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Determination of genetic diversity of *Gluconacebacter hansenii* with RAPD and ERIC-PCR

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Abstract
Cellulose production is typical for *Gluconacebacter* species. Bacterial cellulose is mainly used in paper and textile industry, high-performance speaker diaphragms, dietary foods, artificial skin, paint thinner, medical underwear, wound covering material, cosmetic makeup pads and facemask making. In this study, RAPD-PCR and ERIC-PCR method, which are PCR-based methods, was used to determine the genetic diversity of *Gluconacebacter hansenii*. Nine *Gluconacebacter hansenii* strains, ten RAPD primers and one pair of ERIC primers were used. The RAPD-PCR method produced 106 bands. Out of 106 bands that obtained from RAPD primers, 86 of them were polymorphic which indicates 81.13% polymorphism ratio. ERIC primers have also shown high polymorphism and distinguished species on strain level. As a result of study, the similarities of the strains formed by deriving from the data obtained are seen. Taking notice of the calculated polymorphism rate, the genetic diversity of the strains appears to be at the expected level.

Keywords: *Gluconacebacter* hansenii, genetic variation, bacterial cellulose, PCR

1. Introduction

Acetic acid bacteria (AAB) are Gram-negative, ellipsoidal to rod-shaped cells and obligate aerobic bacteria. Taxonomically, AAB is classified in the family Acetobacteraceae [1, 2]. They are widespread in nature and these bacteria are mainly responsible for producing acetic acid. In addition some acetic acid bacteria produce exopolysaccharide known as bacterial cellulose [3]. Bacterial cellulose is produced as a pure polymer that does not contain lignin and hemicellulose. The high water retention capacity of bacterial cellulose, its durability and flexibility have made it more advantageous to use the industry compared to plant cellulose. Because of these properties, bacterial cellulose is used in the paper, textile industry, high-performance speaker diaphragms, dietary foods, artificial deep, paint thinner, medical underwear dressing material, cosmetic underwear makeup pads and facemask making. Producing cellulosic bacteria, one of the indispensable products of industry, in a short time, efficiently and in good quality is important in biotechnological applications. The production of cellulosic bacteria will stop the abundance of trees in the country, which will help protect nature and the environment [4]. The taxonomy of AAB has undergone many changes in recent years. Several genera and species of AAB have been newly described. AAB is currently classified into ten genera and 44 species, namely *Acetobacter* (16 species), *Gluconobacter* (5 species), *Acidomonas* (1 species), *Gluconacetobacter* (15 species), Asaia (3 species), *Kozakia* (1 species), *Saccharibacter* (1 species), *Swaminathania* (1 species), *Neosaia* (1 species), and *Granulibacter* (1 species), in the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α-subdivision of the Proteobacteria [5, 6, 7]. The identification of the AAB species has traditionally been performed by studying physiological and chemotaxonomic properties [1], but the methods are not completely reliable and are often time-consuming. Therefore, they have been complemented or replaced by different molecular techniques, in particular DNA: DNA hybridizations [5,8] and PCR-based genomic fingerprinting techniques such as Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-amplified 16S rRNA [9] or 16S– 23S rRNA intergenic spacer regions [9, 10, 11, 12], Randomly Amplified Polymorphic DNA (RAPD) fingerprinting [10], PCR amplification of repetitive bacterial DNA elements (repPCR) using the REP (Repetitive Extragenic Palindromic sequences) or ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) primers [9] and Differentiation of species of the family Acetobacteraceae using AFLP DNA fingerprinting [8].

The aim of this study was to determinate the genetic diversity and phylogenetic relationships of nine *Gluconoacebacter hansenii* strains using RAPD-PCR and ERIC-PCR methods.

