Iranian Journal of Microbiology
Volume 14 Number 2 (April 2022) 186-193

ORIGINAL ARTICLE

Combination and improvement of conventional DNA extraction methods in Actinobacteria to obtain high-quantity and high-quality DNA

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Received: March 2021**, Accepted:** March 2022

ABSTRACT

Background and Objectives: DNA extraction is an important step of any molecular experiment. DNA could not be easily extracted from members of actinomycetes by the usual methods of lysis. Due to the low efficiency of the conventional DNA extraction methods, development of an effective technique for DNA extraction of actinobacteria in emergency cases seems to be necessary. Since most of the DNA extraction techniques and commercial kits are not efficient enough to extract DNA from actinobacteria, this study was conducted to improve an efficient method obtained from conventional one to extract DNA from this group of bacteria.

Materials and Methods: DNA extraction was performed using five methods (an improved method, Invisorb Spin Plant Mini Kit, EZ-10 Spin Column, Sarrbrucken method (HZI, Germany) and Kirby Bauer's method). To evaluate the quantity and quality of extracted genomic DNA, UV absorbance of all samples and efficiency of polymerase chain reaction (PCR) were evaluated.

Results: Overall, the results revealed that the highest quantity (up to 4000 ng/µl) and good quality of DNA was obtained using introduced DNA extraction method.

Conclusion: Results indicated that recently introduced improved method was more efficient for extraction of DNA from actinobacteria for DDH (DNA–DNA hybridization) test and for those require the high concentration of DNA.

Keywords: Actinobacteria; Antimicrobial analyses; DNA amplification technique; DNA extraction method; DNA–DNA hybridization; Whole genome sequencing

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INTRODUCTION

Extraction of high-quality nucleic acids from different biological sources for subsequent molecular analyses is the first and most important step in molecular biology and molecular genetics studies (1). High-quality DNA extraction procedure depends on the concentration and high purity of extracted DNA as well as removal of inhibitors. Inhibitors, especially in organisms which produce high levels of secondary metabolites, cause many problems in molecular analyses (2). Nowadays, a wide range of different bacterial DNA extraction protocols such as phenol-chloroform, CTAB (Cetyl Trimethyl Ammonium Bromide), liquid nitrogen, salting out and commercial kits (3-6) are commonly in use. However, most of these techniques and commercial kits are not efficient enough to extract DNA from a different group of actinobacteria.

Actinobacteria and myxobacteria, especially *Streptomyces* strains are known as the most important sources to find bioactive compounds (7, 8). During the last decades, isolation of new actinobacterial strains and producing novel bioactive metabolites has become increasingly difficult (9). In case of this group of bacteria, molecular identification using sequencing and DDH (DNA–DNA hybridization) test is critical. Most of the methods employed for genomic DNA extraction from actinomycetes have faced many problems such as poor yield of DNA and high polysaccharides contaminants. Low yield is due to incomplete lysis because of the tendency of actinomycetes to grow as compact masses or pellets of mycelium (10). Due to the low efficiency of most conventional DNA extraction techniques and commercial kits which yield low concentration, low quality and low quantity DNA that are inappropriate for DDH test, development of efficient techniques to extract actinobacteria DNA seems to be necessary. In this study, the conventional DNA extraction methods were combined and improved in order to introduce an alternate method for high-efficiency DNA extraction which is useful for most bacterial groups.

MATERIALS AND METHODS

Strains. To evaluate and compare five different DNA extraction methods used in this study, five different species were used (Table 1).

Cell mass preparation. All of the strains except Mx35Zk (cultured on VY/2 medium) (12) were cultured on GYM culture medium (65. GYM Streptomyces Medium, DSMZ: 0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$; pH 7.2; sterilized for 20 minutes at 121°C) and incubated at 37°C under constant shaking at 140 rpm in darkness. To obtain cell mass, bacterial suspension (100 ml) of each strain were centrifuged at 5000 rpm for 15 minutes and supernatants were discarded completely. For each strain, 150 mg of precipitated cell mass were measured and used for each DNA extraction. Experiments were performed in three replications.

