Research Article

Identification of Microbial Communities in Fermented Food using DNA Barcoding Technique and Development of Novel Method for DNA Isolation from Food Products

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ABSTRACT

The traditional use of fermented foods is based on native knowledge and the use of locally available plant or animal sources as substrates. Several microorganisms participate in fermentation which includes bacteria and fungus. The present research focuses on identifying the micro-organisms present in batter samples using DNA Barcoding technique and determining novel method for isolation of DNA. Serial dilution in nutrient agar was performed for the isolation of bacteria and yeast. Bacterial and yeast colonies were collected from Sample-1 and Sample-2 and characterized using staining techniques. Using PCR technique, the 16S rRNA and ITS genes from isolated bacterial and yeast colonies have been amplified. The amplified products were further sequenced and the sequences obtained were matched using software from Codon and Code. The organisms were found to be *Streptococcus lutetiensis* and *Saccharomyces cf. Paradoxus*. Barcodes were generated and total acidity, volatile acidity and ethanol concentration of batter sample was observed to increase for consecutive rising hours. The ethanol concentration was found to be 10.8±0.2% and 9.6±0.12% for sample 1 and 2. This was found to be significantly higher. A novel approach for DNA extraction was determined and the DNA was isolated and validated using Agarose Gel Electrophoresis. Thus, organisms present in batter food products were identified using DNA Barcoding technique. An easy and reliable method for DNA extraction was also determined.

Key words: DNA Barcoding, Fermented foods, Polymerase Chain Reaction, 16S rRNA, ITS genes.

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INTRODUCTION

Fermentation is a lifestyle of metabolic turning sugar into acids, gases, or alcohol. Probiotics are live micro-organisms that confer a health benefit on the host when administered in sufficient amounts.[1] Idly is traditional fermented rice and breakfast based on black gram and can be a possible source of probiotic micro-organisms.

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There are three main types of micro-organisms associated with typical fermented foods and drinks, i.e., filamentous fungi, yeasts and bacteria. Several of these non-pathogenic micro-organisms, listed in Commonly Recognized as Healthy (GRAS) status, are used as probiotics due to their beneficial effects on host.[2]

Idly remains to be widely popular fermented food in India and the healthiest breakfast all over the world as stated by UNESCO and WHO. The prevalent micro flora present during idly batter fermentation is *Leuconostoc mesenteroides, Streptococcus faecalis, Pediococcus cerevisiae* responsible for the leavening and acidification.[3] Several bacterial micro flora responsible for the fermentation of

batters include *Leuconostoc mesenteroides, Lactobacillus lactis, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus coryneformis, Streptococcus faecalis* and *Pediococcus cerevisiae*. A number of studies of idly batter bacterial micro flora revealed the presence of *Pediococcus pentosaceus*, *Pediococcus dextrinicus* and *Lactobacillus plantarum*. The metabolic components of the fermenting strains, meanwhile, are directly associated with the quality of the ultimate products.[4,5]

At present, a new method technique on DNA, known as DNA barcoding has been developed to species level identification. It involves the usage of shorter chloroplast or nuclear, standardized DNA sequences, typically consisting of 400–800 bp. DNA barcoding has been recommended for taxonomic identification of flowering plants and fungi, studying evolutionary changes among related organisms, forensics and also in the identification of species in processed commercial plant products.[6,7] DNA barcoding helps to evaluate the origin and quality of raw materials as well as to identify adulteration, such as adding of products from different taxonomic groups. It has been effectively applied to distinguish plant based adulteration in many commodities such as medicinal plants, commercial tea packets, olive oils etc. The technique has a combined advantage of three vital innovations, i.e. molecularization of identification process, standardization of the protocol and computerization, wherein the data submitted to sequences databases serve as standard for comparing the unknown samples under study. Nowadays, DNA barcoding is widely used in various fields.[8]

Thus the present work focuses on the detection of micro-organisms present in the batter samples using techniques for molecular identification using DNA barcoding technique. Maximum acidity, volatile acidity and concentration of ethanol were calculated for the batter samples. A novel DNA isolation method was performed and verified with the PCR technique.

MATERIALS AND METHODS

Collection of sample

A batter sample was purchased from departmental store in Coimbatore district, Tamil Nadu, India. Totally two sample were collected and named as sample 1 and sample 2.

Isolation of micro-organisms from batter

One gram of batter from each sample (S-1 and S-2) was homogenized to get a standardized suspension in a sterile distilled water. They were further serially diluted and plated on nutrient agar, then incubated for Bacteria at 36°C for 24 h and inoculated for 2-4 days at room temperature on PDA agar for the isolation of the yeast. Colonies were measured and noted after incubation.

