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DNA EXTRACTION OF *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* OBTAINED FROM SELECTED YOGHURTS, YOGOURMET AND SOY WARA BOUGHT FROM VARIOUS MARKETS IN SOUTH WESTERN NIGERIA

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Abstract–*Streptococcus thermophilus* and *Lactobacillus bulgaricus* are important members of Lactic acid bacteria, because of their enormous technological and commercial importance in the production of various dairy products, and because they are generally recognized as safe (GRAS) microorganisms. The aim of the study was to optimize the molecular detection and quantification of DNA of various *Streptococcus thermophilus* and *Lactobacillus bulgaricus* obtained from selected Yoghurts, Yogourmet as well as Soy wara using Zymo genomic kits. The result confirmed that Zymo genomic kits are superior to use of other methods. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strain B_{37}, B_{35}, B_{36} and B_{38} had a DNA purity ranging from 1.78 and 2.1 , while *Lactobacillus bulgaricus* strain B_{39}, B_{40} and B_{41} had a DNA Purity ranging from 1.93 and 1.97. The result justifies that higher purity and yield is characteristic of the method. The Agarose Gel Electrophoresis confirmed the DNA of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* at 1500 bp are obtained from Commercial yoghurts and Yogourmet, while the starter cultures obtained from Soy wara did not show any amplification bands.

INTRODUCTION

Lactic acid bacteria are important group of bacteria because of their technological commercial importance in the production of Yoghurts, butter, and other food products. They are also significant to health care due to production of antimicrobial substances and ability to inhibit pathogenic bacteria.

Moreover, these bacteria are also suitable starter cultures for the production of various food products (Abed, 2013). The aim of this research is to evaluate Starter cultures from selected Yoghurts, Yogour met and compare with that obtained from Locally fermented foods such as Soy-wara as alternatives for Yoghurt production in Nigeria.

MATERIALS AND METHODS

DNA Extraction and Purification

The extraction of DNA from the prepared 0.5ml suspension was done using Bacterial DNA kit Zymo USA according to manufacturer's Instruction

Appendix. It is a spin column loading, washing and elution of DNA as follows.

Cell resuspension

2000µl of an overnight culture of bacteria was centrifuged 10,000g for 10 minutes at -4°C each of the sample was removed and decanted.

Cell Lysis

750µl of genomic lysis buffer and two steel balls were added to pellets.

Then balance the Eppendorf tubes on the Ohaus weighing machine balance. Then each of the Eppendorf tubes were placed on the genogrinder and centrifuged at 1500g for 1 minute.

Each of the Eppendorf tubes was opened and steel ball was removed by using the magnetic ball remover.

The Eppendorf tubes were then arranged inside the (Cent5424R) refrigerated centrifuge and runned for 1 minute.

The lysis buffer in each of the Eppendorf tubes were decanted.

Column Activation

A spin column was placed into 2 ml collection tube.

200µl of DNA prewash buffer was added into the spin column.

Centrifuged at 10,000g for 1minute at -4°C

The prewash buffer was decanted

Column Loading

The supernatant was pipetted directly into the spin column.

Centrifuged for 1minute at 10,000g

The flow through was discarded

Primary Washing

200µl of Genomic DNA wash buffer was added.

Centrifuged at 10,000g for 1miute at -4°C.

The flow through was discarded.

Elution of DNA

100µl of Elution buffer was added into the centre of the column

Incubated at temperature for 1 minute.

Centrifuged at 10,000g for 1 minute at -4 °C

The DNA was stored at 4°C or -20 °C

The purified DNA suspension obtained were subjected to Realtime PCR technique.

DNA Amplification Using PCR Machine

The two-stage DNA amplification involves the running of the purified DNA sample in automated Thermocyclers.

After the DNA extraction 3 µl was amplified in 22µl reaction mixture containing 1 µl of each primer (Universal Reverse and Forward), Master Mix (Appendix) ready to load, DNA polymerase 1 µl. DNTPS 2 µl, in water (13.4 µl).

