Combination and Improvement of Conventional DNA Extraction Methods in Actinobacteria

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Abstract

I. **Background:** DNA extraction is an important step of any molecular experiment, in conjunction with the members of Actinomycetes, DNA couldn't be quickly extracted by the usual methods of lysis. Due to the low efficiency of most of the conventional DNA extraction techniques, development of an effective techniques for DNA extraction of Actinobacteria in emergency case seems to be necessary. Since, most of the known DNA extraction techniques and commercial kits do not have sufficient efficiency in the extraction of DNA from a different group of Actinobacteria, the objective of this study was to improvement an efficient method from conventional methods for DNA extraction from Actinobacteria.

II. **Methods and Results:** For this purpose, DNA extraction was performed by five methods (an improvedmethod, Invisorb Spin Plant Mini Kit, EZ-10 Spin Column, phenol-chloroform method 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. Overall, the results showed the highest quantity of DNA (up to 4000 ng/µl) was obtained by employing introduced DNA extraction method, also yielding good quality.

III. These results indicate the recently introduced improved method is more efficient for extraction of DNA from Actinobacteria for DDH (DNA–DNA hybridization) test and for those they require the high concentration of DNA.

Introduction

Isolating high-quality nucleic acids from different biological sources for subsequent molecular analysis is the first and most important step in molecular biology and molecular genetics [1]. Extraction of DNA with high-quality depends on the removal of inhibitors and high concentration and high purity of extracted DNA. Inhibitors, especially in organisms which produce high levels of secondary metabolites, cause many problems in molecular analyzes [2]. Nowadays, a wide range of different DNA extraction protocols is commonly in use for molecular analyses of bacteria, such as phenol-chloroform, CTAB (Cetyl Trimethyl Ammonium Bromide), liquid nitrogen, salting out and commercial kits [4, 5, 6]. But, most of the known DNA extraction techniques and commercial kits do not have sufficient efficiency in the extraction of 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, producing novel bioactive metabolites, became increasingly difficult [9]. In the case of this group of bacteria, molecular identification using sequencing and DDH (DNA–DNA hybridization) test is critical. Most of the methods employed for isolating genomic DNA from Actinomycetes face 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 of the conventional DNA extraction techniques which yield low

concentration DNA and low quality and quantity of recovered DNA using commercial kits that are inappropriate for DDH test, development of eficient techniques for DNA extraction of Actinobacteria seems to be necessary. In this study, the conventional DNA extraction methods were combined and improved to introduce an alternate method for high-efficiency DNA extraction which is useful for most bacterial groups.

Material And Methods

Strains

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

Table 1
Strains name with accession number.

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

Cell mass preparation

All of the strains except Mx35Zk (culture on VY/2 medium [11] were cultured on GYM 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 min at 121 °C) and incubated at 37 °C under constant shaking at 140 rpm in darkness. To obtain cell mass, bacterial suspension (100 ml) were centrifuged at 5000 rpm for 15 min and discarded the supernatant completely. For each strain, 150 mg of precipitated cell mass were measured and used for each DNA extraction method. The experiment was performed in three replications.

DNA extraction

DNA extraction by the 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 bead (0.5 mm diameter) was added for each vial. The samples were homogenized with crushing machine (6 m/s/ for 2*40 s). Samples were incubated at 100 o C for 5 min, then were frozen in liquid N2 for 3 min. tubes were incubated at 100 ^{o}C for 5 min. container were transferred into new 15 ml falcon, 300 µl SDS 20 %, 300 µl proteinase K (10 mg/ml in 50 mM Tris

HCL pH 8, 1 mM Cacl2) and 300 µl Lysozyme (10 mg/ml) were added and incubated at 55 ^{O}C for 2 hours. The container was inverted at least once every 15 min. 50 ml Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added and swing the falcon for 1 hour. The mixture was centrifuged at 8000 rpm for 5 min at room temperature. Withe layer must be significantly reduced or disappeared. If this is not the case, repeat the extraction step one more time and swing the tube for 1 hour. After centrifugation, the upper phase was transferred into a new falcon. 50 ml of Chloroform/Isoamyl alcoholIsoamyl alcohol (24/1) were added and the falcon fluctuated for 30 min. The mixture was centrifuged at 8000 rpm for 5 min 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. falcons were incubated in the freezer (-20 ^{O}C) for 10 min. Samples were centrifuged at 11000 rpm at 4 °C for 10 min. The upper phase was transferred into a new falcon and equal volume cold Isopropanol was added. Samples were incubated at freezer (-20 °C) for 10 min. Then, they were centrifuged at 11000 rpm, 10 min, at 4 °C. The upper phase was discarded. The pellet was washed with cold ethanol 70 % and centrifuged at 11000 r.p.m, at 4 ^{O}C for 10 min (this step was repeated one more time). Pellet was resuspended in deionized distilled water or TE buffer and stored at -20 °C.