DNA extraction: improved method. 150 mg of precipitated cell mass was suspended in 5 ml SET buffer in 1.5 ml tube (75 mM NaCl, 25 mM EDTA pH8, 20 mM Tris HCL, pH 7.5) and 15 glass beads (0.5 mm diameter) were added to each vial. The samples were homogenized with crushing machine (6 m/s/ for 2*40 s). Samples were incubated at 100°C for 5 minutes, then were frozen in liquid N_2 for 3 minutes. Tubes were incubated at 100°C for 5 minutes. Then Containers were transferred to falcon tubes containing 300 µl SDS 20%, 300 µl proteinase K (10 mg/ml in 50 mM Tris HCL pH 8, 1 mM $CaCl_2$) and 300 µl Lysozyme (10 mg/ml) and were incubated at 55°C for 2 hours. The containers were inverted at least once every 15 minutes. 50 ml Phenol: chloroform: isoamyl alcohol (25:24:1) were added and falcon tubes were swung for 1 hour. The mixture was centrifuged at 8000 rpm for 5 minutes at room temperature. White layer must reduce significantly or disappeared. Otherwise extraction step was repeated and tubes were swung for 1 hour. After centrifugation, the upper phase was transferred into a new falcon. The volume of 50 ml of chloroform/isoamyl alcohol (24/1) were added and tubes were fluctuated for 30 minutes. The mixture was centrifuged at 8000 rpm for 5 minutes at room temperature. The upper phase was transferred into a new falcon. 1/10 volume of 1 M NaOAC (pH 4.8) was added into the mixture and mixed properly. Falcons were incubated in freezer at -20°C for 10 minutes. Samples were centrifuged at 11000 rpm at 4°C for 10 minutes. The upper phase was transferred into a new falcon and equal volume of cold isopropanol was added. After incubation at -20°C for 10 minutes samples were centrifuged at 11000 rpm, for 10 minutes at 4°C. The upper phase was discarded. DNA pellet in each tube was washed with cold etha-

nol 70% and centrifuged at 11000 rpm, at 4°C for 10 minutes (this step was repeated twice). Pellets were re-suspended in deionized distilled water or TE buffer and finally stored at -20°C (Fig. 1).

Kirby Bauer's method. 150 mg of precipitated cell mass of each strain was suspended in 15 ml tube containing 3 ml of TE25S buffer (25 Mm Tris-HCl, pH8, 25mM EDTA pH8, and 0.3 M sucrose). The

Table 1. Name and accession number of the bacterial strains used in this study.

Species	Strain designation
Streptomyces sp. (new species) (11)	Act4Zk (MK418597)
Streptomyces roseolilacinus	DSM 40173
Streptomyces cinereoruber subsp. *cinereoruber*	Act39Zk (MK518390)
Streptomyces viridodiastaticus	Ac43Zk (MK518441)
Myxococcus xanthus	MX35ZK (MT446238)