1. 3µl of the Master Mix was added into each PCR tubes.
2. 3µl of extracted DNA was added.
3. 1µl of forward primer was added.
4. 1µl of reverse primer was added.
5. 13.4µl of Distilled water was added.
6. Total volume for each PCR tubes was then 25µl.

The volume is sufficient for one reaction quantities were scaled to allow enough for the number of samples to be analyzed at a time including the control.

Primers used were directed at the thermocyler profile as this denaturation at 94 °C for 5 minutes 36 cycles, of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 45 seconds, and final elongation at 72 °C for 10 °C.

The status of the control was confirmed through the agarose gel electrophoresis and also the 1500bp fragment indicating the presence of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* genomic DNA.

For the second PCR, 3 µl of the first amplicon was used in a 25 µl reaction mixture with forward and reverse primers other reaction mixtures were added at a similar concentration with the first PCR.

The thermocycling profile was identical with the first except for the number of cycles was changed to 35.

The loading into the PCR was carried out as follows.

The wells in the PCR microplate were identified as samples and controls appropriately.

The microplate well was then sealed tightly.

The machine was started for initialization.

The corresponding well position for sample and controls computed into the machine.

The sealed plate was placed properly inside the machine (automated thermal cycler).

The reaction mixture was runned for 3 hours for the first step and 1 hour 30 minutes (second PCR step).

The PCR reaction products were ready for identification and visualization by Agarose gel electrophoresis.

Gel Electrophoresis

Preparation of Agarose Gel-

1.0 gram of agarose was dissolved in 100µl of buffer.

The agarose and buffer were cooled under the tap.

Preparation of Buffer

1 litre of NaBorate buffer was diluted with 19 litres of water.

14 µl of Ethidiumbromide was added to the buffer to form an homogenous mixture.

The Ethidiumbromide buffer mixture was poured into the tray or plate.

Loading of DNA and Loading Dye

Three microliters of each PCR products/sample DNA was loaded into the wells of the gel.

Then three microliters of loading dye was also loaded into the wells of the gel and runned.

On the first row on the gel were the molecular

standard (markers) both +ve ad -ve controls and sample amplicons.

The first lane (well) of each row on the Agarose gel in for the marker.

The gel were run at 100V for 40minutes and were viewed in a photo documentation chamber ENDURO GDS using U.V. Light to capture on a Desktop.

Real Time PCR Amplification

Real-time PCR was performed as described by the manufacturer using 1429R and 27F and probe.

The primers and Taqman probes Zymo U.S.A for detecting the *Lactobacillus bulgaricus* and *Streptococcus thermophilus* specific sequence were already optimized.

The forward and reverse primer PCR mixture contained.

- Taq man containing DNA plus DNA polymerase, deoxyribonucleotide triphosphate (DNTPS) mix and PCR buffers.
- 2.5µl of extracted template DNA
- 1.0µl of each primers
- 0.2µM probe
- 0.1µl Taq Monexogenous internal positive
- Control (1PC)
- Probe Reagent

The total volume of the mixture was 25µl

Amplification and detection were performed with Adenine (thermocycler) using the following program

- 1 cycle of 95 °C for 5minutes
- 34 cycles of 95 °C for 15seconds

DNA Purification

The DNA purification kit(zymo) was used according to manufacturer instruction for all DNA preparations. Cells were grown in MRS broth. An overnight culture of 2 ml was pelleted by centrifugation at 10,000 r.p.m for 10minutes at 4 °C. Each sample was removed and decanted, two steel ball was added into each of the Eppendorf tube, then 750µl of Genomic lysis buffer was added. Balance your Eppendorf tube on Ohaus weighing balance. Each of the Eppendorf tube were placed on the Genogrinder Geno 2010 & run at 1500gf for 1minute. Each of the Eppendorf tube was removed and opened then steel ball was removed using magnetic ball remover. The Eppendorf tubes were placed inside the refrigerated centrifuge (Eppendorf Cent 5424R) and run at 10,000gf for 1minute. The lysis buffer in each of the Eppendorf tube was decanted. Then 200µl of DNA prewash buffer was added into each of the spin column and centrifuged at 10,000gf for 1minute at 4°C then the DNA prewash was decanted from each Eppendorf tube 200µl of genomic DNA wash buffer was added into each Eppendorf tube and centrifuged at 10,000gf for 1minute at 4°C. the spin column in each Eppendorf tube was removed and placed in corresponding 1.5ml Eppendorf tube and 100µl of Elution buffer was added into each of the 1.5ml Eppendorf tube and centrifuged at 10,000gf for 1minute at 4°C.