2. Materials and methods

2.1 Sample collection and isolation of microorganisms

Samples were isolated from home-made vinegar and wine samples. Ten milliliter of wine samples were taken under aseptic conditions and added on 90 ml of HS Broth (2 % glucose, 0.5 % yeast extract, 0.5 % polypeptone, 0.675 % Na_2HPO_4, 0.115 % citric acid) [13]. 100 ppm cycloheximide was added into the medium for inhibition of yeasts and fungi. Under static conditions, the bacterium was incubated at 30 ^{0}C for 7 days. After incubation, the pellic formation was observed. Pellic was suspended in 0.85 % PSW (NaCl) solution under aseptic conditions and ensured that pellic was completely fragmentized. After complete fragmentization, it was diluted up to 10^{-3} with a sterile saline solution. After dilution, from every dilute it was distributed homojenically to HS (1.5 % agar) and GYC agar (5% glucose, 1% yeast extract, 3% $CaCO_3$, 2 % agar) and incubated at 30 ^{0}C for 7 days. At the end of the time period the little, white or beige, high or convex and compact mucoid colonies were selected.

All isolates were preserved by freezing in glycerol at −20 ^{0}C and monthly checked for strain stability [14].

2.2 Morphological and biochemical identification of microorganisms

Morphological and biochemical identification of isolates was performed using Gram staining and standard biochemical tests (catalase, oxidase, indole production, carbohydrate fermentation, nitrate reduction etc.) as outlined in Bergey's Manual of Systematic Bacteriology [15].

2.3 Molecular identification of microorganisms

DNA isolation of the nine isolates was realized using the classical phenol-chloroform method according to Flanagan [16] with a few changes. Bacterial samples were suspended in 1ml of sterile distilled water at 0.5 McFarland and centrifuged at 10000g for 5 mins. Pellets were dissolved in 700 µl GET buffer (50 mM Glucose- 10 mM EDTA- 25 mM TrisHCl) with vortexing. Then 10µl 25% SDS and 5µl 25 mg/ml Proteinase K were added to the suspension and mixed by inverting the tubes. Then tubes were incubated in a 60ºC water bath for 20 mins. After taking tubes from water bath 500 µl of PCI (Phenol-Chloroform-Isoamyl Alcohol) were added and mixed by inverting the tubes for 5 mins. Tubes were centrifuged at 10000g for 5 mins. 200 µl of the supernatants were transferred to new tubes and 200 µl KAc and 400 µl cold isopropanol was added. Tubes were incubated at -20ºC 10 mins. Then tubes were centrifuged at 10000g for 5 mins. Supernatants were discarded and pellets were washed with 500 µl 70% cold ethanol. After centrifugation at 10000g for 2 mins alcohol was discarded and evaporated. After the pellets were completely dried 50 µl of sterile distilled water were added to the tubes and dissolved at RT overnight. DNA concentration and purity were measured with Nanodrop spectrophotometer (Thermo Scientific). DNA samples were run on 0.8% agarose gel at 90 V 30 mins for the control of DNAs.

16S rDNA primers (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-CGG TTA CCT TGT TAC GAC TT-3') were used for molecular identification. PCR reactions were carried out at initial denaturation 95 ºC 5 min, denaturation 94 ºC 40 sec, annealing 50 ºC 40 sec, extension 72 ºC 40 sec with 35 cycles and a final extension at 72ºC 10 min. Reagents concentrations were 10X Taq Buffer, 0.5M dNTP mix, 10 pM from each primer, 7.5 mM MgCl2 and 1U Taq polymerase with the final volume of 25 µl. PCR products were sent to Macrogen (Holland) for sequencing. Sequences were compared with those present in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.4 RAPD-PCR

RAPD-PCR method, which is a PCR-based method, was used to determine the genetic diversity of *Gluconacetobacter hansenii* used as a model organism in the production of bacterial cellulose. 10 PCR primers were used in PCR experiments (Table 1) which were obtained from firma. In reactions 5X Firepol Mastermix (Solis Biodyne) was used. Reactions were set up as instructed in the manual of the master mix with the final volume of 25 µl. As a negative control dH_2O was used instead of DNA template. Reaction conditions are as follows: Initial Denaturation 95ºC 4 mins, with 45 cycles of denaturation at 94ºC 1 min, annealing 30-40ºC 1 min, extension 72ºC 45 secs and final extension was held at 72ºC 5 mins. [17].

