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Effect of Sodium Nitrite mutagen on curdlan producing by Agrobacterium tumefaciens and RAPD analysis of mutated strains

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Abstract: In this study an attempt was carried out to produce mutants by using chemical mutagen sodium nitrite which could produce curdlan with improvement in yield and productivity than the wild type strain. Mutagenesis of *Agrobacterium tumefaciens* by using sodium nitrite occurred at pH 5.5 using five concentrations (0.0, 10, 15, 20 and 25 mg/ml) for one and two hours at 30°C. Auxotrophic mutants were selected and identified for requirements. The curdlan produced by mutants and wild strain were determined after 3 days. The mutants that produced higher levels of curdlan than the wild strain *Agrobacterium tumefaciens* were isolated. The mutant (A.T9) strain produced 54.2 g/L of curdlan while, the wild strain produced 18.2 g/L after 3days. Four mutants which produced the highest levels of curdlan were biologically examined as antiviral activity. Random amplification of polymorphic DNA (RAPD) were used to confirm, on molecular level, that the change in DNA changing in production of curdlan. Genetic relationships were also studied to identification and assigning the eight strains into different group according to similarity and differences between them.
Keywords: mutation, sodium nitrite, *Agrobacterium tumefaciens*, curdlan, RAPD-PCR.

Introduction

Mutagenesis" is one of the source of all genetic variations. Strain improvement is an essential part of process development for microbial products. It can reduce costs by developing strains with increased productivity. Strain improvement can be carried out by such techniques as rational screening and genetic engineering or by the traditional method of mutagenesis and selection on the basis of direct titre measurement. The last method, also referred to as random screening, is still a reliable and cost effective procedure. Consequently, 'it is frequently the method of choice for short-term strain development.

Curdlan is a high molecular weight, water insoluble (alkali soluble) extracellular polysaccharide composed only of β-(1→3) glucose residues[1]. Curdlan is a secondary metabolite synthesized by *Alcaligenes faecalis* var. *myxogenes* and *Agrobacterium radiobacter* under nitrogen-limiting conditions[2]. Since its discovery by[3], curdlan production has drawn considerable interest because of its unique rheological and thermal gelling properties. Curdlan is one of the FDA approved biopolymer used in food industries such as jelly, noodles, edible fibers manufacturing process. Curdlan is extensively used as an ingredient in animal feed since it acts as immune stimulator[4]. It is used as concrete admixture and increases the water absorbing capacity of the concrete[5]. Curdlan sulphate is developed as an antiviral agent against human immunodeficiency virus infections[1,6].

(RAPD) - PCR techniques were used to distinguish differences between strains within a species. One or a few short primers of arbitrary sequence are allowed to bind under low stringency conditions to various sites

on both strands of the template DNA. The PCR reaction yields a series of products of varying size, which may be separated by gel electrophoresis. The band pattern represents a "genetic fingerprint" characterizing a particular bacterial strain[7]. This study was carrying out by cooperation between genetical lab faculty of agriculture botany department and genetic engineering.

The present investigation was made to in biotechnological aspects, cheap substrates, mutations and genetically modified high curdlan yielding bacteria can be used in antiviral technique to improve the strains by using chemical mutagen. In this tendency the sodium nitrate was used as chemical mutagens[8]. And after treatment they were analyzed by RAPD technique to confer the potential changes occurred on their chromosomal elements similarly observed the quality and quantity of curdlan.

2. Material and Methods

a- Bacterial strains:

Agrobacterium tumefeceins strain donated from assist. prof. of pathology I. H. Tolba Al-azhar university, Botany department. The strain was patterned on MM and CM plats and incubated for 3 days to be sure that is wild type.

b- Viruses strain antiviral activity:

The influenza virus Type A (H1N1) and (H3N2) were isolated from patients and characterized by PCR and standard anti influenza sera obtained from central laboratory for evaluation of veterinang biologics.

2.1.1 Media

1- Complet media

a- Trypton yeast extract (TY)[9].

b- Luria broth Medium (CM) (LB)[10].

2- Minmal medium (MM)[11].

3- DMEM medium (Sigma) was used for cell line (MDCK) cultured containing penicillin (100 U/ml), Streptomycin (100 µg/ml) and 10% heat inactivated fetal bovine serum (GIBCO)[12].