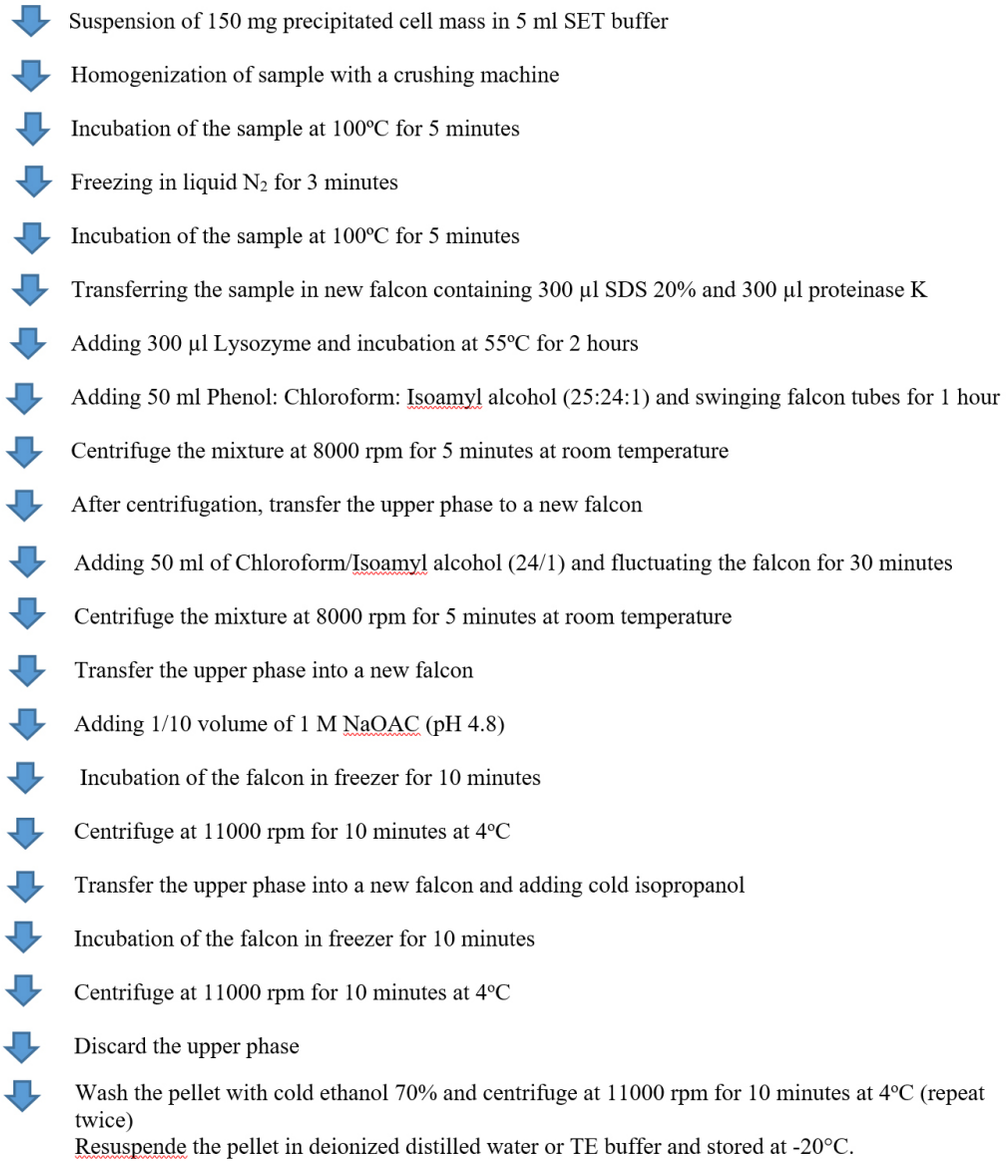

Fig. 1. Flow chart of improved DNA extraction method.

volume of 100 µl lysozyme solution was added (60 mg ml-1 in water, final lysozyme concentration is 2 mg ml-1) to each tube and they were incubated for 10 minutes at 37ºC. Then 4 ml 2× Kirby mix (2× Kirby mix: 2 g TPNS (sodium tri-isopropylnaphtalene sulphate, Kodak P3513; SDS can be used instead), 12 g sodium 4-aminosalicilate (BDH), 5 ml Tris-HCl pH 8, 6 ml phenol pH 8, make up to 100 ml with water) was added to each tube and tubes were agitated for 1 minutes on a vortex mixer. After that 8 ml phenol/chloroform/isoamyl alcohol (25:24:1) was added into the tubes and tubes were agitated for 15 seconds as above and then centrifuged for 10 minutes at 3500 rpm. After centrifugation, the upper phase was transferred to a new falcon containing 3 ml phenol/chloroform/isoamyl alcohol (25:24:1) and 600 µl of 3 M sodium acetate, then falcons were agitated as in step 2 and centrifuged as in step 4. Then 6 ml isopropanol was added into the falcons and mixedd, spooled DNA onto a sealed Pasteur pipette, and finally, DNA was washed in 5 ml of 70% ethanol. DNA Pellets were dried and re-suspended in deionized distilled water or TE buffer and stored at -20ºC (13).