Table 1: List of RAPD primers

No	Primer Name	Primer Sequence
1	RAPD1	5'-TGGTCAGTCA-3'
2	RAPD2	5'-CGGTCACTGT-3'
3	RAPD3	5'-AGCGGGGCGTA-3'
4	RAPD4	5'-CGCGTGCCCA-3'
5	RAPD5	5'-GACAGACAGACAGACA-3'
6	RAPD6	5'-GTGGTGGTGGTGGTG-3'
7	RAPD7	5'-ACGGCGCTTC-3'
8	RAPD8	5'-GAAACGGGTG-3'
9	RAPD9	5'-AATCGCGCTG-3'
10	RAPD10	5'-GTGGCTCTCC-3'

2.5 ERIC-PCR

ERIC-PCR were carried out with 5X Firepol Mastermix (Solis Biodyne). Companies instructions were used with the final volume of 25 µl. 95ºC 7 mins, with 45 cycles of denaturation at 90ºC 30 sec, annealing 55ºC 1 min, extension 72ºC 8 mins and final extension was held at 72ºC 16 mins. ERIC-PCR primers were given in Table 2 [18].

Table 2: List of ERIC-PCR primers

Primer Name	Primer Sequence
ERIC1	5'-ATGTAAGCTCCTGGGGATTCAC-3'
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'

2.6 Agarose Gel Electrophoresis

The results of the PCR reactions were run on 1.4% agarose gel at 90 V 60 mins in 1X TBE buffer. The gel was stained with SafeView Classic (ABM). The gels were visualized under UV light and photographed.

2.7 Data analysis

Evaluation of the bands in RAPD-PCR was carried out according to the presence (1) or absence (0) of the bands. Genetic variety index was calculated using POPGENE 32 software. The dendrogram was automatically generated by the software [19].

ERIC-PCR banding pattern was coded as 1 (present) and 0 (absent) and the data obtained was subjected for statistical analysis by Squared Euclidean Distance (SED) (Ward's Method) using the software IBM SPSS v22 [20].

3. Results & Discussion

Isolated bacteria are a rod-shaped, aerobic, Gram-negative, non-spore forming, catalase-positive. The sequence analysis of the 16S rDNA gene of the isolate *G. hansenii* was submitted to BLAST at the GenBank to find similar nucleic acid sequences (Table 3).

Table 3: *Gluconacetobacter hansenii* isolated from home-made vinegar and wine samples

Strain number	Name of Samples	Name of The Species	Accession No
1^{2b}	Cider Vinegar	*Gluconacetobacter hansenii*	KF155166.1
1^{1}	Cider Vinegar	*Gluconacetobacter hansenii*	KF155166.1
2^{1}	Wine	*Gluconacetobacter hansenii*	AB166735.1
2^{3}	Wine	*Gluconacetobacter hansenii*	AB166735.1
3^{1}	Wine	*Gluconacetobacter hansenii*	KF155166.1
3^{2}	Wine	*Gluconacetobacter hansenii*	AB166734.1
3^{3}	Wine	*Gluconacetobacter hansenii*	AB166735.1
6^{2}	Grape Vinegar	*Gluconacetobacter hansenii*	KF155166.1
6^{1k}	Grape Vinegar	*Gluconacetobacter hansenii*	KF155166.1

A total of 106 of which 86 polymorphic bands were obtained from 10 RAPD primers used in the study (Figure 1). The number of bands formed by the primer ranges from 4 to 20. The average number of bands was found to be 10.6. The polymorphism rate of the primers ranged from 50% to 100% and the average polymorphism rate was calculated as 81.13%. When matrices (Table 4) and dendrograms (Figure 2) are evaluated, the most similar strains 1^{1} and 6^{1k} are seen as the least similar strains 1^{2b} and 1^{1}, respectively

