Optimisation of DNA extraction from the Algerian traditional date Product « Btana » for bacterial diversity analysis by Pyrosequencing technology

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Study objectif:

In food microbiology, a handful of protocols have been developed for the purification of crude DNA from food matrices to study their microbial diversity. The effectiveness of DNA extraction depends on food properties, particularly their contents of complex molecules that often co-extracted with DNA or inhibiting its amplification like organic carbon, proteins, pigments. Other contaminants might arise with the extraction method undergone. So it is crucial to choice adequate methods that provide an unbiased isolated DNA for reflecting true representation of the microbial community.

Therefore we have 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, QIAGEN). DNA extracts were further purified with Nucleospin purification kit, amplified with 16S rRNA primers by conventional PCR and run on agarose gel electrophoresis. Extracted DNA was used for downstream analysis with 454 Roch pyrosequencer.

Material and methods

Eleven samples of date product "Btana" were collected from different localities in South of Algeria. 25 g of date past was homogenized in sterile bag with 250 ml of alkaline phosphate buffer using Stomacher apparatus, then 1 ml of each sample was centrifuged for 10 min at 14,000 rpm to harvest bacterial cells.

Protocols A: DNeasy kit

Cell pellet

Chemical and enzymatic lysis according to the manufacturer instructions

Incubation 56°C / 1 h

Mechanical lysis with 3 g of 710-1180µm + Protenase K

Incubation 56°C/ 30 min

Binding and elution,

ADN pellet

Preheated 56°C (500µl)

Protocols B: CTAB

Extraction buffer : 100mM Tris, pH 8, 1.4M NaCl, 30mM EDTA, pH 8. 2% of polyvinylpyrrolidone (PVP; Sigma,) 2% CTAB (cethyltrimethyl ammoniumbromide). Before use 1% (v/v) b-mercaptoethanol and 40mg/ml of lysozyme were added.

Incubation at 56°C/1 h

Mechanical lysis with 3 g of 710-1180µm + Protenase K

Mechanical lysis with 3 g of 710-1180µm

supernatant + 1V Alcohol isoamilic:chloroform (1:24)

12,000 rpm /for ten minutes

The aqueous phase 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.

12,000 rpm /10 min

washed with 200µl of cold 70% ethanol

ADN pellet

re-suspended in sterile ARN free water

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

DNA purification: with the genomic DNA-clean-up NucleoSpin kit (Macherey Nagel GmbH, Düren, Germany) according to the manufacturer's recommendations.

DNA: recovery — DNA concentration and purity were checked by spectrophotometric measure using NanoDrop 2000 spectrophotometer

PCR reaction — The extracted DNA with the four protocols was further checked as template in PCR for amplification of the region V7 to V8 of the 16S rRNA gene.

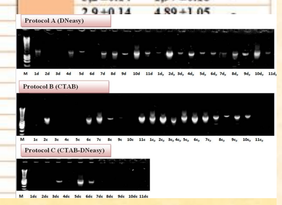
Electrophoresis: After migration, DNA fragments were visualized by a UV transillumination system.

Statistical analysis — ANOVA and Post hoc analysis (Tukey 's multiple comparison test) PRISM SAS

Results and discussion

Table; DNA yield and purity of the eleven Btana sample recorder with three protocols A, B, C

