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Optimization of DNA extraction from the Algerian traditional date's product "Btana"

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Abstract –Btana is traditional preservation method that can sustain date's supply during many years in comparaison to the commercial storage methods. However no scientific informations are available about biological factors that contribute to the successful of this method. Bacterial communities are a major key in preservation of many foods. Culture independent techniques are the most powerful tools to enhance bacterial diversity studies, but their efficiencies start with DNA extraction step. Therefore we have studied 3 protocols of DNA extraction from 11 Btana samples to evaluate their yield in total microbial DNA recovery. Protocols were based on a commercial kit DNeasy (QIAGEN, Germany) and two modified CTAB extraction methods (combined CTAB-DNeasy protocol, modified CTAB protocol) using polyvinyl pyrrolidone (PVP) and treatment with high salt solution (5M NaCl). Protocols were compared for quantity of DNA extracted using NanoDrop® ND-1000 Spectrophotometer and quality of DNA by 260/280 nm absorption ratio. The total extracted DNA was cheeked by PCR amplification of the 16S rRNA and visualized by electrophoresis on agarose gel (0.8%). Results showed that CTAB modified method provide the best DNA yield; however purification with NucleoSpin® Kit (Clontech, UK) was mostly needed for amplifying the DNA template. DNeasy kit protocol gave an amplified high quality DNA, but poor yields were obtained from date samples.

Keywords: Traditional foods, Dates; *Btana*, DNA extraction, CTAB.

1. Introduction

Date palm (Phoenix dactylifera) is a monocotyledon tree grown in many regions of the world. The edible parts of P. dactylifera is the ripe fruit which has a high nutritional value and contains a remarkable content of vitamins, fiber, sugars, salts and trace amount of fat and proteins (Vayalil 2012; Al-Shahib and Marshall 2003). In South Algerian rural cities, dates represent a major source of income for many families and are daily consumed practically during the holy month of Ramadan, popular festivities, funerals and to welcome guests. The local population has developed a number of date preservation methods to overcome the post harvesting lost. The basic method is named Btana which involve transformation and storage of date at ambient temperature.

Preparation of this product is still performed by deep rooted food culture of the rural Algerian communities and is highly appreciated. A necessary need urge us to study this traditional date products because it represent a local knowledge that should be maintained and scientifically understood to find out all factors that might contribute to the preservation of this local heritage. In recent years several high throughput molecular techniques have been successfully used to detect and survey bacterial flora in traditional foods (Abriouel et al. 2006; Greppi et al. 2013; Mukisa et al. 2012; Nam et al. 2012). Nevertheless, some difficulties have severely limited the depth characterization of microbial diversity by theses new edge techniques (Pinto et al. 2007). The ability to extract a representative and sufficient amount of total DNA is one of the most concerns of microbiologist. The most pitfalls for microbial diversity studies arise frequently from low DNA yield, no suitable purity, and degradation that lead to altered informations on diversity richness (Thakuria et al. 2008). DNA extraction has therefore been highlighted as a limitation of culture-independent methods (Abriouel et al. 2006). Choosing an extraction method often involves a trade-off between cost (materials and labor), the optimal yield of DNA and the removal of substances that could interfere with the PCR reaction (Cankar et al. 2006). Like other fruits, date presents many drawbacks for DNA extraction due to its complex composition and the presence of a broad range of polysaccharides, carbohydrates, polyphenolics, fibers, and a high content of salts which can interfere with enzymatic and chemical reactions during DNA extraction (Boudries et al. 2007; Vayalil 2012). Many authors revealed that the presence of these molecules affects negatively the classical extraction protocols and many commercial kits (Probeski et al. 1997; Gudenschwager et al. 2012; Mamlouk et al. 2011). The use of the solid polymer PVP (polyvinyl pyrrolidone) and the ionic detergent CTAB (hexadecyltrimethyl ammonium bromide) was proposed as a powerful alternative to remove polyphenols and polysaccharides respectively (Probeski et al. 1997; Jara et al. 2008). CTAB protocols which often combine the use of treatments with PVP and high salt solution are cheap methods of extraction compared to commercial kits. However, many modifications were undergone on the initial protocol proposed by Doyle and Doyle (1990) to suit the particularities of each food matrices (Michiels et al. 2003; Jara et al. 2008, Mamlouk 2011). Here we compared a commercial kit of DNA extraction; DNeasy Blood & Tissue kit (QIAGEN, GmbH, Hilden, Germany) with two modified CTAB protocols to assess their efficiencies in bacterial DNA extraction from the Algerian traditional date's product "Btana". The protocol's efficiency was verified by DNA yield, purity, and feasibility of 16sRNA gene amplification.