Identification of microbes using staining techniques

Gram's staining for Bacteria

Isolated pure cultures were spread on the glass slide and heat fixed. 5 drops of crystal violet was added to the slide and left undisturbed for 60 sec. Slides were then washed with distilled water and iodine solution is poured. After 30 sec decolouriser was added and the slides were washed. Few drops of safranin was added for 20 sec and washed. Finally the slides were washed on running water, dried and observed under microscope.

Lacto Phenol Cotton Blue staining for Yeast

Two drops of Lacto Phenol Cotton Blue was added on a clean wax-free slide and a loop of yeast culture was added. Cover slip was placed over the slide and observed under microscope.

Identification of microbes using 16s rRNA sequence, ITS sequence and generation of Barcode

The complete bacterial Universal-16S rRNA was amplified with the first UF 5"-GAGTTTGCTGCTCAG- and UR 5"-ACGGCTACCTTGTACGACTT- 3'. Yeast samples were amplified with the first ITS1F 5" -TCCGTAGGTGCGGG- 3' and ITS4R 5" -TCCTCGCTTATTGC- 3' for yeast. The PCR reaction mixture was performed in 20 µL with 2 µL 10x PCR buffer, 0.2 µL Taq polymerase [2U / µL], 2 µL of each [4pmol / µL] main. A single colony of yeast was picked and used for colony PCR. The amplification began at 94°C for 10 min, followed by 30 cycles of 94°C for 1 min, 48°C for 1 min for bacteria and 58°C for yeast, 72°C for 2 min and a final extension step at 72°C for 10 min. The PCR products (approx. 1400 bp for bacteria and 750 for yeast) were segregated for 60 min with a 1% (wt / vol) agarose gel at a persistent capacity of 50 V in 1 TAE-buffer. For identification purposes the amplified items were visualized under the UV Trans illuminator. Using Codon and Code program, coding sequence found using BLAST tool, this amplified product sequenced and sequence matched after. Finally, Barcodes were generated using BOLD database.

Assessment of acidity and ethanol content in batter

Total acidity and volatile batter acidity analyzed by titration method and batter content of ethanol was measured using potassium dichromate method.

Isolation of DNA directly from the food samples

DNA was extracted from batter directly using modified CTAB process. Collected batter sample was centrifuged for 7 min under 5000 rpm then collected the supernatant. Collected supernatant was again centrifuged for 6 min, this time concentrating the pellets under 7000 rpm. Pellet was resuspended using CTAB buffer (5% CTAB, 5 M NaCl, 25mM Tris-HCl and 50mM EDTA, 2% beta Mercaptoethanol) and then incubated for 2 hr at 48°C. After 10 min incubation centrifuge at 10,000 rpm and gather supernatant in fresh pipeline. Apply Chloroform volume equal to: Isoamyl alcohol (24:1) and blend well. Centrifuge for 10 min, at 12,000 rpm. Load the upper aqueous layer into a fresh tube and blend well with 1 ml of Isopropanol. Incubate this overnight, at -20°C. Following 10 min of incubation centrifuge, at 12,000 rpm. Pick and wash the pellet with 70 per cent ethanol. Lastly, pellet with 1x TE buffer was resuspended.

PCR amplification of isolated DNA for confirmation of Bacteria

Isolated DNA has been amplified with the bacterial Universal-16S rRNA with the main UF 5" -GAGTTTGCTGCTCAG- 3' and [illegible] 5'-ACGGCTACCTTGACTTT- 3". The PCR reaction mixture was performed in 20 µL containing 2 µL DNA, 2 µL 10x PCR buffer, 0.2 µL Taq polymerase [2[illegible] / µL], 2 µL of each [4pmol / µL] main. The amplification started for 10 min at 94°C, followed by [illegible] cycles of 94°C for 1 min, 48°C for 1 min, 72°C for [illegible] min and a final extension stage at 72°C for 10 min. The PCR products (approx. 1400 bp) were isolated for 60 min with an agarose gel of 1 percent (wt / vol) at a persistent power of 50 V in 1x TAE-buffer. For identification purposes the amplified items were visualized under the UV Transilluminator.

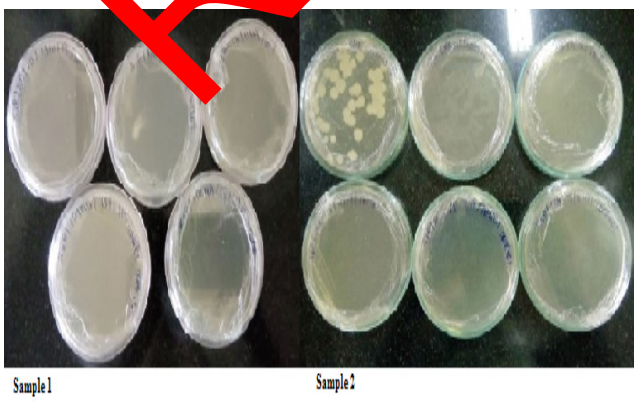


Figure 1: Isolation of bacterial colonies from batter.

