 20min.

Mutant code	CGTase U/mL	% from W.T
WT3	76.15	100.0
WT3-20-1	81.03	106.4
WT3-20-2	80.42	105.6
WT3-20-3	80.86	106.1
WT3-20-4	81.19	106.6
WT3-20-5	81.24	106.7
WT3-20-6	80.93	106.2
WT3-20-7	80.69	106.0
WT3-20-8	79.72	104.7
WT3-20-9	77.71	102.0
WT3-20-10	78.09	102.5

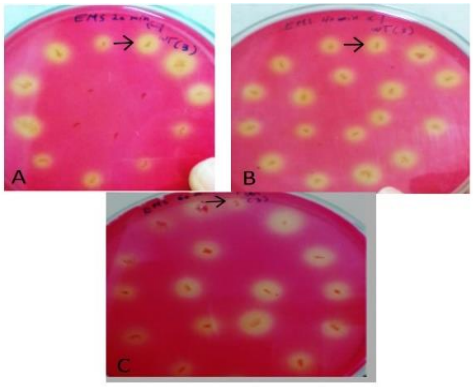

Fig. 4. Screening assay of high CGTase producing mutants after 20 min (A), 40 min (B) and 60 min (C) of EMS-mutagenesis in comparison of *B. hunanensis* (WT3) which is indicated by the arrow.

Table 3. CGTase production of selected mutants after treatment of *B. hunanensis* (WT3) with EMS-mutagen for 40min.

Mutant code	CGTase U/mL	% from W.T
WT3	76.15	100
WT3-40-1	81.01	106.3
WT3-40-2	80.98	106.3
WT3-40-3	81.63	107.2
WT3-40-4	83.61	109.8
WT3-40-5	80.98	106.3
WT3-40-6	79.31	104.1
WT3-40-7	79.92	105
WT3-40-8	81.50	107
WT3-40-9	80.48	105.7
WT3-40-10	80.54	105.8

Table 4. CGTase production of selected mutants after treatment of *B. hunanensis* (W.T3) with EMS-mutagen for 60min.

Mutant code	CGTase U/mL	% from W.T
WT3	76.15	100
WT3-60-1	81.72	107.3
WT3-60-2	80.70	106
WT3-60-3	78.56	103.2
WT3-60-4	83.87	110.1
WT3-60-5	77.23	101.4
WT3-60-6	79.02	103.7
WT3-60-7	83.33	109.4
WT3-60-8	84.19	110.6
WT3-60-9	81.65	107.2
WT3-60-10	80.06	105.1

Table 5. Antibiotic susceptibility of the 3 selected CGTase hyper-producing EMS-mutants.

Strain code	Antibiotic disc									
	*AM +10	AMC 30	AML 25	C 30	CN 10	CT 25	DO 30	RF 30	SAM 20	S 10
WT3	**18	28	35	22	31	18	35	43	32	44
WT3-40-4	20	R	48	52	49	15	47	50	R	58
WT3-60-4	R	R	31	16	R	10	20	12	R	38
WT3-60-8	R	R	R	R	40	R	37	35	R	39

*AM, ampicillin; AMC, amoxycillin; AML, amoxycillin/clavulanic acid; C, chloramphenicol; CN, gentamycin; CT, colistin; DO, doxycycline; RF, rifampicin SV; SAM, ampicillin/sulbactam; S, streptomycin.
+ Concentration (µg/disc). R= resisted (no clear zone).
**Means of diameter of inhibition zones (mm).

Results in Table (6) revealed that all obtained fusants, from cross1 between (WT3-60-4 and WT3-60-8 mutants) in the presence of AML+CN as selective markers, exhibited higher CGTase productivity than the original strain (WT3). Moreover, fusant F1-8 exhibited higher CGTase productivity of about 122.4% in comparison of the original strain (WT3). CGTase productivity of the fusants; F1-8 and F1-9, represents about 110.7% and 108.8% of the higher parent 2, respectively. It's worth noting that the CGTase productivity of all fusants was higher than that of the higher parent 2.

Fusants developed from cross 2 between (WT3-60-4 and WT3-60-8 mutants) in the presence of C+CN as selective markers (Table 7) demonstrated that all fusants had higher CGTase productivity than the parent strain (WT3). However, 5 of the 8 fusants had higher CGTase productivity than the greater parent 2. F2-5 was the best fusant, producing 87.38 U/mL of CGTase, or 114.7 percent of the parent (WT3) production while also representing roughly 103.8 percent of the higher parent 2.