2. Materials and methods

2.1 Preparation of samples

Eleven samples of date products "Btana" were collected from different localities in the South of Algeria. Twenty five grams of date past was homogenized in sterile bag with 225 ml of phosphate bufferd saline (8.5 g NaCl, 1.1 g K_2HPO4, 0.32 KH_2PO4, pH 7.4) using Stomacher apparatus, then 1 ml of each sample was centrifuged for 10 min at 14,000 rpm to harvest bacterial cells. Pellets were treated

according to three protocols: DNeasy(A), CTAB(B), DNeasy-CTAB (C). Crude DNA from each protocol was purified with Nucleospin (Clontech, UK) kit according to the manufacturer's instructions.

2.2 Protocols A: DNeasy kit

Qiagen DNeasy Plant kit (QIAGEN, Germany) was used following the manufacturer's recommendations with introduction of a grinding step as mentioned below.

2.3 Protocols B: CTAB (modified by Probeski et al. 1997)

The extraction buffer contained [100mM Tris, pH 8, 1.4M NaCl, 30mM EDTA, pH 8] was autoclaved and 2% of polyvinylpyrrolidone (PVP, Sigma-Aldrich, St Louis, USA), 2% CTAB (cethyltrimethyl ammoniumbromide, Sigma-Aldrich, St Louis, USA) were added after filter sterilization. Before use 1% (v/v) b-mercaptoethanol and 40mg/ml of lysozyme were added to the solution.

The buffer was preheated (56°C) and added (500µl) to the pellet, then homogenized by vortexing for 5 s. Microtubes were then incubated at 56 °C in a Thermomixer (Eppendorf AG, Hamburg, Germany) for 1h. The lysis step was followed by grinding with approximately 3 g of 710-1180µm diameter acid-washed glass beads (Sigma, USA) in a bead beater instrument (Vortex Genie2, Scientific Industries) for 10s. Then 20µl of Proteinase K were added to the pellet, and incubated at 56°C for 1 h.

The solution obtained after enzymatic digestion was centrifuged (12,000 rpm) for 5 minutes. Afterword, supernatant was cooled at room temperature, overlapped with 1V Alcohol isoamilic:chloroform (1:24) and mixed thoroughly by inverting the tubes to form an emulsion. Then a centrifugation was performed (12,000 rpm) for ten minutes. The aqueous phase was recuperated, treated with ½ V of 5M NaCl , precipitated with 2V of absolute cold ethanol (-20°C) and stored at -20°C during 10 min.

Crude ADN pellet was obtained by centrifugation at 12,000 rpm for 10 min then washed with 200µl of cold 70% ethanol, and re-suspended in sterile RNA free water.

2.4 Protocols C : CTAB-DNeasy

Samples were treated as mentioned in the early step of CTAB protocol. The upper aqueous phase obtained after alcohol isoamilic:chloroform treatment was directly transferred to a DNeasy column and treated according to the manufacturer instructions.

2.5 DNA recovery

DNA concentration and purity were checked by spectrophotometric measure using NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington DE) according to the manufacturer's instructions (the absorption ratio 260/280 nm was considered to assess purity of DNA). The extracted DNA was further checked in PCR for amplification of the 16S rRNA gene.