Agarose Gel Electrophoresis of DNA

All glass ware used were sterilized in microwave operating at 650W for 2minutes.

1.0 gram of Agarose was dissolved in 100µl of buffer.

Cool the Agar + buffer under the tap.

14µl of Ethidium bromide was added to the buffer to form a gel homogenous mixture.

Pour the Ethidium bromide buffer mixture into the tray/plate.

1L of Na Borate Buffer + 19 Liters of distilled water was prepared.

Pipette 3µl of each sample DNA into the PCR plate. Add 3µl of loading dye into each wall in the PCR plate.

2 litters of dilute Na Borate buffer was added into the Electrophoretic tank.

Switch on the power to run at 100V for 40minutes.

After 40 minutes the Gel was placed in the ENDURO GDS and closed for ultraviolet radiation.

The image was then captured and the alignment between the ladder and those of the samples were compared.

Qualitative and Quantitative Assessment of DNA

Purity and Concentration of each DNA sample were measured using computerized Nanodrop Spectrophotometer result of DNA purity and concentration (µg/ml) were recorded and plotted automatically. The Nanodrop Spectrophotometer measures DNA purity and concentration according to the following equations.

$$\text{DNA Purity} = \frac{\text{Absorbance at 260nm}}{\text{Absorbance at 280nm}}$$

DNA yield (µg) = DNA concentration (µg/µl) x Total Sample volume (ml).

Polymerase Chain Reaction and Gel

Electrophoresis

The PCR products was separated by electrophoresis on 1.5% (w/v) Agarose gel containing Ethidium bromide (0.5µg/ml). 3 µl of each DNA sample was placed into the corresponding well and 22 µl of the PCR product was also loaded into each well in the PCR machine and run for 3hours.

Agarose Gel Electrophoresis

Transfer 3 µl of PCR products into new well add 3µl of loading dye into each well.

Transfer 6µl of PCR products and loading dye into the Electrophoretic tank containing Agar Ethidium bromide + dilute Na Borate in the Electrophoretic tank.

Switch on the Electrophoretic tank and run at a constant voltage of 100volt for 1 hour 30 minutes.

RESULT AND DISCUSSION

The result obtained revealed that DNA extraction with Zymo Genomic kits produced the highest purity (between 1.78 and 2.1) and highest DNA yield between 112.5 and 230.1µg/µl for *Streptococcus thermophilus* while it produced the highest purity between (1.93 and 1.97) and highest DNA yield between 115.9 and 181 µg/µl for *Lactobacillus bulgaricus* (Table 3). The molecular identification of Target *Streptococcus thermophilus*and *Lactobacillus bulgaricus* (Lactic acid bacteria) using DNA extracted directly from various Yoghurts, Yogourmets and Soywara samples indicated that all the samples contained DNA bands at 1500 bp (Figure 1). On the contrary real time PCR for *Streptococcus thermophilus* and *Lactobacillus bulgaricus* revealed that Eight out of Ten samples showed signs of amplification at 1500 basepair. The detection of *Streptococcus thermophillus* and *Lactobacillus bulgaricus* in Soy wara samples was negative, However, the detection of *Streptococcus thermophillus* and *Lactobacillus bulgaricus* in selected Yoghurts, as well as Yogourmets was positive (Figure 2).