Furthermore, the effect of intra-specific protoplast fusion technique on CGTase productivity was assessed on 10 fusants selected from cross 3 between (WT3-60-4 and WT3-60-8 mutants) in the presence of CN+CT as selective markers (Table 8) and revealed that all obtained fusants had higher CGTase productivity than the original strain (WT3). However, 7 of the ten fusants had higher CGTase productivity than the greater parent 2. F3-4 was the best fusant, producing 92.81U/mL of CGTase, or 121.9 percent of the parent (WT3) production while also representing roughly 110.2 percent of the higher parent 2.

Table 6. CGTase productivity of fusants obtained after protoplast fusion between WT3-60-4 and WT3-60-8 mutants (cross 1, AML+CN).

Parents and fusants code	CGTase production (U/ml)	% To P2 (higher parent)	% To W. T
WT3	76.15	90.5	100
P1-WT3-60-4	83.87	99.6	110.1
P2-WT3-60-8	84.19	100	110.6
F1-1	85.17	101.2	111.8
F1-2	87.56	104	115
F1-3	89.96	106.9	118.1
F1-4	89.13	105.9	117
F1-5	86.72	103	113.9
F1-6	88.60	105.2	116.3
F1-7	87.20	103.6	114.5
F1-8	93.17	110.7	122.4
F1-9	91.63	108.8	120.3

Table 7. CGTase productivity of fusants obtained after protoplast fusion between WT3-60-4 and WT3-60-8 mutants (cross 2, C+CN).

Parents and fusants code	CGTase production (U/ml)	% To P2 (higher parent)	% To W. T
WT3	76.15	90.5	100
P1-WT3-60-4	83.87	99.6	110.1
P2-WT3-60-8	84.19	100	110.6
F2-1	82.81	98.4	108.7
F2-2	84.80	100.7	111.4
F2-3	85.55	101.6	112.3
F2-4	83.20	98.9	109.3
F2-5	87.38	103.8	114.7
F2-6	85.13	101.1	111.8
F2-7	85.62	101.7	112.4
F2-8	83.73	99.5	110

Table 8. CGTase productivity of fusants obtained after protoplast fusion between WT3-60-4 and WT3-60-8 mutants (cross 3, CN+CT).

Parents and fusants code	CGTase production (U/ml)	% To P2 (higher parent)	% To W. T
WT3	76.15	90.5	100
P1-WT3-60-4	83.87	99.6	110.1
P2-WT3-60-8	84.19	100	110.6
F3-1	85.20	101.2	111.9
F3-2	82.16	97.6	107.9
F3-3	83.97	99.7	110.3
F3-4	92.81	110.2	121.9
F3-5	82.70	98.2	108.6
F3-6	87.62	104.1	115.1
F3-7	92.36	109.7	121.3
F3-8	89.49	106.3	117.5
F3-9	85.18	101.2	111.9
F3-10	85.45	101.5	112.2

In general, mutation has been shown to be an efficient approach for increasing CGTase production. Furthermore, improved CGTase-producing fusants were developed using intra-specific protoplast fusion between higher CGTase-generating mutants. As a result, protoplast fusion has proven to be an effective method for increasing CGTase synthesis. This method can be used to obtain recombinant strains **[44, 45, 46, 47, 39].**

3.6. Molecular characterization of the superior CGTase-producing mutants and fusants:

To determine molecular variabilities following various mutagenesis treatments and protoplast fusion crosses, three random primers, two tested EMS-mutants and four of recombinant fusants as well as *B. hunanensis* (WT3) (parental), were used. When the RAPD primer (RP1) was employed, the banding profile of the tested mutants, fusants, and parental strain was obtained using the RAPD assay, as shown in (Figure 5). Two bands (1300, 500bp) were found in the parental-strain (lane 1). The mutant WT3-60-4 (lane 2) gave two bands of molecular weights (1250, 750bp). One band (2500bp) was detected for the mutant WT3-60-8 (lane 3). It was found that fusants F1-8 and F1-9 (lanes 4, 5) share the presence of the band of molecular weight (2500bp), but fusant F1-8 has another band with molecular weight (1200bp), while fusant F1-9 has another band with molecular weight (1300bp). One band (1200bp) was detected for the fusant F3-4 (lane

6). Finally, the fusant F3-7 (lane 7) gave two bands of molecular weights (700, 400bp).