2.2 Methods

2.2.1 Chemical mutagen sodium nitrite ($NaNO_2$).

The wild type strain *Agrobacterium tumefeceins* was treated with sodium nitrite as a mutagenic agent with five concentrations: 0.0, 10, 15, 20 and 25 mg⁄ml of liquid MM. Each concentrations was applied for one and two hours at 30 ^{o}C[13].

2.2.2 The isolation and characterization of auxotrophic mutation.

Isolation of mutants; The mutants classified according to methods described by[14] and Characterization.

2.2.3 The isolation and determination of curdlan productivity mutants.

were detected by [15,16].

2.2.4 Biological measurements (Antiviral activity).

2.2.4.1 Evalution of the cytotoxic effect of polysaccharide extracts on MDCK cell cultures[12].

2.2.4.1 Evalution of *in-vitro* antiviral effect of polysaccharide extracts on H1N1 and H3N2 viruses replication in MDCK cell cultures were modified by[12]. The assays were performed in 96 well tissue culture plates and 50ml tissue culture flasks following the role of[12]. Virucidal = virrses + polysaccharide extract + MDCK cell cultures.

Post-Exposure = virrses + MDCK cell cultures + polysaccharide extract.Pre- Exposure = polysaccharide extract + MDCK cell cultures + virrses.

2.3 Molecular marker.

2.3.1 Isolation of bacterial DNA.

DNA was extracted and isolated from strains by modified cetyltrimethyl ammonium bromid (CTAB) protocol[17].

2.3.2 (RAPD) - PCR techniques was carried out according to[18] with slight modifications.

2.3.3 RAPD-PCR Reactions:

A set of five random 10-mer primers (Table 1) was used in the detection of polymorphism. The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM primer, 1 U Taq DNA polymerase and 25 ng template DNA.

2.3.4 Thermocyling Profile and Detection of the PCR Products:

PCR technique was carried out according to[17]. PCR products were visualized on UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

Table 1. Sequence of the five decamer arbitrary primers assayed in RAPD-PCR.

Primer	Sequence (5´-3´)
OPA-03	AGTCAGCCAC
OPA-06	GGTCCCTGAC
OPB-11	GTAGACCCGT
OPB-12	CCTTGACGCA
OPD-03	GTCGCCGTCA

2.3.5 Data Analysis

The banding patterns generated by RAPD-PCR marker analyses were compared to determine the genetic relatedness of the 8 strain . Clear and distinct amplification products were scored as ‘1’ for presence and ‘0’ for absence of bands. Bands of the same mobility were scored as identical.

The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient[19].

Dice formula: GSij = 2a/(2a+b+c)

Where GSij is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.

The similarity matrix was used in the cluster analysis. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA)[19]. And relationships between groups were illustrated as dendograms.

3. Results and Discussion

The present study was planned to improve the efficiency of *Agrobacterium tumefeceins* to produce curdlan by genetic approach such as chemical mutagen. The Biological measurements (Bio-effect) of curdlan were studied. Random Amplified Polymorphic DNA (RAPD) is PCR based method which can be used to distinguish differences between strains within a species.

Induction mutation by sodium nitrite ($NaNo_2$).

Cell suspension was treated with five concentrations: 0.0, 10, 15, 20 and 25 mg of $NaNo_2$/ml cell suspension on shaker for one and two hours at 30 ^oC incubation for 3 days. Table (2) showed that survival percentages decrease gradually by increasing $NaNo_2$ concentration and also decreased gradually by increasing the time of treatment to $NaNo_2$ at 30 ^oC. The results obtained in table (2) that 10, 15, 20 and 25 mg sodium nitrite/ml gives 2, 3, 2 and 3 mutants after 1h respectively and also gave 2, 6, 2 and 2 mutants after 2h with the same concentration of sodium nitrite. These previous results are a gree with[8].

Table 2. *Agrobacterium tumefeceins* cell survived different $NaNo_2$ concentrations and mutant number.