PCR reactions were carried out using 20 µl volumes each containing: 2 µl extracted DNA, 2 µl of dNTPs (2mM), 1.6 µl of $MgCl_2$ (25 mM), 2 µl PCR buffer (10×), 0.8 µl of each primer (10 µM) 16S-1500 F: 5'-GAG-TTT-GAT-CMT-GGC-TCA-G-3'

(Eurogentec, 3968107, Tm=60°C) 16S-1500 REV: 5'-TAC-GGT-TAC-CTTGTTACGAC-3' (Eurogentec, 3968108 Tm=58 °C.) and 0.2 µl of Taq

DNA polymerase (2.5U, Fastastart, Roch, Germany) and 2µl Bovine Serum Albumin (BSA, Invitrogen, USA). The PCR program was performed with a Mastercycler gradient (Eppendorf, Westbury, NY) under the following conditions: Initial denaturation at 94 ºC for 5 min, 35 cycles at 94 ºC for 30 s, 56 ºC for 30 s and 72 ºC for 1 min with a final extension at 72 ºC for 5 min.

2.6 DNA purification

The extracted DNA samples were purified with the genomic DNA-clean-up NucleoSpin kit (Macherey, Nagel GmbH, Düren, Germany) according to the manufacturer's recommendations. Then, PCR products were run in 0.8% agarose gel and visualized by UV transillumination system (Eastman Kodac Company, Scientific Imaging Systems, Rochester).

3. Statistical analysis

Values of different parameters were expressed as the mean of duplicate determinations ± standard error. ANOVA analysis was used to analysis data within and between protocols (A, B, C) with α value selected at 0.05. Post hoc analysis was performed with Tukey multiple comparison test to see the difference of DNA yield and purity between the methods.

For comparison of the effect of purification on DNA quality and yield, repeated measures ANOVA were used. The statistical analyses were performed using GraphPad Prism 5.01 software.

4. Results

In the present study, we have assayed 3 different DNA extraction protocols from the traditional date products "Btana" to see the most suitable extraction method that yield best quality and quantity of the total microbial DNA for metagenomics or other downstream applications. The PCR products of 16S rRNA gene visualized by electrophoresis were further used to confirm the effectiveness of the protocols (**Figure 1** and **2**). Table 1 summarized the yield of DNA and A260/280 obtained with 11 Btana samples by the various extraction methods using Nanodrop spectrophotometer. Results are presented as mean of duplicate measure ± standard deviation.