Invisorb spin plant mini kit and EZ-10 spin column genomic DNA miniprep kit. Genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Stratec Molecular, Germany) and EZ-10 Spin Column Genomic DNA Miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada) following the manufacturer's protocol.

Saarbrucken method (HZI, Germany). 150 mg of precipitated cell mass was suspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8, 20 mM Tris HCL, pH 7.5) and incubated at 100ºC for 30-60 minutes. After that, 300 µl of SDS 20%, 300 µl of proteinase K (10 mg/ml in 50 mM Tris HCL pH 8, 1 mM CaCl2) and 300 µl of Lysozyme (10 mg/ml) were added into the suspension and tubes were incubated at 55ºC for 2 hours. Each container was inverted at least every 15 minutes. 50 ml of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added into the mixture and falcon tubes were swung for 1 hour. The mixture was centrifuged at 8000 rpm for 5 minutes at room temperature (this step was repeated twice). White layer must be reduced significantly or disappeared. Otherwise, extraction step was repeated again and tubes were swung for 1 hour. After centrifugation, the upper phase was transferred into a new tube. 1 volume of Chloroform/Isoamyl alcohol (24/1) was added and the falcons fluctuated for 30 minutes. The mixture was centrifuged at 8000 rpm for 5 minutes at room temperature. The upper phase was transferred into a new falcon. 1/10 volume of 1 M NaOAC (pH 4.8) was added and mixed properly. 2 volumes of absolute ethanol were added and falcon tubes were shaken. The gDNA was Fished and placed in Eppendorf tube. DNA pellets were washed with ethanol 70% (twice) and then they were dried. Pellets were re-suspended in deionized distilled water or TE buffer and stored at -20ºC.

Assessment of the DNA concentration and quality. To evaluate the quantity and quality of extracted genomic DNA, UV absorbance of all samples were measured by Nano-drop spectrophotometer (IMPLEN Nano Photometer UV / VIS Spectrophotometer). Generally, the intensity of UV absorbance of DNA solution at wavelengths of 260 nm and 280 nm indicates the purity of DNA. DNA absorbs UV light at 260 nm and 280 nm, while aromatic proteins absorb UV at 280 nm. Normally, 260/280 absorbance ratio for pure DNA is 1.8, that it is relatively proteins free. Lower 260/280 absorbance ratio means extracted genomic DNA contains protein (14). For this purpose, the absorbance of extracted DNA was measured at 260 nm and 280 nm by nanodrop spectrophotometer with this ratio: 4 µL/ 1 µL (distilled water/ DNA).

Evaluation of polymerase chain reaction (PCR) amplification. For this purpose, the 16S rRNA gene region was amplified. Amplification was performed with universal forward (F27- 5'-GAGTTTGATCCTGGCTCAGGA-3') and reverse (R1492-5'-TACGGYTACCTTGTTACGACTT-3') primer pair in a total reaction volume of 25 µl consisted of the following components: 12.5 µL JSRM, 10 µL water, 1 µL forward and reverse primer dilution as well as 0.5 µL of the template DNA (15). Polymerase chain reaction within the thermocycler (Mastercycler gradie) started with an activation temperature of 95°C for 5 minutes. The first cycle of the touchdown PCR started with a denaturation step of 94°C for 1 minute and a primer annealing at 70°C for 1 minute, followed by two minutes of elongation at 72°C. During the next nine cycles, the annealing temperature was decreased by 1°C per cycle to 60°C. Finally, for the replication of the template, 28 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 2 minutes and a final extension at 72°C

for 5 minutes were performed. Afterwards, the samples were mixed with loading buffer charged with the DNA-dye SYBR-Green and were loaded on a 0.8% agarose gel. After 90 minutes at 70 V, the bands were detectable under UV light.