Concentration $NaNo_2$ (mg/ml)	Time (h.)	No. of cell survived/ml (10^5)	Survival (%)	Number of Mutants
0.0	0.0	238.33	100	0.0
10	1	87.33	36.64	2
15	1	69.00	28.95	3
20	1	52.33	21.95	2
25	1	25.00	10.48	3
10	2	62.33	26.15	2
15	2	36.00	15.10	6
20	2	16.00	6.71	2
25	2	9.00	3.77	2

A mutant with a single biochemical requirement when inoculated on all twelve plates will grow on two of them, one of plates 1-6 and one of plates 7-12. This technique is more efficient than previous methods in which the field of search is progressively reduced. With one or a few mutants auxanograghic techniques are preferable[14]. The genotypes of *Agrobacterium tumefeceins* mutant's no. characterization and curlan production were presented in table (3).

Results in table (3) showed that the biochemical mutants and their characterization were gave the highest frequency of methionine requiring mutants (4 mutants; A.T1, A.T12, A.T13 and A.T21). While the uracil (3 mutants; A.T4, A.T6 and A.T9) and histidine (3 mutants; A.T7, A.T8 and A.T19) requiring mutants were followed methionine requiring mutants, tryptophan (A.T10 and A.T11), valine(A.T14 and A.T22) were gave two mutants for each, and alanin (A.T16 and A.T17). The lowest requiring were; lysine (A.T2), inositol (A.T3), proline (A.T5), ornithine (A.T15) and glutamic acid (A.T20) requiring mutants.

Table 3. Classification of mutations genotype of *Agrobacterium tumefeceins* and curdlan production.

Mutant code	$NaNo_2$ (mg/ml)	Time (h.)	Genotype	Mean of (curdlan) g/L	
Wild type	0.0	0.0	Wild type	18.2	100%
A.T1	10	1	Methonine	21.2	116
A.T2	10	1	Lysine	19.4	106
A.T3	10	2	Inositol	20.2	111
A.T4	10	2	Uracil	39.8	219
A.T5	15	1	Proline	34	187
A.T6	15	1	Uracil	40.2	221
A.T7	15	1	Histidine	24.6	135
A.T8	15	2	Histidine	35.6	196
A.T9	15	2	Uracil	54.2	248
A.T10	15	2	Tryptophane	22.6	124
A.T11	15	2	Tryptophan	20.4	112
A.T12	15	2	Methonine	37.6	206
A.T13	15	2	Methonine	31.4	172
A.T14	20	1	Valine	19	104

A.T15	20	1	Ornithine	25	137
A.T16	20	2	Alanin	26.2	144
A.T17	20	2	Alanin	26	143
A.T18	25	1	Tryptophane	22.2	122
A.T19	25	1	Histidine	19.8	109
A.T20	25	1	Glutamic acid	19.2	106
A.T21	25	2	Methonine	19.6	108
A.T22	25	2	Valine	18.4	101

Curdlan production from *Agrobacterium tumefeceins* strain

Results in Table (3) showed also curdlan productivity of the parental strain *Agrobacterium tumefeceins* and twenty two selected higher producer mutants.

The curdlan productivity of the industrial strain *Agrobacterium tumefeceins* was 18.2 g/L. The mutant strain (A.T9), which obtained from sodium nitrite dose of 15 mg/ml after 2h, recorded the highest curdlan productivity (54.2 g/L) exceeding 2.5-folds the productivity of its original strain. The mutant (A.T22), which obtained from sodium nitrite dose of 25 mg/ml after 2h found to be lowest curdlan productivity 18.4 g/L, nearly about wild type

In general, for curdlan productivity all of the twenty two selected mutants proved to be highly producer strains comparing to the corresponding original strain. These results agree with[15].

Antiviral activity

Cytotoxicity of polysaccharide extracts on MDCK cell culture

The mean cytotoxic concentration 50 (CC_{50}) of the five samples (W.T, A.T4, A.T6, A.T9, A.T12) preparation of curdlan extracts was 2mg (2000 µg⁄ml) medium. Cell toxicity might render to high alkaline, alkalinity of the extracts at high concentrations. The other used (tested) concentrations of extracts were safe for MDCK cell cultures based on their morphological features and potentiality for growing and maintenance.

Antiviral activity for each of polysaccharide were studied in MDCK cells infected with (H1N1) & (H3N2) viruses concentrations from the previous five samples W.T, A.T4, A.T6, A.T9 and A.T12) extracts 1000, 500 and 250µg⁄ml were used in this study, results obtained were represented in Fig.(1). The obtained results showed effect on virus replication inhibitory effect of each of (H1N1) and (H3N2) viruses in MDCK cell cultures using the higher available concentration of the previous strains. These results agree with[1,6] on *Agrobactrium sp* and [12]on *Eugenia jambolana* Lam.