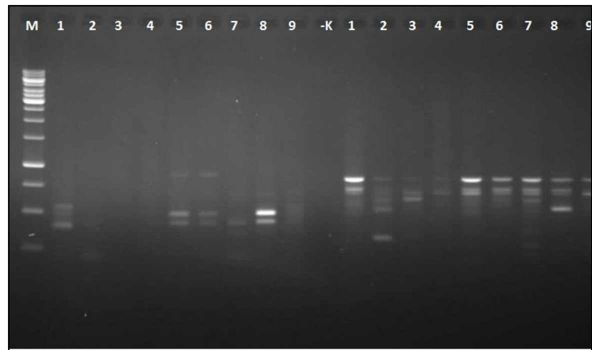

Fig 1: Agarose gel electrophoresis of RAPD-PCR results of two primers

Table 4: Similarity matrix of the strains based on RAPD-PCR profile ($1:1^{2b}$, $2:1^{1}$, $3:2^{1}$, $4:2^{3}$, $5:3^{1}$, $6:3^{2}$, $7:3^{3}$, $8:6^{2}$, $9:6^{1k}$)

pop ID	1	2	3	4	5	6	7	8	9
1	****	0.7075	0.6792	0.6792	0.6226	0.6226	0.5943	0.5000	0.5566
2	0.3460	****	0.8396	0.6698	0.5943	0.6132	0.6038	0.5472	0.6226
3	0.3868	0.1748	****	0.7736	0.6226	0.6226	0.6132	0.5943	0.6698
4	0.3868	0.4008	0.2567	****	0.6038	0.6226	0.6321	0.5755	0.6698
5	0.4738	0.5203	0.4738	0.5046	****	0.8113	0.7075	0.6698	0.6132
6	0.4738	0.4891	0.4738	0.4738	0.2091	****	0.7264	0.6321	0.6698
7	0.5203	0.5046	0.4891	0.4587	0.3460	0.3196	****	0.7736	0.7358
8	0.6931	0.6030	0.5203	0.5526	0.4008	0.4587	0.2567	****	0.8302
9	0.5859	0.4738	0.4008	0.4008	0.4891	0.4008	0.3067	0.1861	****

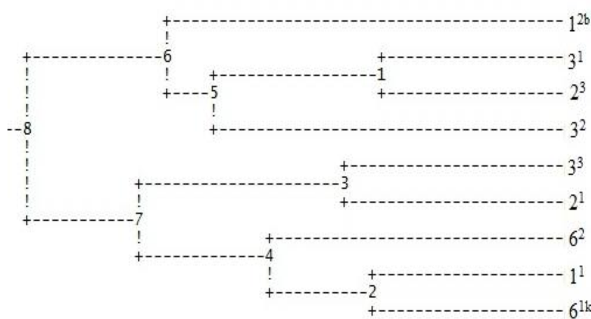

Fig 2: Similarity tree of the strains drawn by Popgene 32 software

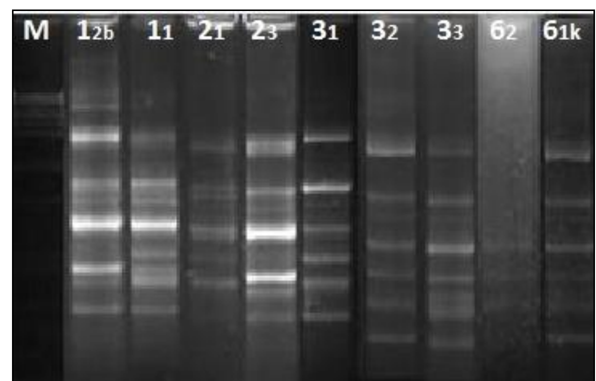

Fig 3: Agarose gel electrophoresis of Eric-PCR results.