RESULTS

The results obtained in this study revealed that the average amounts of recovered DNA by the improved recommended method using 150 mg wet cell mass, was ranged from 1500 ± 500 to 4000 ± 1000 ng/µl for actinomycetes and 150 ± 30 ng/µl for myxobacteria with purity measured at A260/280 of 1.22 ± 0.06 to 1.5 ± 0.19 and 1.7 ± 0.02, respectively. DNA obtained using this method was comparable to other methods as shown in Table 2. The highest concentration of DNA was obtained with the improved recommended method (method 5) and Kirby Bauer's method (method 3). In contrast, the highest purity of DNA was found when using the EZ-10 Spin Column Genomic DNA miniprep kit and the Saarbrucken methods. The inverse relationship between quality (measured as A260/280 absorbance ratio) and quantity of the extracted DNA is shown in Fig. 3. The comparison of the extraction methods studied, i.e. the improved method, the Kirby Bauer, Saarbrucken and commercial kits methods, showed different efficiency in DNA extraction and purity. As already stated, the highest quality of DNA was obtained by the EZ-10 Spin Column Genomic DNA Miniprep kit with a 260/280 absorbance ratio of 1.67, which also yielded the lowest quantity of DNA (0.355 µg/ml). Quantity and quality of DNA obtained from different methods of DNA extraction are shown in Fig. 3. The Saarbrucken method and improved method have the most similar procedure as compared to other methods but their results were almost different particularly in quantity of DNA (Fig. 3: Method 4 and 5). The highest concentration of DNA was obtained with the improved method, 2.124 µg/ml DNA, which also yielded good quality as compared to other methods. PCR results demonstrated that 16S rRNA gene was amplified using DNA templates of all five different bacterial strains obtained by improved recommended method and EZ-10 spin column, while other methods were efficient in case of some bacterial strains. For example, in case of DNA that was extracted by Invisorb Spin Plant Mini Kit, only, 16S rRNA gene of *Myxococcus xanthus* (Mx35Zk), *Streptomyces* sp. (Act4Zk.) and *Streptomyces roseolilacinus* (DSM 40173) was amplified (Fig. 2). At the initial steps of DNA extraction by the improved recommended method and EZ-10 Spin Column, physical destruction was employed. The results revealed that a combination of physical, chemical and thermal shocks for lysis of cell wall has high efficiency. According to the results,

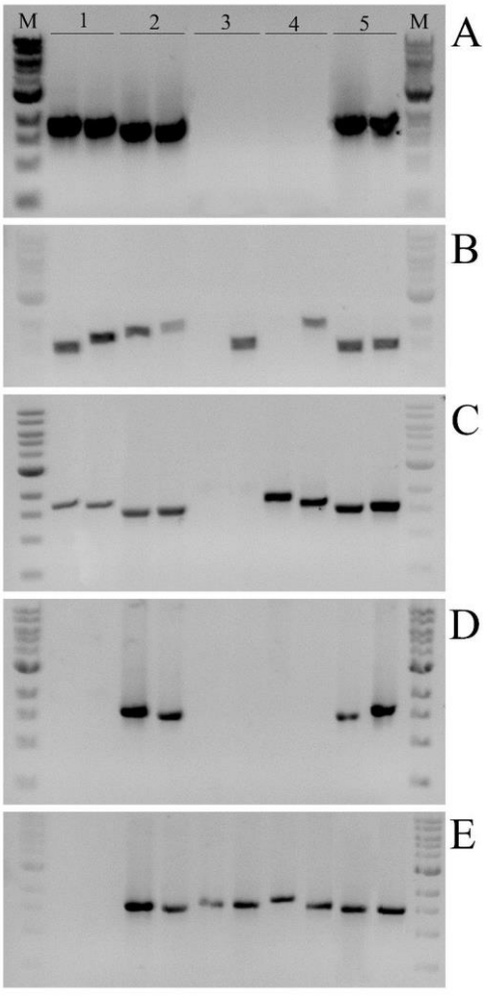

Fig. 2. Amplification of 16S rDNA for A: *Myxococcus xanthus* (Mx35Zk), B: *Streptomyces* sp. (Act4Zk.), C: *Streptomyces roseolilacinus* (DSM 40173), D: *Streptomyces cinereoruber* subsp. *Cinereoruber* (Act39Zk), E: *Streptomyces viridodiastaticus* (Act43Zk). Lane M: Marker 1000 bp; Lane 1: PCR product amplified with DNA template obtained from Invisorb Spin Plant Mini Kit; Lane 2: PCR product amplified with DNA template obtained from EZ-10 Spin Column Genomic DNA Miniprep kit, Lane 3: PCR product amplified with DNA template obtained from Kirby Bauer method; Lane 4: PCR product amplified with DNA template obtained from Sarbrucken method and Lane 5: PCR product amplified with DNA template obtained from improved method.