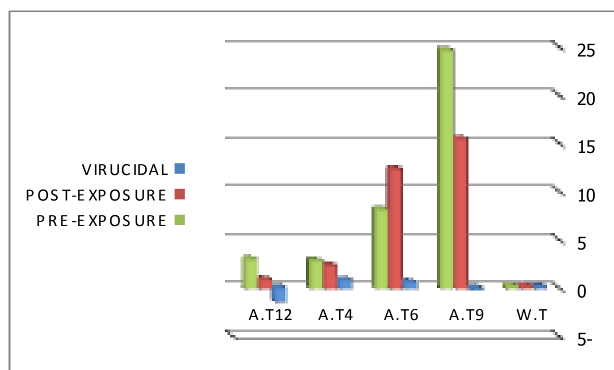

Fig 1: virus publication inhibitory effect of curdlan extract on (H1N1) and (H3N2) viruses on MDCK.

Fingerprinting RAPD PCR technique

Polymorphism as detected by RAPD analysis

DNA markers such as randomly amplified polymorphic DNA have extensively been used recently for identification and evaluation of genetic relationships among strains. RAPD molecular marker has been assist selected desirable genotype. RAPD analysis involves the implication of DNA segments, commonly using arbitrary 10 base primers to find polymorphic regions within the genome. Five RAPD primers were screened with the DNA of the 8 strain; W.T, A.T4, A.T5, A.T6, A.T8, A.T9, A.T12 and A.T13.

In general, data in Table (5&6) and Figure (3) revealed that the size of amplified fragments ranged from 1600 bp for primer B11 to 70 bp for primer A06. The higher total numbers of bands were 21 bands of both primers; B11 and D03, while the lower total number of bands was 9 bands for primerA06. The least number of RAPD-PCR strain-specific marker (monomorphic bands) was detected for the two primers; B-12 and D-3(3 markers out of 16 and 21 makers, respectively), while the largest specific markers was detected for primer A-03(7 markers out of 10 bands).

Table 5. Total number of amplicons monomorphic, polymorphic and percentage of polymorphism as revealed by RAPD markers among 8 strain (W.T, A.T4, A.T5, A.T6, A.T8, A.T9, A.T12 and A.T13).

Primer	Total No. of amplicons	Monomorphic amplicons	Polymorphic amplicons	% of polymorphism
A03	10	7	3	30.05%
A06	9	4	5	55.55%
B11	21	4	17	81.00%
B12	16	2	14	81.25%
D03	21	1	20	85.71%
Total	**77**	**18**	**59**	**72.72%**
Average	**15.4**	**4.2**	**11.2**	

Also, data in Table (5&6) and Figure (3) showed that the primer B11 indicated the amplification of 21 bands with size ranged from 150 to 1600 bp, all of them were polymorphic, in which 4 of them were specific markers at 150, 280, 750 and 1000 bp. the Primer A06 indicated the amplification of 9 bands with size ranged from 70 to 1100 bp all of them were polymorphic, in which 4 of them were specific markers at 70, 150, 380 and 400 bp. At same trend, the primer B12 indicated the amplification of 16 bands with size ranged from 140 to 1300 bp, all of them were polymorphic, in which 2 of them were specific markers at 420 and 500 bp. the Primer D03 indicated the amplification of 21 bands with size ranged from 200 to 1500 bp all of them were polymorphic, in which one of them were specific markers at 230 bp. On the contrary, primer A03 indicated the amplification of 10 bands with size ranged from 80 to 1100 bp, all of them were polymorphic, in which 7 of them were specific markers at 80, 100, 180, 210, 300, 700 and 1100 bp.

On the other hand, the total number of fragments produced by the five primers was 77 with an average 15.4 fragments/primer (Table 5), while the number of polymorphic fragments ranged from 21 to 9. A maximum number of fragments (21) were amplified with two primers; B11 and D03 while, the minimum number of fragments (9) was amplified with primer A06. The highest number of polymorphic bands (18) was obtained with primer D03, which exhibited the highest percentage (85.7%) of polymorphism while, the lowest number of polymorphic bands (3) was obtained with primer A03, which exhibited the lowest percentage (30%) of polymorphism. This corresponds to a level of polymorphism of 72.72% and an average number of polymorphic fragments/primer of 11.2.