method	Samples	Crud DNA			Purified DNA(Nucleospin)				method			
		DNA (ng/µl)	A260/280	AMP	DNA (ng/µl)	A260/280	Recovery rate	AMP	C	DNA (ng/µl)	A260/280	AMP
A	1	12,6 ± 0,85	1,13 ± 0,05	+	3,45 ±0,07	3,43 ±1.24	27,38%	+		3,95 ±0,64	2,15 ±0,25	-
	2	12,70 ±0,14	1,49 ±0,04	-	5,50±0.14	2,20 ±0.02	43,31%	+		9,00 ±0,00	1,24 ±1,02	-
	3	13,20 ± 0,57	1,42 ±0,00	-	5,05 ±0.07	3,29 ±0.40	38,26%	+		9,55 ±0,21	2,26 ±0,00	+
	4	17,45 ± 0,35	1,83 ±0,14	+	7,30 ±0.42	3,69 ±0.17	41,83%	+		9,00 ±0,14	2,35 ±0,14	-
	5	13,05 ± 0,07	1,98 ±0,86	+	7,90 ±0,42	1,62 ±0.01	60,54%	+		4,40 ±0,28	1,98 ±0,04	+
	6	13,20 ±0,85	1,24 ±0,01	-	6,10 ±0,28	2,11 ±0.03	46,21%	+		6,85 ±0,21	2,08 ±0,20	+
	7	5,70 ±3,39	2,05 ±1,44	+	1,45 ±0.21	9,88 ±8.97	25,44%	+		1,7 ±0.28	/	-
	8	7,45 ±0,07	1,62 ±0,11	+	1,65 ±0.64	2,59 ±0.82	22,15%	+		2,2 ±0.0	/	-
	9	17,90 ±0,28	1,05 ±0,06	+	1,75 ±1.06	1,26 ±0.11	9,78%	+		2,25 ±0.07	1,95 ±0.35	-
	10	28,60 ±0,42	1,58 ±0,01	+	4,80 ±1.98	2,05 ±0.28	16,78%	+		5,2 ±0.14	1,97 ±0.18	-
	11	7,80 ±0,00	1,74 ±0,10	+	0,90 ±0.57	1,53 ±0.40	11,54%	+		2,9 ±0.14	4,89 ±1.05	-
B	1	145,80 ±2,26	1,90 ±0,01	-	82,10 ±0,85	2,23 ±0,03	56,31%	+				
	2	83,80 ±1,13	2,04 ±0,00	+	37,20 ±0,57	2,35 ±0,01	44,39%	+				
	3	437,10 ±1,13	2,03 ±0,00	-	344,40 ±1,84	2,15 ±0,00	78,79%	+				
	4	36,40 ±0,00	1,66 ±0,01	-	21,20 ±0,28	2,65 ±0,05	58,24%	+				
	5	57,50 ±0,57	1,76 ±0,00	-	40,80 ±1,56	2,28 ±0,02	70,96%	+				
	6	168,50 ±8,06	2,28 ±0,13	+	108,90 ±0,28	2,25 ±0,00	64,63%	+				
	7	43,30 ±1,27	1,71 ±0,04	+	22,75 ±1,06	2,50 ±0,03	52,54%	+				
	8	44,30 ±0,71	0,98 ±0,02	-	4,05 ±0,92	6,09 ±2,05	9,14%	+				
	9	30,60 ±2,83	0,93 ±0,01	+	5,05 ±2,19	1,55 ±0,10	16,50%	+				
	10	40,85 ±0,07	1,33 ±0,00	-	14,85 ±0,35	2,59 ±0,11	36,35%	+				
	11	18,80 ±0,42	1,06 ±0,01	+	0,3 ±0.00	3,84+-0,00	0,00%	-				

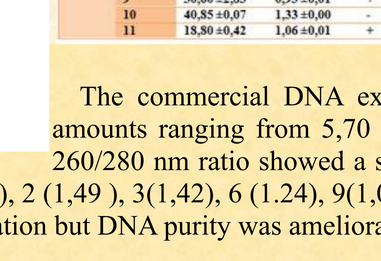


The commercial DNA extraction kit Denasy (A) allowed a moderate DNA amounts ranging from 5,70 to 28,60 ng/µl (P<0.05). DNA quality verified with 260/280 nm ratio showed a satisfactory purity, however 6 samples present a very low values; 1 (1,13), 2 (1,49), 3(1,42), 6 (1.24), 9(1,05.), 10 (1,58). A substantial amount of DNA (69.8%) was lost by purification but DNA purity was ameliorated as all samples were successfully amplified.

Table 2 . DNA yield and purity means of the eleven Btana sample recorder with three protocols A, B, C

	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

DNA yields using CTAB protocol (B) along with NaCl treatment record a concentration ranged from 18,80 ng/µl to 437 ng/µl and varied strongly according to the samples (P<0,05). The resulted yield is higher than those obtained using all other methods. A great lost of yield (55.65%) was observed after purification as stated by ANOVA matched pairs test (P<0.05). The ratio A260/A280 was slightly increased after purification in some samples.
Protocol C produced the most poor yields ranged between 1,7-9,55 ng/µl (P=0.0002). Amplification was partially successful with 3 samples only (3, 5, 6). DNA purification brought no efficiency in amplification (except for sample 2) by contrast a substantial DNA yields were lost as well as failure in amplification of the previously amplified sample before purification (3, 5, 6).



DNA yield (means (ng/µl) ± SEM) from 11 Btana samples extracted by 3 methods

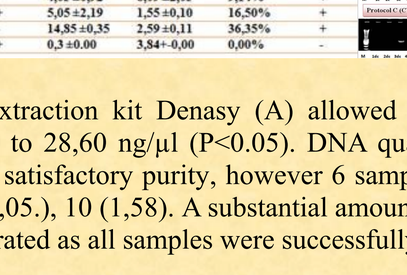


Figure -2- showing absorbance ratios at 260/280 for 11 Btana samples using 3 protocols (A:DNeasy, B: CTAB, C: CTAB-DNeasy). Median values are indicated by the line within the boxplot. The box whiskers indicate the minimum and maximum values

The commercial kit Dneasy was expected to perform better in DNA yield and quality as well as for PCR reaction. Nevertheless, it was failed in isolating DNA with purity and yield as high as the modified CTAB method (B). It seemed to be better than the previous protocol (B and C) 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.
CTAB-DNeasy protocol gave low recoveries may be because of a lack of efficiency of the cell lysis, decrease of the efficiency of spin columns by polysaccharide contamination.
purification with Nucleospin enhanced the quality of the DNA thanks to the elimination of interfering substances that could be coextracted with DNA and could affect PCR, however it seemed to be also deleterious for DNA recovery.
The differences in range of DNA yield, from Btana samples using the same protocol, could be caused by variations in the number of microbial cell present per sample or by the range of complex contaminants that are present.