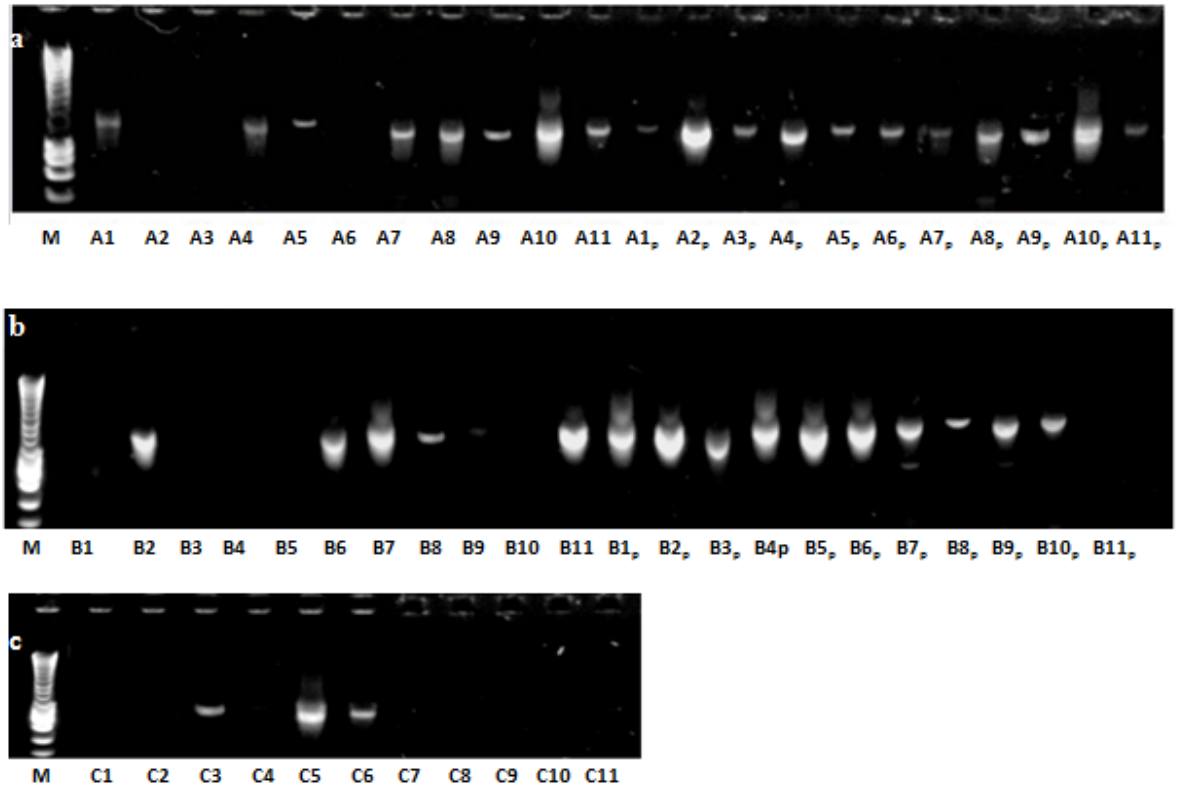

Figure 1. 0.8% Gel electrophoresis of 16S RNA amplicon products extracted from 11 Btana samples with three DNA extraction protocols (a: DNeasy, b: CTAB, c: CTAB-DNeasy) and purified with Nucleospin kit (P).

The commercial DNA extraction kit DNeasy (A) allowed a moderate DNA amounts ranging from 5.70 to 28.60 ng/µl ($P>0.05$). However, DNeasy kit was the most efficient in DNA amplification as eight (1, 4, 5, 7, 8, 9, 10, and 11) out of the eleven samples were amplified after this protocol, although DNA quality was not satisfactory. After purification, a substantial amount of DNA (69.8%) was lost but DNA purity was almost ameliorated and all samples were successfully amplified.

DNA yields using CTAB protocol (B) record a concentration ranged from 18.80 ng/µl to 437 ng/µl and varied strongly according to the samples ($P<0.05$). Samples (1, 3, 4, 5, 8, and 10) were strongly inhibited before purification. A great lost (55.65%) in DNA yield was observed after purification as stated by ANOVA matched pairs test ($P<0.05$). The ratio A260/A280 was slightly increased after purification leading to an improvement in amplification and signal intensity (**Figure 2, 3**).

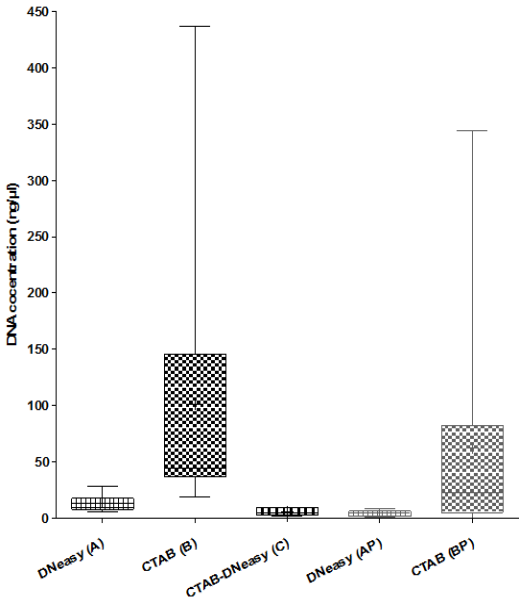

Figure 2. Mean values of DNA yield from three protocols A: DNeasy, B: CTAB, C: CTAB-DNeasy) and yield after DNA purification from protocols DNeasy (AP) and CTAB (BP). The box whiskers indicate the minimum and maximum values.