Kirby Bauer’s method

150 mg of precipitated cell mass were resuspended in 3 ml TE25S buffer (25 Mm Tris-HCl, pH8, 25 mM EDTA pH8, 0.3 M sucrose), 100 µl lysozyme solution was added (60 mg ml-1 in water, final lysozyme concentration is 2 mg ml-1) and incubated 10 min at 37 ºC. Then 4 ml 2x Kirby mix (2x 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.) were added and agitated for 1 min on a vortex mixer. Then, 8 ml phenol/chloroform/isoamyl alcohol (25:24:1) was added and agitated for 15 secs as above then centrifuged 10 min at 3500 r.p.m. 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, falcons were agitated as in step 2 and centrifuged as in step 4. Then 6 ml isopropanol was added, mixed, spooled DNA onto a sealed Pasteur pipette, and finally, DNA was washed in 5 ml 70% ethanol. Pellets were dried in air and redissolved in deionized distilled water or TE buffer and stored at -20 ºC [12].

DNA extraction with 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 of 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 100 ºC for 30–60 min. 300 µl SDS 20 %, 300 µl proteinase K (10 mg/ml in 50 mM Tris HCL pH 8, 1 mM Cacl2) and 300 µl Lysozyme (10 mg/ml) were added and incubated at 55 ºC for 2 hours. The container was inverted at least every 15 min. 50 ml Phenol:

Chloroform: Isoamyl alcohol (25:24:1) were added and swing the tube for 1 hour. The mixture was centrifuged at 8000 rpm for 5 min at room temperature (this step was repeated one more time). The withe layer must be significantly reduced or disappeared. If this is not the case, repeat the extraction step one more time and swing the tube 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 min. The mixture was centrifuged at 8000 rpm for 5 min 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 volume of absolute ethanol were added and gently were shaked. The gDNA was Fished and placed in Eppendorf tube. The pellet was washed with ethanol 70 % (twice) and dried. Pellet was resuspended in deionized distilled water or TE buffer and stored at -20 ºC.

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. Generally, the intensity of UV absorbance of DNA solution at wavelengths of 260 nm and 280 nm showing the purity of DNA as an indicator. 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 [13]. 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 PCR (Polymerase Chain Reaction) 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 and 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 [14]. The PCR within the thermocycler started with an activation temperature of 95 °C for 5 min. The first cycle of the touchdown PCR started with a denaturation step of 94 °C for 1 min and a primer annealing at 70 °C for 1 min, 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 min, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min and a final extension at 72 °C for 5 min 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 min at 70 V, the bands were detectable under UV light.

Results

The results obtained in this study revealed 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 is 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. 2. The comparison of the extraction methods studied, i.e. the improved method, the Kirby Bauer, Saarbrucken and commercial kits methods, has shown a different efficiency in DNA and purity. As already stated, the highest quality of DNA was obtained by the EZ-10 Spin Column Genomic DNA Miniprep kit method, a 260/280 = 1.67, also yielding the lowest quantity, 0.355 µg/ml. In fact, in the other methods, DNA quantity and quality was almost the same (Fig. 2). The Saarbrucken method and improved method have the most similar procedure in compare with other methods but their results were almost different particularly in quantity of concentration of DNA (Fig. 2: Method 4 and 5). The highest concentration of DNA was obtained with the improved method, 2.124 µg/ml also yielding good quality in comparison with other methods. PCR results demonstrate that *16S rRNA* gene was amplified by using DNA templates of all five different bacterial strains that obtained by using improved recommended method and EZ-10 Spin Column, while other methods were efficient in the case some bacterial strains. For example, in the case of DNA that 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. 1). 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 methods for lysis of cell wall has high efficiency. According to the results, both the improved recommended method and EZ-10 Spin Column are efficient DNA extraction methods but, the quantity of extracted DNA was low using the EZ-10 Spin Column (Table 2).