Table 2. Quality (OD260/OD 280 ratio) and quantity (ng/µl) of extracted genomic DNA using five different methods.

Methods	Strain	DNA concentration (ng/µl)	260/280 absorbance ratio
Method 1	Act4Zk	3750 ± 250	2.45 ± 0.15
(Invisorb Spin Plant Mini Kit)	DSM 40173	1250 ± 250	1.22 ± 0.09
	Act39Zk	-	-
	Act43Zk	750 ± 400	1.5 ± 0.3
	Mx35Zk	29.5 ± 3.5	2.23 ± 0.03
Method 2	Act4Zk	1750 ± 250	1 ± 0
(EZ-10 Spin Column Genomic DNA	DSM 40173	77 ± 3	1.86 ± 0.06
Miniprep kit)	Act39Zk	33.5 ± 3.5	1.91 ± 0.09
	Act43Zk	32.5 ± 1	1.82 ± 0.13
	Mx35Zk	150 ± 10	2.03 ± 0.01
Method 3	Act4Zk	755 ± 350	1.42 ± 0.2
Kirby Bauer method	DSM 40173	3750 ± 1300	1.05 ± 0.05
	Act39Zk	3500 ± 200	0.87 ± 0.03
	Act43Zk	2274 ± 1000	1.68 ± 0.2
	Mx35Zk	48.3 ± 16	1.56 ± 0.3
Method 4	Act4Zk	3750 ± 1000	1.15 ± 0.12
Sarbrucken method	DSM 40173	11.25 ± 1.25	1.50 ± 0.03
	Act39Zk	750 ± 500	1.50 ± 0.4
	Act43Zk	31 ± 5.5	2.4 ± 0.06
	Mx35Zk	43.5 ±	1.4 ± 0.15
Method 5	Act4Zk	1500 ± 500AA	1.4 ± 0.05
improved Method	DSM 40173	4000 ± 1000	1.5 ± 0.19
	Act39Zk	4000 ± 500	1.22 ± 0.06
	Act43Zk	3500 ± 500	1.4 ± 0.16
	Mx35Zk	150 ± 30	1.7 ± 0.02

both the improved recommended method and EZ-10 Spin Column were efficient DNA extraction methods but, the quantity of extracted DNA was low using the EZ-10 Spin Column (Table 2). It should be noted that DNA extraction using the methods mentioned in this study and amplification of the 16S rRNA gene was performed several times at different time intervals, which showed high reproducibility of the results of improved method compared to others methods. Also, the DNA obtained by the improved method after 20 months of storage in deionized distilled water at -20ºC, was still of good quality and could be used in molecular studies.

DISCUSSION

The importance for appropriate extraction methods in order to acquire exceptionally purified nucleic acids without inhibitors has been explained previously (16, 17). Extraction and concentration of DNA are important in molecular analytical techniques. Hence, any efficient DNA extraction method must provide sufficient quantity and inhibitor-free DNA that leads to disruption of enzymatic and ion activity. To obtain high quality and quantity genomic DNA, several DNA extraction methods, also ready-to- use kits, have been developed. Some of the well-known methods like CTAB and SDS (Sodium Dodecyl sulfate) chloroform methods are used in molecular analytical methodologies. The efficiency, speed and application of inexpensive facilities and non-toxic chemicals are important factors of extraction methods. The improved recommended method in this study is time-consuming and needs toxic chemicals. However, in emergency cases that other methods are not useful it can be considered as an alternative as well as effective method of DNA extraction as compared with the existing ones. Some of the molecular biology techniques, such as whole genome sequenc-