ERIC-PCR bands ranged from 300 to 3000 bp (Figure 3). The obtained banding profile showed high polymorphism. DNA band profile data were analyzed by SED using software IBM SPSS v22 (Table 5). This empowered the plotting of dendrogram demonstrating the level of genetic similarity among the strains (Figure 4).

Phylogenetic analysis of ERIC-PCR results generated two clusters, one consists six strains and other consists three strains. Cluster analysis indicated, although they are species, high variability among strains. Therefore, ERIC-PCR distinguished different strains of the same species.

Table 5: Proximity matrix of strains according to ERIC-PCR data

Case	Squared Euclidean Distance								
	1^{2b}	1^1	2^1	2^3	3^1	3^2	3^3	6^2	6^{1k}
1^{2b}	,000	3,000	9,000	8,000	7,000	11,000	11,000	10,000	12,000
1^1	3,000	,000	6,000	7,000	4,000	10,000	10,000	9,000	11,000
2^1	9,000	6,000	,000	5,000	8,000	6,000	6,000	5,000	5,000
2^3	8,000	7,000	5,000	,000	9,000	3,000	3,000	6,000	4,000
3^1	7,000	4,000	8,000	9,000	,000	8,000	8,000	9,000	9,000
3^2	11,000	10,000	6,000	3,000	8,000	,000	,000	3,000	1,000
3^3	11,000	10,000	6,000	3,000	8,000	,000	,000	3,000	1,000
6^2	10,000	9,000	5,000	6,000	9,000	3,000	3,000	,000	2,000
6^{1k}	12,000	11,000	5,000	4,000	9,000	1,000	1,000	2,000	,000

This is a dissimilarity matrix

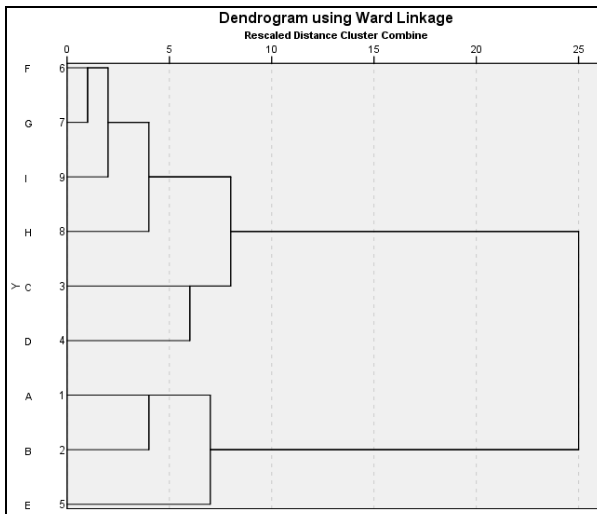

Fig 4: Dendrogram based on the ERIC-PCR profile. (A:1^{2b}, B:1^1, C:2^1, D:2^3, E:3^1, F:3^2, G:3^3, H:6^2, I:6^{1k})

If we compare the two dendrograms, we can see both have two main clusters. RAPD-PCR dendrogram has four strains on one cluster and five on the other while ERIC-PCR dendrogram has three strains on one cluster and six strains on the other cluster. ERIC-PCR dendrogram puts 1^{2b}, 3^1 and 1^1 together while RAPD-PCR dendrogram separates 1^1 from the other two. ERIC-PCR dendrogram branches 1^{2b} and 1^1 from the same nod and binds 3^1 to these two, RAPD-PCR dendrogram separates these two, branches 1^1 and 6^{1k} from the same nod and binds 6^2 to these two strains while putting 1^{2b} to a different cluster. These huge differences can be explained by two different software that was used to evaluate the data.