RESULTS

Isolation of micro-organisms and Staining

Serial dilution was carried out for the isolation of bacteria and yeast in nutrient agar and Potato dextrose agar (Figure 1). Bacterial and yeast colonies were obtained from Sample 1 and Sample-2 (Figure 2). The colonies from the plates were picked and streaked on a fresh media to obtain pure colonies. The isolated pure colonies were subjected gram staining and LCB staining. Via Gram' staining Gram Negative rod shaped bacterial cells were observed and in Lacto[illegible]l Cotton Blue staining yeast cells were observed (Figure 3). Further the species identification of colonies were performed by 16srRNA sequencing and ITS sequencing.

Identification of microbes using 16s rRNA sequencing

The 16S rRNA and ITS genes of the isolated bacterial and yeast colonies were amplified using PCR technique. The PCR products were separated on a 1% (wt/vol) agarose gel and visualized under UV-Trans illuminator for identification. PCR products showed approx. 1400 bp for bacteria and 750bp for yeast (Figure 4). The amplified products were further sequenced and the

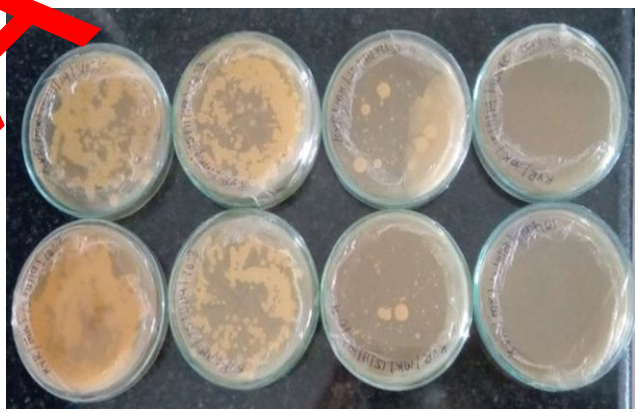


Figure 2: Isolation of yeast from batter samples.

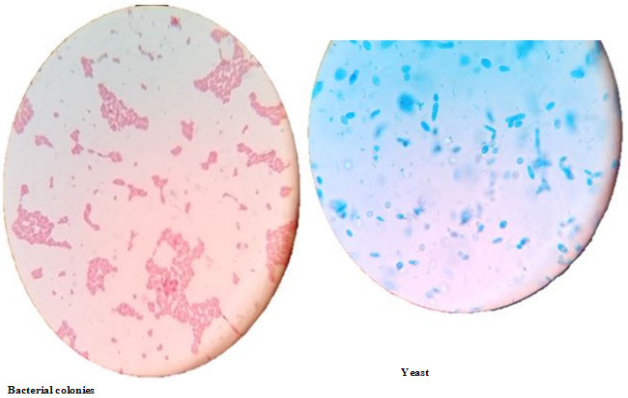
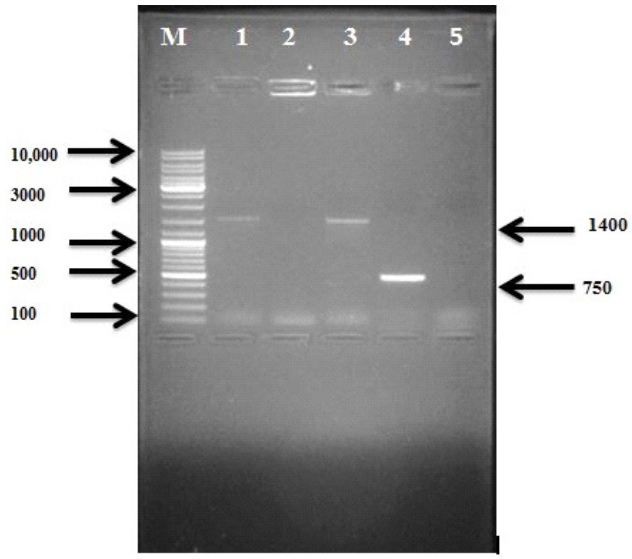


Figure 3: Microscopic images of stained cells.