Overall, Randomly Amplified Polymorphic DNA (RAPD) analysis showed significant variation between the five primer patterns. These results described here confirm the reliability of the variation of curdlan production of mutants and are in agreement with[20].

Genetic Relationships and Cluster Analysis as Revealed by ScoT within and among 8 strains.

Package SPSS system. Significant different were determine at p‹0.05. Data from PCR product gel were pooled into 1 and 0, there were interred into the input of the program as shown in the dendrogram Figure 4. the statistical analysis data were carried by[21]. The dendrogram generated by Gel works 1D analysis confirmed the above pattern of diversity using PCR product gel.

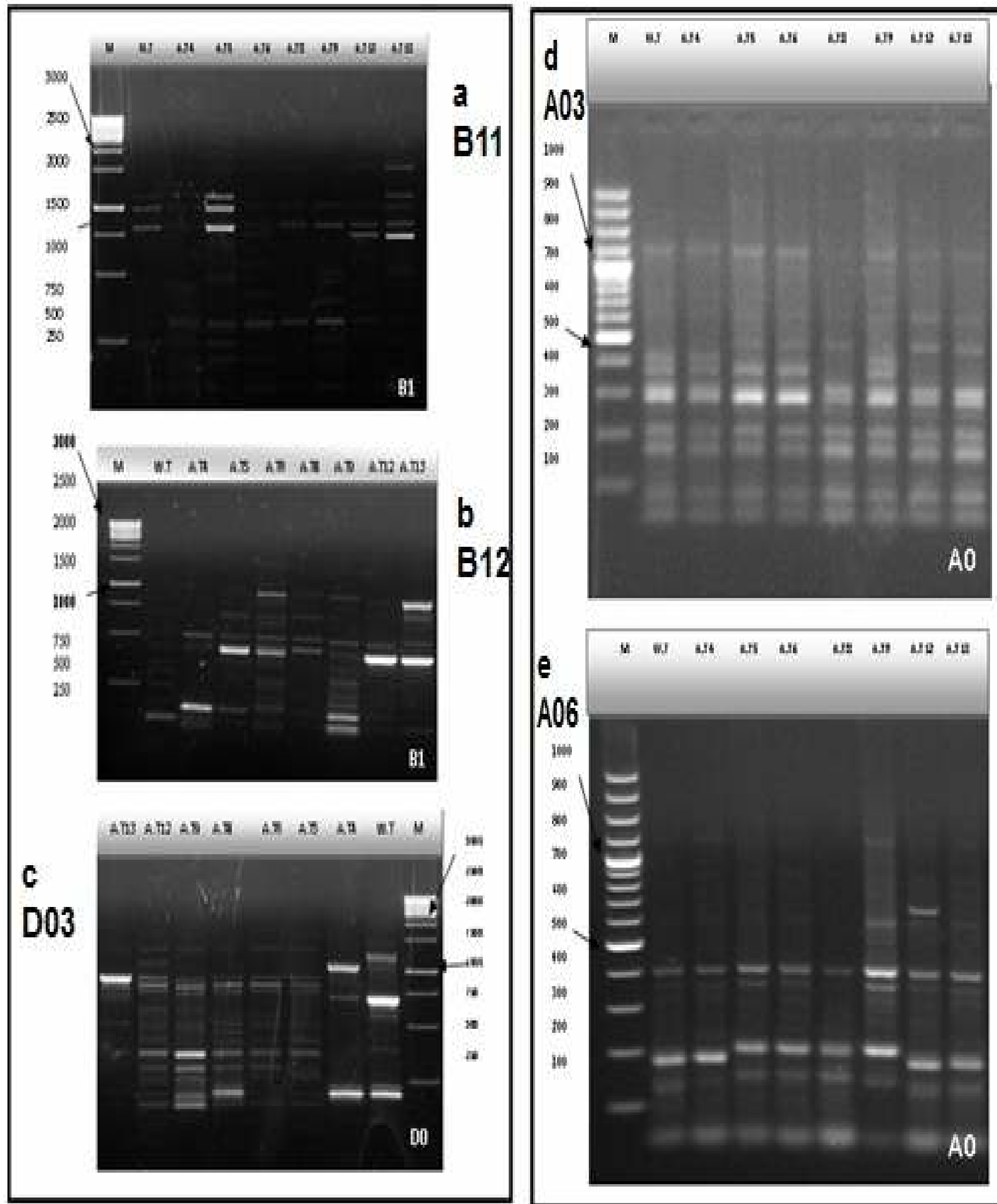

Fig 3: (a, b, c, d and e): RAPD profiles for the eight strain (W.T, A.T4, A.T5, A.T6, A.T8, A.T9, A.T12 and A.T13). as detected with primers B11, B12, D03, A03 and A06 Lanes 1 to 8 (W.T, A.T4, A.T5, A.T6, A.T8, A.T9, A.T12 and A.T13). M: 100bp ladder DNA marker.

Table 6. Binary data obtained for absence (0) and presence (1) from RAPD gel electrophoresis among eight strains from five primers B11, B12, D03, A03 and A06.

				B12				
A.T13	A.T12	A.T9	A.T8	A.T6	A.T5	A.T4	W.T	M
0	0	0	0	0	0	1	0	140