In recent years RAPD-PCR and ERIC-PCR methos have been applied to a determined taxonomic grouping of acetic acid bacteria. Treck *et al.* [11] performed phenotypic characterization by RAPD-PCR metod of Acetobacter sp. isolated from spirit vinegar production. They identified seven different RAPD profilies of *Acetobacter europaeus*. Nanda *et al.* [21] demostrated that intraspecific variation of *Acetobacter pasteurianus* by RAPD-PCR and ERIC-PCR. Gonzales *et al.* [22] showed that during fermentation *Gluconobacter oxydans*, *Acetobacter aceti*, *Gluconacetobacter hansenii* and *Gluconacetobacter liquefaciens* were isolated and fingerprinted using RAPD-PCR and ERIC-PCR methods. Gonzales *et al.* [9] isolated acetic acid bacteria from wine and carried out typing of acetic acid bacteria using ERIC-PCR. Lisdiyanti *et al.* [23] reclassified *Gluconacetobacter hansenii* and *Acetobacter pasteurianus* by RAPD-PCR and ERIC-PCR. Prieto *et al.* [24] realized that *Acetobacter cerevisiae* and *Gluconobacter oxydans* was isolated and analysed using RAPD-PCR. Gullo and Giudici [25] isolated *Acetobacter pasteurianus* in traditional balsamic vinegar and characterized by ERIC-PCR. Gullo *et al.* [26] isolated strain AB0220 on GYC medium and tested subcultures of *A. pasteurianus* AB0220 by ERIC-PCR.

Hidalgo *et al.* [27] appointed that acetic acid bacteria isolated from wine were genotyped by ERIC-PCR technique and two species as *Acetobacter pasteurianus* and *Gluconacetobacter intermedius* were identified. Wu *et al.* [28] carried out that 21 acetic acid bacteria were isolated and characterized using ERIC-PCR fingerprinting. Fernández-Pérez *et al.* [29] studied strain typing of 103 acetic acid bacteria isolates from vinegar using the ERIC-PCR method and found dominant species as *Gluconacetobacter europaeus*. Vegas *et al.* [30] isolated 265 isolates from two different culture media and carried out population dynamics of acetic acid bacteria in traditional vinegar production and characterized strain level by ERIC-PCR. Valera *et al.* [31] carried out the identification of acetic acid bacteria (AAB) from sound grapes from the Canary Islands. *Acetobacter pasteurianus*, *Acetobacter tropicalis*, *Gluconobacter japonicus* and *Gluconacetobacter saccharivorans* were identified and charecterized using ERIC-PCR method. Mohanraj *et al.*, [32] RAPD (Random amplified polymorphic DNA) analysis were performed to establish the phylogenetic relationship between *Acetobacter pasteurianus* (NCIM 2522), *Acetobacter xylinum* (NCIM 2526). Polymorphism was analyzed based on the dendrogram of RAPD patterns using UPGMA (Unweighed Pair GroupMethod with Arithmetic Mean). RAPD analysis in our study showed that there is an 80% similarity between these bacterial strains. Gullo *et al.* [33] researched cultivability and phenotypic stability of *Acetobacter pasteurianus* over 9 years of preservation. Wu *et al.*, [34] carried out that yeasts, lactic acid bacteria and acetic acid bacteria in traditional Chinese vinegar isolated and the genotypic diversity of the isolates was characterized by ERIC/PCR on strain level. Hidalgo *et al.* [35] isolated *Acetobacter* strains in blueberry and identified genotypes of *Acetobacter cerevisiae* using ERIC-PCR method. Mateo *et al.* [14] show that acetic acid bacteria (AAB) diversity was analyzed by to fingerprinting techniques of ERIC-PCR and nine different AAB species were identified from the 624 isolates.

4. Conclusions

As a result of this study, the similarities of dendrogram species formed by the way of obtained data are seen. When the calculated polymorphism rate is taken into

consideration, it is seen that the genetic diversity of the strains is at the expected level. The yield of the strains of the strains should be checked, and the strains producing more efficient and higher quality cellulose by molecular cloning will be obtained. By producing high-quality and high-yielding bacterial cellulose on this count, paper-medical will become an alternative industrial material in all aspects of the industry.

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