Quality and yield of extracted DNA are basic requirements for PCR based detection assay. The selection of a suitable extraction method is essential for a successful and valid PCR analysis. The only limiting problems with PCR method is lowyieds of nucleic acids or the presence of inhibitory substances that are sometimes extracted along with the DNA of samples, which often causes failure of amplication reactions, hence lead to false or negative result, as is in the case of Soy wara a locally fermented food product, which contains low yields of nucleic acids or may contain inhibitory Protein substances, accounting for negative result. The presence of proteins in Soy wara samples may also interfere with amplification process.

The quality and yield of DNA extracted was confirmed by Agarose gel electrophoresis. The Zymo genomic kits gave a successful extraction

Table 1. Sequence of Universal primers 1429R and 27F used in colony PCR

Universal primers	
1429R	GGTTACCTTGTTACGACTT
27F	AGAGTTTGATCCTGGCTCAG

Table 3. Quality and yield of extracted DNA from Selected yoghurts and Soy wara

S/N	Sample ID	Nucleic Acid (µg/µl) DNA yield	260/280 DNA quality
1	B_{35}	25.3	1.98
2	B_{22}	11.4	1.78
3	B_{36}	112.5	1.95
4	B_{37}	21.2	1.93
5	B_{12b}	90.5	1.64
6	B_{38}	230.1	2.1
7	B_{39}	181.6	1.97
8	B_{40}	244.5	1.93
9	B_{41}	115.9	1.94
10	B_{26}	12.4	1.24

Table 2. Amount of aliquot of reaction mixture used and conditions followed in PCR

Reaction mixture	Amount	Conditions		
DNA template	2.5ul	Initial denaturation	94^0C	5
Reaction buffer	1ul	36 cycles of		
Magnesium chloride	1ul	Denaturation	94^0C	30 sec
Primers ReverseForward	1ul1ul	Annealing Extension	56^0C72^0C	30 sec45 sec
Taq DNA polymerase	01ul			
dNTPx	2ul	Final extension	72^0C	7 mins
Water	13.4	Hold and stored	10^0C	……

method for amplification of the target *Streptococcus thermophilus* 16Sr DNA and *Lactobacillus.*

bulgaricus 16Sr DNA (Figure 2) which shows five amplicon for *Streptococcusthermophillus* and three amplicons for *Lactobacillus bulgaricus*, molecular size of 1500 basepair associated with the calculated size of target gene of *Streptococcusthermophillus*and *Lactobacillus bulgaricus* and show a single band of PCR. Successful amplification of target gene of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* was a useful tool in diagnostic of Lactic acid bacteria, which indicted that the primers were specific for the DNA extraction method and that the PCR protocols were optimized.

The quality of the extracted DNA was assessed by spectroscopy using the 260/280 absorbance ratios. Where O.D. 260/280 value from 1.78 to 2.1 for *Streptococcus thermophillus* was considered as high purity.

The results obtain in this present investigation maybe due to the use of high concentration of 750µl of genomic lysis buffer and steel balls, and to additional step of protein precipitation using 100µl of elution buffers, which help to remove contaminants and increases purity.

Rantakokko – Jalava and Jalava (2002) reported that the use of higher concentration of genomic lysis buffers or lysozyme resulted in higher yield and gave the highest recovery of genomic DNA from all isolates tested. The cell walls of Gram positive bacteria can be efficiently broken by use of peptidoglycan degrading enzyme, lysozyme (Rantakkko-Jalava and Jalava, 2002). Enzymes that digest the peptidoglycan of bacteria are known as Murien Hydrolases. The best known example of Muramidasesin lysozyme which binds to the bacteria surface and attacks peptidoglycans (Touch *et al.*, 2000).