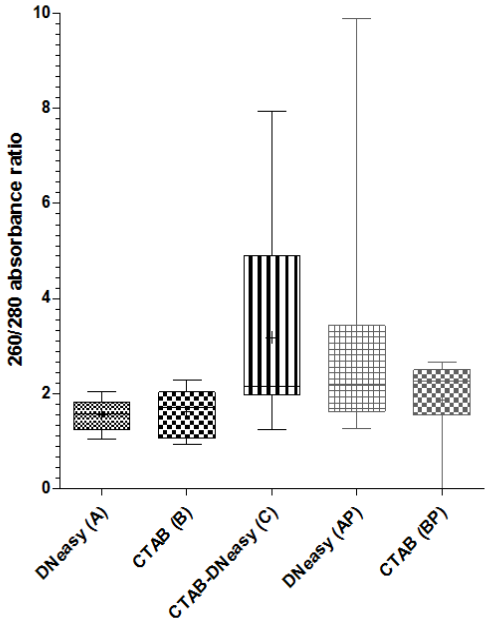

Figure 3. Mean values of absorption ratio (260/280) used to check DNA purity from the three protocols A:DNeasy, B: CTAB, C: CTAB-DNeasy) and after DNA purification from DNeasy (AP) and CTAB (BP). The box whiskers indicate the minimum and maximum values.

Table 1. Concentration and purity of crude and purified DNA from Btana samples with protocols (A, B, C) and PCR efficiency. Different letters in the same row indicates significant different values.

	Protocol A (DNeasy)	Protocol B (CTAB)	Protocol C (CTAB-DNeasy)	Protocol A P (DNeasy)	Protocol B P (CTAB)
DNA (ng/µl)	**13,60 ± 1.9a**	**100,6 ± 63.65b**	**5,182 ± 0.9a**	**4,168 ± 0.74a**	**61,94 ± 30.04a**
DO260/280	**1,56 ± 0.1a**	**1,607± 0.14a**	**2,61 ±0.63a**	**3,059 ± 0.72a**	**2,422 ± 0.42a**
Amplification (positive samples/11)	**8**	**5**	**3**	**11**	**10**

Protocol (C) produced the poorest yields as amplification was partially successful for only 3 samples (3, 5, and 6). DNA purification lost a substantial DNA yields and brought no efficiency in amplification, except for sample 2 (result not shown). Statistical results, indicated a significant difference ($P<0.01$) in DNA extracted by the various protocols. Post hoc analysis using Tukey's analysis showed that the statistically significant difference detected was between the CTAB Protocol (B) and both [DNeasy (A), CTAB-DNeasy (C)] protocols ($P<0.05$). However no significant difference was found between Protocol A and C ($P>0.05$). DNA purity analysis showed no significant difference between the tested protocols ($P>0.05$) according to ANOVA analysis (Table.1). A significant effect ($P<0.05$) was found between the amplification and used extraction protocol, however no relationship was found between DNA concentration and 260/280 ratio and DNA amplification ($P>0.05$).

5. Discussion

In food microbiology, many protocols were developed for DNA extraction from food matrices to study their microbial diversity by independent culture methods. The effectiveness of DNA extraction depends on food properties, particularly their contents of complex molecules that often co-extracte with DNA or inhibit its amplification like organic carbon, proteins, pigments (Jara et al. 2008). Other contaminants arise with the extraction method undergone (Gudenschwager et al. 2012). So it is crucial to choose adequate methods that provide an unbiased isolated DNA for reflecting true representation of the microbial community.