1	1	1	0	1	0	1	1	150
1	1	1	1	1	1	1	0	180
0	0	0	0	1	0	0	1	200
0	1	1	0	1	0	0	0	230
0	1	1	1	0	0	0	1	250
0	0	1	0	1	0	0	0	300
0	0	1	0	1	0	0	1	350
0	0	0	0	0	0	0	1	380
1	1	1	1	1	1	1	1	420
1	1	1	1	1	1	1	1	500
0	0	0	0	1	0	0	0	620
1	0	0	1	1	1	0	1	680
1	0	0	0	1	0	0	0	750
1	1	1	1	1	1	0	0	850
0	0	0	0	1	0	0	0	1300

				B11				
A.T13	A.T12	A.T9	A.T8	A.T6	A.T5	A.T4	W.T	M
1	1	1	1	1	1	1	1	150
1	0	0	0	0	0	0	0	160
0	0	0	0	0	0	0	1	200
0	0	1	0	1	1	1	0	210
1	0	1	0	1	1	1	1	250
1	1	1	1	1	1	1	1	280
0	1	0	0	0	0	0	0	310
1	0	1	0	0	0	0	0	320
0	1	0	0	1	0	1	0	350
0	0	0	0	0	0	1	1	400
0	0	1	0	0	1	0	0	420
1	0	0	0	0	1	0	0	500
1	0	1	0	0	0	0	0	530
1	0	0	0	1	0	0	0	560
1	1	0	0	0	0	0	0	600
1	1	0	0	1	0	0	0	700
1	1	1	1	1	1	1	1	750
1	1	1	1	1	1	1	1	1000
1	0	0	0	1	1	1	1	1150
1	1	0	1	0	0	0	0	1500
1	0	0	0	0	0	0	0	1600

				D03				
A.T13	A.T12	A.T9	A.T8	A.T6	A.T5	A.T4	W.T	M
0	0	1	1	1	1	1	1	200
1	1	0	0	0	1	0	0	220
1	1	1	1	1	1	1	1	230
1	1	0	0	1	0	0	0	250
0	0	1	0	1	1	1	0	300
0	0	1	1	1	1	1	1	340
1	0	1	1	1	1	1	1	380
0	0	1	1	1	0	1	0	420
0	0	0	0	1	0	0	0	500
1	1	1	1	1	1	1	0	550
1	0	0	0	0	0	1	1	600
1	1	0	1	1	1	1	0	680
1	1	0	0	0	1	1	1	700
1	1	0	0	0	0	0	1	750
0	0	1	1	1	1	1	1	750
0	0	1	1	1	1	1	1	800
0	0	1	1	1	1	1	1	900
1	1	0	0	0	0	0	0	1000
1	0	0	0	0	0	1	0	1200
0	0	1	1	1	1	1	1	1400
1	1	0	0	0	0	0	0	1500