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

Methods	strain	DNA concentration (ng/µl)	260/280 absorbance ratio
Method 1 (Invisorb Spin Plant Mini Kit)	Act4Zk	3750 ± 250	2.45 ± 0.15
	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 (EZ-10 Spin Column Genomic DNA Miniprep kit)	Act4Zk	1750 ± 250	1 ± 0
	DSM 40173	77 ± 3	1.86 ± 0.06
	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 Kirby Bauer method	Act4Zk	755 ± 350	1.42 ± 0.2
	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 Sarbrucken method	Act4Zk	3750 ± 1000	1.15 ± 0.12
	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 improved Method	Act4Zk	1500 ± 500AA	1.4 ± 0.05
	DSM 40173	4000 ± 1000	1.5 ± 0.19
	Act39Zk	4000 ± 500	1.22 ± 0.06

Methods	strain	DNA concentration (ng/µl)	260/280 absorbance ratio
	Act43Zk	3500 ± 500	1.4 ± 0.16
	Mx35Zk	150 ± 30	1.7 ± 0.02

Discussion

The requirement for appropriate extraction methods to acquire exceptionally purified nucleic acids without inhibitors has been explained [15, 16]. Extraction and concentration of DNA are the main important steps 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 the fact that neither expensive facilities, nor toxic chemicals are required, however, the improved recommended method is time-consuming and need toxic chemicals but in emergency cases that other methods are not useful can be an alternative and effective to the existing methods of DNA extraction. Indeed, extraction methods are evaluated based on their yield and the quality of results.

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. The improved method described here considerably increases the concentration of DNA in comparison with other methods and is particularly useful for Actinomyces species.

Declarations

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Conflicts of interest/Competing interests

Zahra Khosravi babadi, Abolfazl Narmani, Gholam hossein Ebrahimipour and Joachim Wink declare that they have no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

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Authors contributions

methodology, Zahra Khosravi babadi and Abolfazl Narmani; investigation and writing –original draft preparation, Zahra Khosravi babadi; writing—review and editing, Zahra Khosravi babadi; Abolfazl Narmani and Joachim Wink; project administration, Joachim Wink and Gholam hossein Ebrahimipour All authors have read and agreed to the published version of the manuscript.

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Figures

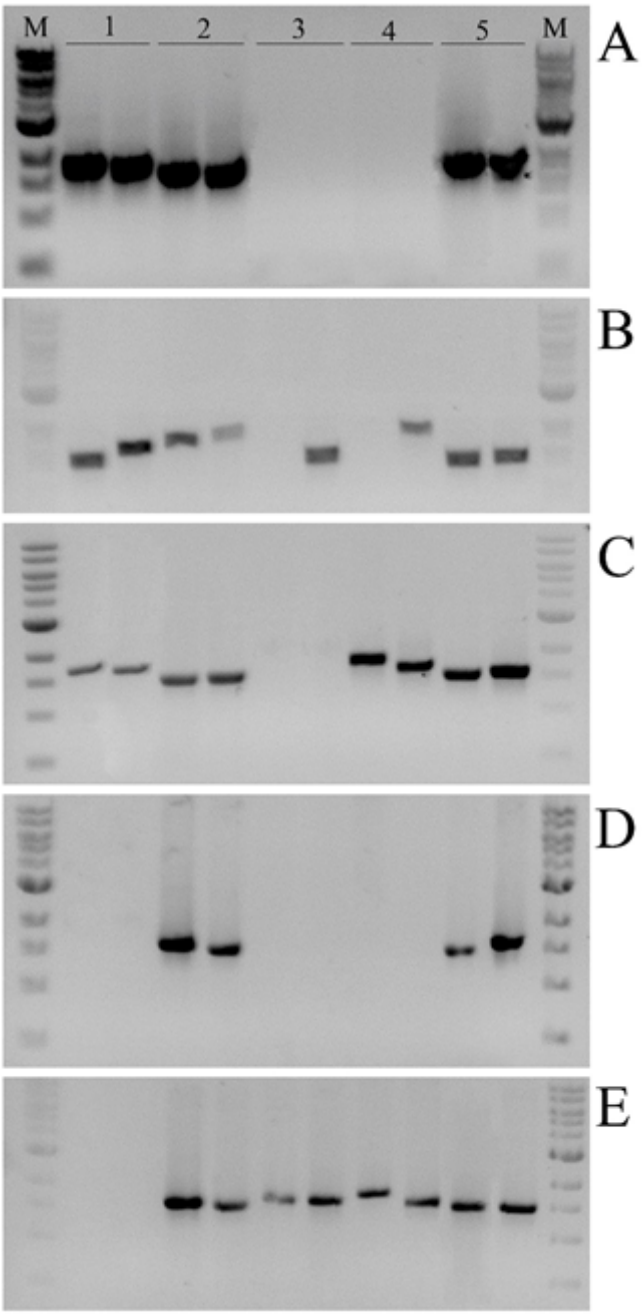

Figure 1

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 1000bp; 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.