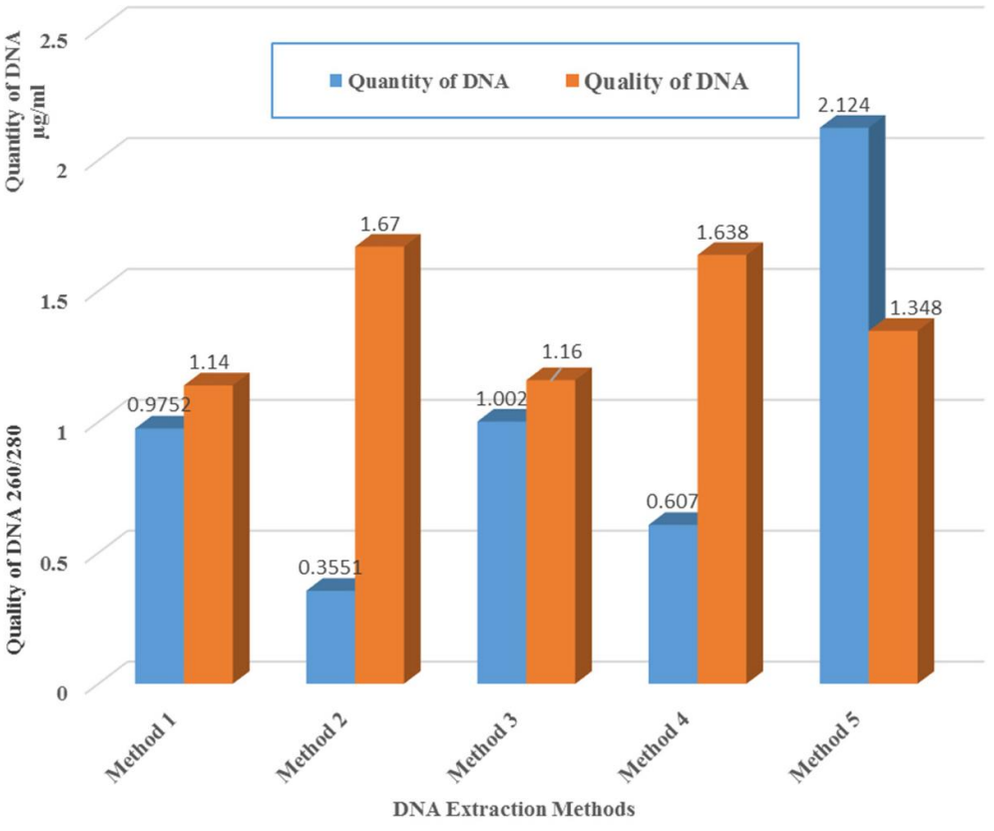

Fig. 3. Relationship between purity and concentration of the DNA extracted by various methods. Method 1: Invisorb Spin Plant Mini Kit, method 2: EZ-10 Spin Column Genomic DNA Miniprep kit, method 3: Kirby Bauer method, method 4: Sarbrucken method and method 5: improved Method.

ing (WGS) and DNA–DNA hybridization (DDH) require high quality DNA (high molecular weight with an A260/280 ratio between 1.8 and 2.0). Indeed, DNA extraction methods are evaluated based on their yield and the quality of results. DNA-DNA hybridizations still is a major tool in species delineation in actinobacteria. Considering all the points, the improved method in this study is sufficiently efficient in extracting of high quality gDNA for DDH and WGS. Using different shocks to lysis of the cell wall has made the improved method time-consuming, but considering the acceptable results, the time consuming nature of the improved method for DNA extraction can be ignored.

CONCLUSION

Extraction of DNA from most strains of actinomycetes is very time-consuming because a long pretreatment is needed before the cell wall becomes sensitive to the usual lytic enzymes. On the other hand, high quality DNA is required for DDH, which is a common method in species delineation in actinobacteria. The improved method described here considerably increases the concentration of DNA in comparison with other methods and is particularly useful for Actinomyces species.

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