The main criteria for validation of these methods are based on quantitative and qualitative analysis (Jara et al. 2008). Some studies were conducted on total genomic of date's part (leaves, fruits, roots) for botanic and phytopathologic purposes (Arif et al. 2010). However, no relevant studies hitherto intended to study the microbial diversity of dates or traditional date's products (like Btana) by direct extraction of microbial DNA. We therefore evaluated three methods for quality and yield of the extracted DNA from 11 Btana samples. The DNA extraction protocols followed involved combinations of mechanical, chemical and

enzymatic lysing procedures. Two protocols (B, C) were a modified CTAB protocol, and one protocol (A) was a commercial kit widely used in DNA extraction (DNeasy). Chemical composition of Btana is not yet known; by contrast date's composition is well defined. They are rich in carbohydrates like glucose, fructose and sucrose, and represent a good source of fibers (6.4–11.5%) as well as many essential minerals (0.10–916 mg/ 100 g dry weight), flavonoids, and phenolic compounds (Benmeddour et al. 2012). The presence of the aforementioned elements makes microbial DNA extraction more difficult and can inhibit the activity of enzymes responsible for DNA synthesis or interfere in the quantification of nucleic acids by spectrophotometric methods (Moyo et al. 2008). Protocols (B, C) used the cationic surfactant CTAB that had been increasingly applied to extract DNA and RNA from plant tissues and in particular polysaccharide-rich plants (Wang and Stegemann 2010). β-mercaptoethanol and PVP are thought to forms complex hydrogen bonds with polyphenolic compounds and help their precipitation and separation from DNA by centrifugation (Wang and Stegemann 2010). Many authors brought modification on the CTAB protocol to fit to the sample characteristics by coupling with silica column, adding up the volume of extraction buffer, or treatment with NaCl solution (Sánchez-Hernández and Gaytán-Oyarzún 2006; Ghosh et al. 2009; Sharma and Purohit 2012). NaCl together with CTAB is known to remove polysaccharides that interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases (Arif et al. 2010). However the suitable NaCl concentration mentioned in literature varies between 0.02 M and 6 M (Aljanabi et al. 1999, Ghosh et al. 2009). Therefore we tried to improve DNA extraction with the CTAB method by including a step of treatment with 5M NaCl solution and by elution with column spin DNeasy (Protocol B) using DNA binding methodology (Clements et al. 2008).

The commercial kit DNeasy was expected to perform better in DNA yield and quality as well as for PCR reaction. Nevertheless, it failed to isolate a good yield of pure DNA as high as that achieved with the modified CTAB method (B) (**Figure 3**). Moreover, the positive PCR product analyzed on agarose gels, showed less band's signal in comparison with protocol (B). Regardless these limits DNeasy kit seemed to be better than the previous protocol (B) in elimination of PCR inhibitors compounds from DNA extract and was the most suitable for PCR amplification.

The modified CTAB method (B) gave a much better yield in DNA, compared to the other methods (Fig. 3). Two main steps might be responsible of these efficiencies. Treatment with the high salt solution (5M NaCl) which separate polysaccharides from cell extract and BSA added during amplification, who act as an antioxidants to deal with problems related to residual phenolics responsible for PCR failure according to many authors (Yang et al. 2007). The optimized protocol gave higher yield than those extracted using various other protocols. However some samples were inhibited during amplification indicating that PCR inhibitors were not totally eliminated. The DNA quality was further enhanced by purification that leads to amplification of most DNA extracts. CTAB-DNeasy method lead to a good DNA quality in most samples, however low recoveries were obtained may be

because of a lack of cell lysis, decrease of the efficiency of spin columns by polysaccharide contamination or due to DNA losses during buffer washing (Wang and Stegemann 2010). In other hand purification with Nucleospin enhanced the quality of the DNA thanks to the elimination of interfering substances coextracted with DNA; however it seemed to be also deleterious for DNA recovery (Mamlouk et al. 2011).

In conclusion, we have successfully performed optimization of DNA extraction from the traditional date product "Btana" suitable for downstream analysis. Results, suggest that the CTAB extractions coupled with salt solution treatment yield more DNA than those methods using spin-bind column elution; however there is a need to purification step to remove most PCR inhibitors. It is clear that purification diminishes in somehow DNA recovery from the traditional date product "Btana"; however the yield remain still above the detection limit of next generation sequencing like pyrosequencing that needs as little as pectograme amounts of DNA. More work is still need to assess the impact of the CTAB-DNA extraction protocol on the estimation of the microbial diversity present in "Btana" using a well defined community structure samples.

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