Furthermore, using RP3 primer (Figure 6) with the tested mutants and fusants in comparison of *B. hunanensis* (WT3) parental-strain. For the parental (WT3) strain, five bands (2800, 400, 300, 220,120bp) were observed (lane 1). The mutant WT3-60-4 (lane 2) has the highest bands number (7 bands) and share the presence of the four specific bands (900, 850, 800, 750bp). Six bands were detected for the mutant WT3-60-8 and fusant F1-8 (lane 3, 4) and share the presence of the five specific bands (1500, 1000, 770, 700, 600bp). It was found that fusants F1-9, F3-4 and F3-7 (lanes 5, 6,7) share the presence of the band of molecular weight (2500bp), but fusant F1-8 has another band with molecular weight (1000bp), while fusants F3-4 and F3-7 (lanes 6,7) share the presence of the bands with molecular weights (1500, 1000, 600bp). Finally, fusants F3-4 and F3-7 (lanes 6,7) has another two distinct bands gave two bands of molecular weights 770 and 400bp, respectively.

RP5 primer was also used with the tested mutants and fusants in comparison to the parental-strain of *B. hunanensis* (WT3) to discover molecular differences between mutants, fusants, and the parental-strain (Figure 7). Five bands (850, 650, 550, 400, 200bp) were found in the parental-strain (lane 1). The mutant WT3-60-4 (lane 2) produced five molecular weight bands (1000, 750, 650, 500, 300,200bp). For the mutant WT3-60-8, four bands (1500, 1200, 550, and 350bp) were found (lane 3). Fusant F1-8 (lane 4) has two bands of molecular weights (650, 300bp), whereas fusant F1-9 had five bands of molecular weights (1500, 1400, 850, 750, 400bp). Fusant F3-4 has four molecular weight bands (1500, 1350, 400, 200bp). The fusant F3-7 was found to have five bands (1750, 1100, 1000, 650, and 400bp) (lane 7).

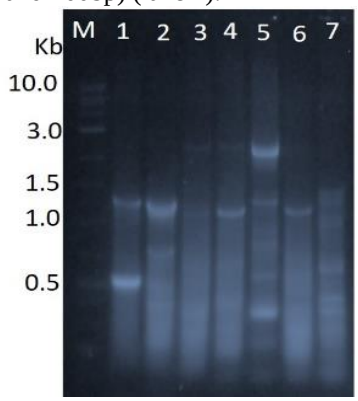

Fig. 5. RAPD-DNA amplified banding profiles using primer (RP1) for two different mutants and four fusants (Lanes 2 to 7) *B. hunanensis* (W.T3) (lane 1) opposite to DNA Marker, iNtRON Biotechnology Inc., South Korea, (lane M). The mutants and fusants sequence as follow: WT3, WT3-60-4, WT3-60-8, F1-8, F1-9, F3-4 and F3-7.

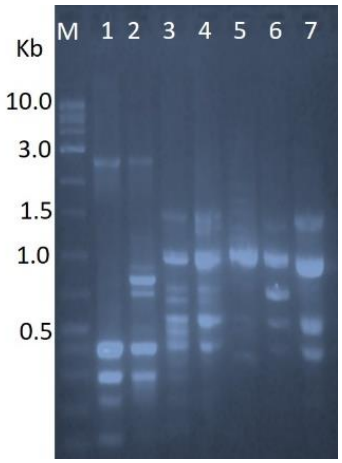

Fig. 6. RAPD-DNA amplified banding profiles using primer (RP3) for two different mutants and four fusants (Lanes 2 to 7) *B. hunanensis* (W.T3) (lane 1) opposite to DNA Marker, iNtRON Biotechnology Inc., South Korea, (lane M). The mutants and fusants sequence as follow: WT3, WT3-60-4, WT3-60-8, F1-8, F1-9, F3-4 and F3-7.

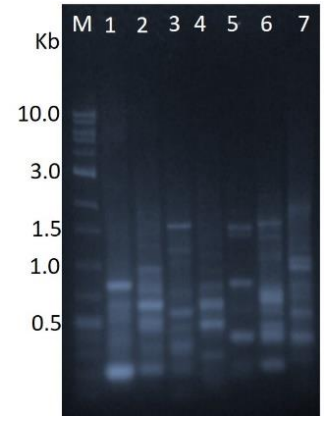

Fig. 7. RAPD-DNA amplified banding profiles using primer (RP5) for two different mutants and four fusants (Lanes 2 to 7) *B. hunanensis* (W.T3) (lane 1) opposite to DNA Marker, iNtRON Biotechnology Inc., South Korea, (lane M). The mutants and fusants sequence as follow: WT3, WT3-60-4, WT3-60-8, F1-8, F1-9, F3-4 and F3-7.