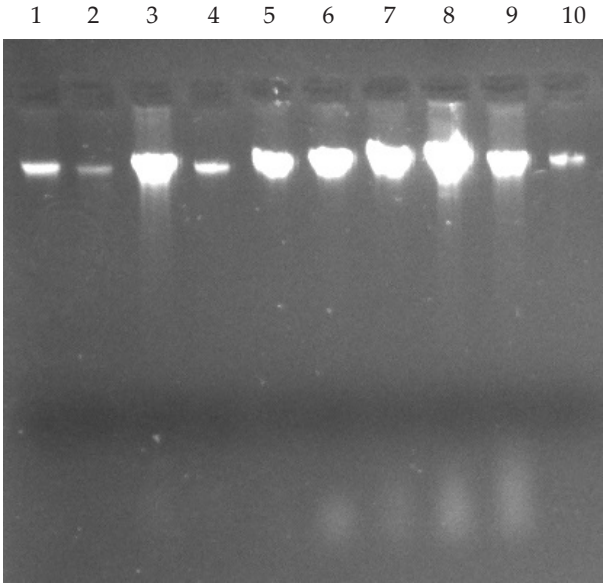

Fig. 1. Agarose gel Electrophoresis of genomic DNA of *Lactobacillus bulgaricus and Streptococcus thermophilus* extracted from (i) farm farm fresh yoghurt (ii) soywara (iii) Good life yoghurt (iv)Habib yoghurt (v) Yogourmet (vi) Dairy day yoghurt (vii) Tito yoghurt (viii) Greek yoghurt (ix) L & Z Yoghurt(x)Soy wara

Ligozzi and Fontana (2003) documented that the recovered nucleic acids have an Absorbance 260/280 ratio of 1.8 to 2.0 are suitable for direct restriction enzyme digestion and the DNA is also suitable for southern blot, molecular cloning, PCR extralong polymerase chain reaction (XL PCR)and other molecular and biotechnological application The amplified PCR products of *Streptococcus thermophilus* and *L bulgaricus* obtained from selected yogurts and yogourmets are better than that obtained from soy-wara.

Ten isolates of *Streptococcus thermophilus* and *Latobacillus bulgaricus* was used for amplifications of 16Sr DNA region using 27F and 1492R Primers. five amplified products of *Streptococcus thermophilus* and three amplified products of *Lactobacillus bulgaricus* were successfully amplified in the amplification procedures in (Fig. 2).

The eight amplified products of the isolates showed single 16Sr DNA band and the length of the amplified DNA fragment were approximately

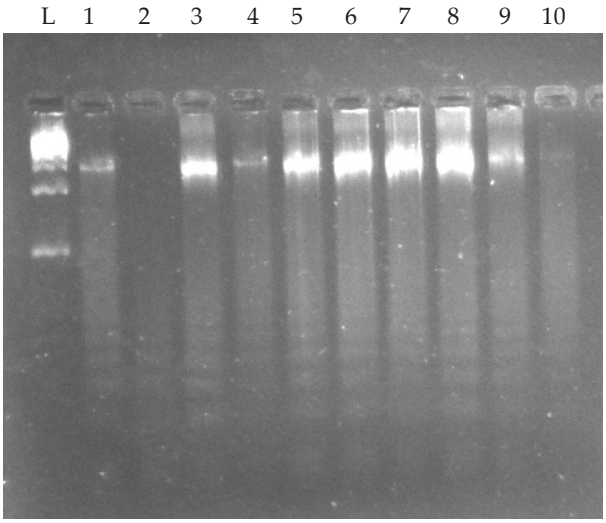

Fig. 2. Agarose Gel electrophoresis PCR products amplified from *Streptococcus thermophilus* and Lactobacillus bulgaricus obtained from selected yoghurts, Yogourmet as well as Soy- wara

1500b.p which were

Preliminary identified as 16Sr DNA (Figure 2)

The phenotypic and molecular techniques confirmed the identity of isolates as *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

The eight Lactic acid bacteria strains were isolated from farm fresh yoghurt, Good life, Habib, Dairy Day, Tito, Greek L&Z yoghurts as well as yogourmet Starter cultures sbought from Osogbo and Ede area of south western Nigeria. Isolate Number B_{35}, B_{36}, B_{37}, and B_{38} belongs to *Streptococcus thermophilus* while, Isolate number B_{39}, $B_{40}$and B_{41} were identified as *Lactobacillus bulgaricus*. In contrary isolate number B_{22} and B_{26} obtained from soywara did not have any amplicon but phenotypically identified as *Streptococcus thermophilus* and *Lactobacillusbulgaricus*.

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