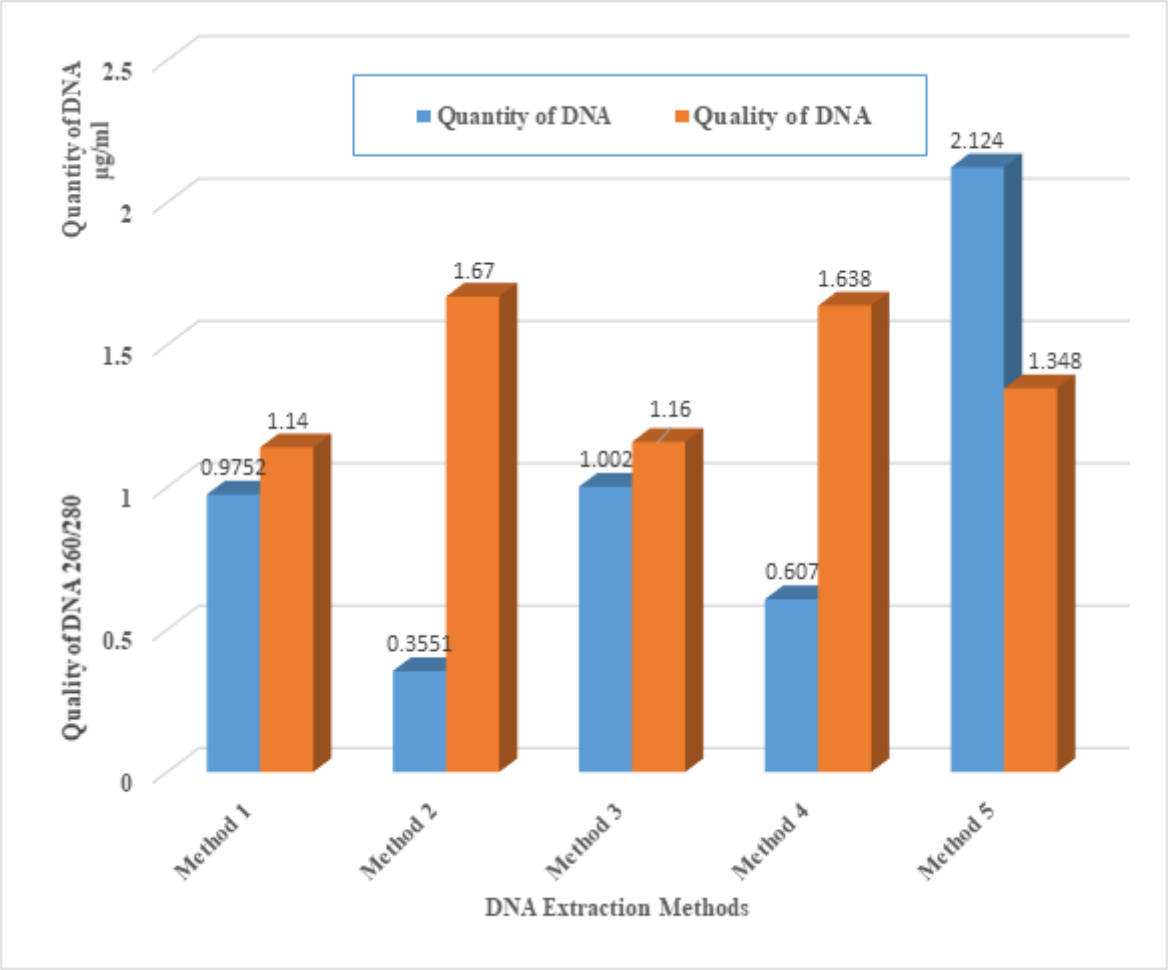

Figure 2

Relationship between purity and concentration of the DNA extracted by various methods