Conclusion

Date's product "Btana" contain high content of polysaccharides, carbohydrates and other metabolites that can co-precipitate with DNA and interfering with enzymatic reactions performed for DNA analysis .
According to our investigation, the best method for DNA extraction from "Btana" is the modified CTAB method, that had a good yield between amplification and recovery when samples were treated with high salt solution (5M NaCl). The improvement could be achieved with purification. It is less expensive than commercial kits but it is more time-consuming and somewhat labor intensive.
DNeasy kit is also very interesting tool, less laborious and time saving, however it should used with caution and more intention to not underestimate the bacterial richness in the date product "Btana".

Pyresequencing analysis

Among the total OUT discovered, firmicutes represent more than 84.79% of total OTUs. Proteobacteria (10.61%) Actinobacteria (3.01%) and Bacteroidetes (1.14%). Minor phyla Cyanobacteria (0,16%), Candidate division TM7 (0,04%), Deinococcus-Thermus (0,03%), Chloroflexi (0,02%) and Acidobacteria (0,01%). (0.18%) of phyla are Unclassified.
Samples (B11. B2 B3 B6 B7 B8 B9 B10 B1) were broadly dominated by firmicutes phylum members (65,22% ; 86,00%; 99,97%; 99,79%; 99,94%; 64,29%; 99,97%; 93,98%; 73,81%), but samples B4, B5 contain almost 46,60% ; 26,63% of firmicutes respectively.
Firmicutes was dominated by Bacilli class mainly Bacailalles (76,81%) followed by Lactobacillales (20.71%) and clostridales (1.85%). two samples only are not dominated by Bacilalles (B6:0.32, B8:2.32%) other samples were highly dominated by Bacilalles (average 90.29%).
Detection of 605 species distributed between 201 genera. However, a small number of species are abundant, others are either singletons (224 singletons) or rare. , and dominant above 5%.
-592 species, constituting an average of 97.85%, were considered rare(<1%), representing 19.85 % of TPo.
-Subdominant subgroup (1% <-<5%) account for 8 species (17.37 %).
-Predominant species (>5%) accounted for 6 species and represent an abundance of 63.44% in TPo.
-Bacillus was the most frequent genera present in all sample accounting for of 57species level phylotypes.
Only 8 Bacillus species were well represented and identified (B. aryabhattai, B.alkalitelluris, B.*cereus, B.endophyticus, B.foraminis,B. licheniformis, B.jeotgali, B. korlensis, B. malacitensis, B. marisflavi, B.psychrosaccharolyticus,* B.safensis, B. sp 13965, B. sp 19490, B. sp A24, B.BM-11 0, B.By231Ydz-fq, B.TDWCW2).

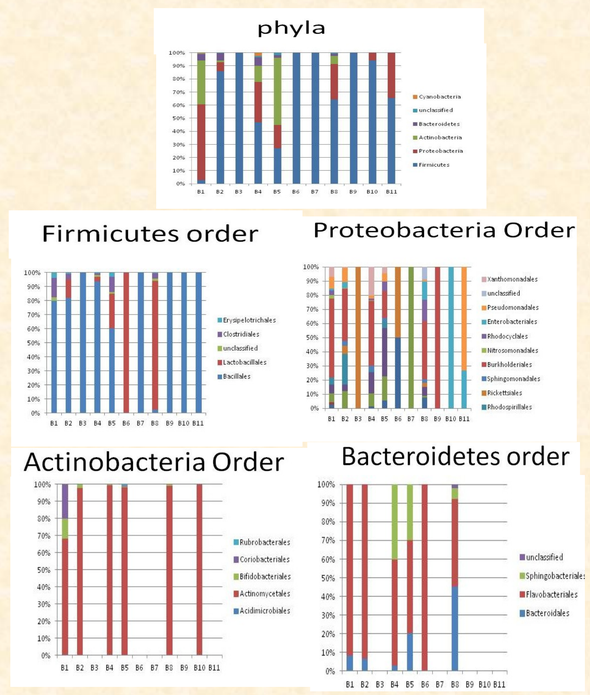


Figure: Histogramme of pyresequencing analysis of Btana sample at various taxonomic level

Non culturing method applied in microbial field have opened an new area in comprehension the dynamic and structure of microbial community in traditional food to sustain their stability and to determine the effective microflora in the microbial actions that undergoing during traditional fermentation, however the study of microbial diversity with this cutting edge technology required at least a high DNA quality and quantity to estimate the real richness microbial. Often it is need to begin with DNA extraction optimization and evaluation of the working protocol to chose the most reliable one for downstream application.

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