				A03				
A.T13	A.T12	A.T9	A.T8	A.T6	A.T5	A.T4	W.T	M
1	1	1	1	1	1	1	1	80
1	1	1	1	1	1	1	1	100
1	1	1	1	1	1	1	1	180
1	1	1	1	1	1	1	1	210
1	1	1	1	1	1	1	1	300
0	0	1	0	1	1	1	1	400
0	0	1	0	0	0	1	1	450
1	1	0	1	1	1	0	0	500
1	1	1	1	1	1	1	1	700
1	1	1	1	1	1	1	1	1100

				A06				
A.T13	A.T12	A.T9	A.T8	A.T6	A.T5	A.T4	W.T	M
1	1	1	1	1	1	1	1	70
1	1	1	1	1	1	1	1	150
1	1	0	0	0	0	1	1	200
0	0	1	1	1	1	0	0	220
1	1	1	1	1	1	1	1	380
1	1	1	1	1	1	1	1	400
1	1	1	0	0	0	0	0	600
0	1	0	0	0	0	0	0	700
1	1	1	0	1	1	1	1	1100

To examine the genetic relationships among higher curdlan production 7 mutants; A.T4, A.T5, A.T6, A.T8, A.T9, A.T12 and A.T13 and their Wiled type (W.T). Based on RAPD-PCR results, the scored data were analyzed using the Dice coefficient to compute the similarity matrices. These similarity matrices were used to generate a dendrogram using the UPGMA method. The dendrogram tree and similarity indices among the eight strains utilizing RAPD-PCR. The UPGMA cluster analysis was carried out to represent graphically the genetic distances among the 8 strain.

As shown in (Figure 4), a phylogenetic tree illustrated that all the eight strains had two distinct groups G1 and G2. This tree which shown in Figure (4) evidenced that mutants; A.T6, A.T9, A.T4, A.T8 and A.T5 are cluster in descending manner according to their relatedness to their Wild type. The first group (G1) includes one cluster with two strains; A.T12 and A.T13 with close distance between them. High genetic similarity ratio between the two strains about 84% with the same genotype methonine. This cluster is so far from the other clusters with low genetic similarity ratio no more than 57 %.

On the other hand, the second group (G2) includes 6 strains; W.T, A.T4, A.T5, A.T6, A.T8 and A.T9 with different distance between them in four pool-clusters; a, b, c and d. It's had one sup-group (cluster a) include two strains; A.T5 and A.T8 with very closely distance and similarity ratio up to 96%. This result is in parallel with the data in Table (4) which showed that the two mutant strains; A.T5 and A.T8 produced equal approximately efficiency of curdlan production 34 and 35.6,respectively. Two mutant strains; A.T6 and A.T9 have second sup-group (cluster b) with close distance between them (about 84% similarity) with the same genotype uracil and far from the cluster (d). It's have distance from them about 78% similarity. Wiled type and mutant strain A.T4 have the rest sup-group (cluster c) with close distance between them (about 88% similarity) and far from the two clusters; d and b (cluster a). It's have distance from them about 56% similarity and have a common ancestor the cluster (a). This result was in agreement with those obtained by[22,23].

Dendrogram using Average Linkage (Between strains)

Rescaled Distance Cluster Combine

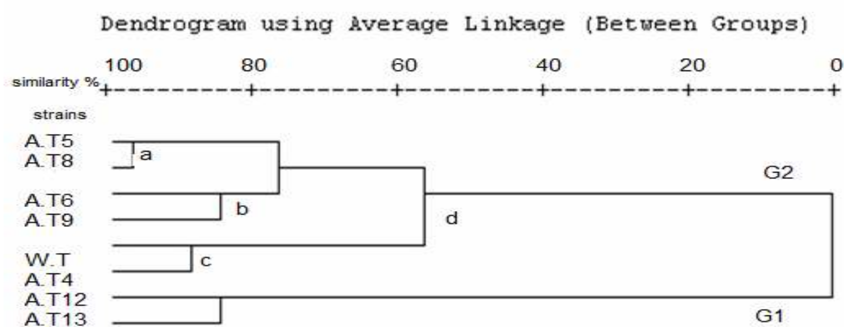

Fig 4. Dendrogram for eight *Agrobacterium tumefaciens* strains constructed from RAODs data using Unweighted Pair-Group Arthnetic Average (UPGMA) and similarity matrices computed according to Dice coefficients.

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