The number of obtained banding profiles when using primer RP1 were 12 bands, primer RP3 were 33 bands, and primer RP5 were 31 bands after RAPD assay, as shown in (Table 9). Furthermore, the primer RP5 produced the most polymorphic bands, while the primer RP3 produced the least polymorphic bands. The RP1 primer was also used to identify the decreased unique bands. After using the primers RP3 and RP5, six distinct bands were obtained. After all primers had been applied, there were no monomorphic bands visible.

Table 9. Total amplified DNA bands and the percentages of polymorphic bands observed using RAPD-assay with three random primers.

Primer code	Unique loci	Polymorphic loci	Monomorphic loci	Total band	Polymorphic percentage
RP1	5	3	0	12	25.00%
RP3	6	8	0	33	24.24%
RP5	6	9	0	31	80.65%
Total	17	20	0	56	-

RAPD banding patterns differ due to variations in DNA nucleotides (fingerprints). Changes in the primer's annealing nucleotide sequences may prevent annealing, as seen by the removal of the identical amplified band. Mutations can induce new annealing sites to appear in the template (genome), resulting in new bands. RAPD assays have been used in a few studies to detect bacterial strains, mutations, and fusants **[48, 49, 50, 51, 52].**

3.7. Phylogenetic tree in relation to PCR-RAPD assay:

The exact relationships between different band types may be acquired using PCR-RAPD data, and genetic links can be derived as in (Table 10). The genetic relationship between the mutant WT3-60-8 and F1-8 was significant (70.0%), and then between fusants F1-8 and F3-4 was low (44.4%). A small genetic relation matrix (8.3%) was established between mutants WT3-60-4 and WT3-60-8, on the other hand.

The artery pattern using RAPD distances revealed that the mutant WT3-60-8, fusant F1-8, and fusant F3-7 are clustered into one first cluster, which is related with the fusants F1-9 and F3-4 in the second cluster, according to the evolution tree (Figure 8). In the third cluster, the link between the new excellent mutant WT3-60-4 and the parental-strain can be shown. In addition, (Figure 8) found that new genotypes (mutant and fusants) thought to be strong CGTase-producers were grouped together in one cluster. The foregoing findings follow the same pattern as **[49**, **50, 51]**.

Table 10. The proximity matrix between the tested mutants, fusants, and the parental *B. hunanensis* (WT3) strain based on RAPD assay.

Strain code	WT3	WT3-60-4	WT3-60-8	F1-8	F1-9	F3-4	F3-7
WT3	1.000	0.316	0.133	0.133	0.167	0.000	0.000
WT3-60-4	0. 316	1.000	0.083	0.250	0.190	0.182	0.174
WT3-60-8	0. 133	0.083	1.000	0.700	0.353	0.333	0.421
F1-8	0. 133	0.250	0.700	1.000	0.235	0.444	0.316
F1-9	0. 167	0.190	0.353	0.235	1.000	0.400	0.250
F3-4	0. 000	0.182	0.333	0.444	0.400	1.000	0.353
F3-7	0. 000	0.174	0.421	0.316	0.250	0.353	1.000

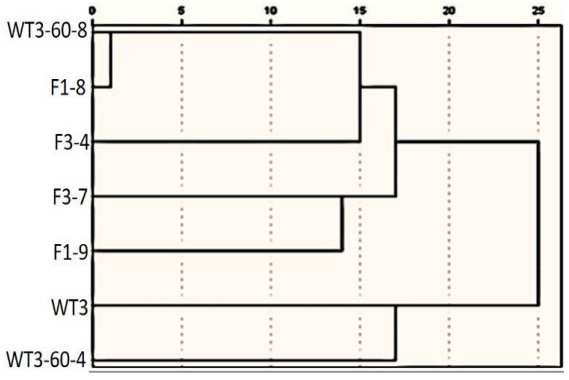

Fig. 8. Phylogenetic tree between the tested mutants, fusants, and the parental *B. hunanensis* (WT3) through RAPD method.

4. Conclusion

The screening of CGTase-producing colonies, their molecular identification, and the improvement of strong *B. hunanensis* (WT3) strain for high CGTase-producing mutants after EMS-mutagenesis and protoplast fusion were all carried out as part of this study. Furthermore, superior mutants and excellent fusants' RAPD-profiles changed in contrast to the wild strain, and these progressions were identified as biomarkers of genetic differences in superior mutants and excellent fusants of *B. hunanensis*. The phylogenetic diversity was used to group the genetically improved CGTase-producing strains into clusters, which reflected the large genetic changes of some improved CGTase-producing strains.

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