M- DNA ladder (1kb);
1, 3- 16s rRNA gene Product:
4- ITS1F and ITS4R gene Product;
2, 5- Blank

Figure 4: Agarose Gel Electrophoresis image of PCR amplified product of 16s rRNA and ITS1F, ITS4R.

obtained sequences were aligned using Codon and Code software. The bacterial and yeast sequences are given as follows:

Bacterial 16s rRNA sequence

TGTTGTCTCAGTCGTGACAAGGTT
AAAGTTGGAAGAGTTGCGAAC
ACGCGTAGGTAACCTGCCTA
TAACTATTGGAAACGATA
AACAGCATTTAACCCAT
GGAGCAATTGCTTC
CGTTGTATTAGC
TCACCAAGGC
AGGGTGATCGG
GGCCCAG
GGAA
GAGCA
CGGATCG
CGTGTGTGA
CGGTAACTTACCAGAAAGGGACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTCCC
GAGCGTTGTCCGGATTTATTGGGCGTAAAGC
GAGCGCAGGCGGTTTAATAAGTCTGAAGTTA
AAGGCAGTGGCTTAACCATTGTTCGCTTTGGA
AACTGTTAGACTTGAGTGCAGAAGGGGAGAG
TGGAATTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAGGAACACCGGTGGCGAAAGCG
GCTCTCTGGTCTGTAACTGACGCTGAGGCTCG
AAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTA
GGTGTTAGGCCCTTTCCGGGGCTTAGTGCCG
CAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGACCGCAAGGTTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGATGT
GGTTTAATTCGAAGCAACGCGAAGACCTTACC
AGGTCTTGACATCCCGATGCTATTCCTAGAGA
TAGGAAGTTTCTTCGCACATCGGTGACAGGTG
GTGCATGGTAGTTTTCAGTTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
CTATTGTTAGTTGCCATCATTAAGTTGGGCACT
CTAGCGAGACTGCCGGTAATAAACCGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGTT
GGTACAACGAGTCGCGAGTAGGTGACGGCAA
GCAAATCTTCAAACCAATCTCAGTTAGGAT
TGTAGGCTGCAATTTGCCTACATGAAGTCGGA
ATTCGTAGTAATAGCGGATCAGCACGCCGCGG
TGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTCACACCACGAGAGTTTGTAACACCCGAAG
TCGGAGTAAGATCCTTCTCGGTGACAGCTA

The identification of the species based on the obtained sequences was done using BLAST. From the BLAST analysis of bacterial 16s rRNA sequence the organism was found to be *Streptococcus lutetiensis* strain 2709. and showed similarity of 98.82%. From the BLAST analysis of Fungal ITS sequence the organism was found to be *Saccharomyces cf. Paradoxus* and the similarity was found to be 97.8%. The barcodes were generated for

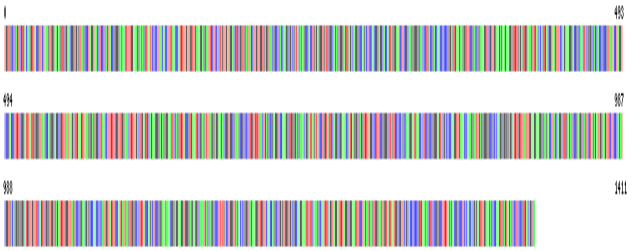


Figure 5: Barcode for *Streptococcus lutetiensis* 16s rRNA sequence.

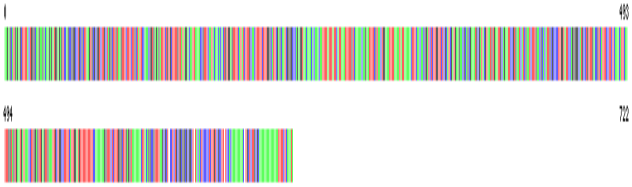


Figure 6: Barcode for *Saccharomyces cf. Paradoxus* ITS sequence.

the *Streptococcus lutetiensis* and *Saccharomyces cf. Paradoxus* sequence and shown in the Figure 5 and Figure 6.

Assessment of acidity and ethanol content in batter

The batter samples (1 and 2) were subjected for the evaluation of Total acidity, Volatile acidity and Ethanol concentration. The total acidity of the batter Sample 1 and Sample 2 at 0 hr was 0.3± 0.02% and 0.38± 0.015%; 0.82 ±0.03% and 0.76 ± 0.04% at 24h; 0.98±0.023% and 0.94±0.025% at 48h. Volatile acidity of the Sample 1 and Sample 2 at 0h was 0.26± 0.013% and 0.32± 0.06%; 0.72 ±0.0012% and 0.65 ± 0.022% at 24h; 0.84±0.043% and 0.81±0.038%. Ethanol concentration of the batter Sample 1 and Sample 2 at 0h was 1± 0.2% and 0.7± 0.04%; 7±0.1% and 6.8 ± 0.2% at 24h; 10.8±0.2% and 9.6±0.12%. The Total acidity, Volatile acidity and Ethanol concentration of the batter samples were found to be increasing for consecutive increasing hours. Surprisingly, the ethanol concentration of the Sample 1 at 48h was found to be 10.8±0.2% and Sample 2 showed 9.6±0.12% which is found to be significantly higher (Table 1).

Isolation of DNA directly from the batter

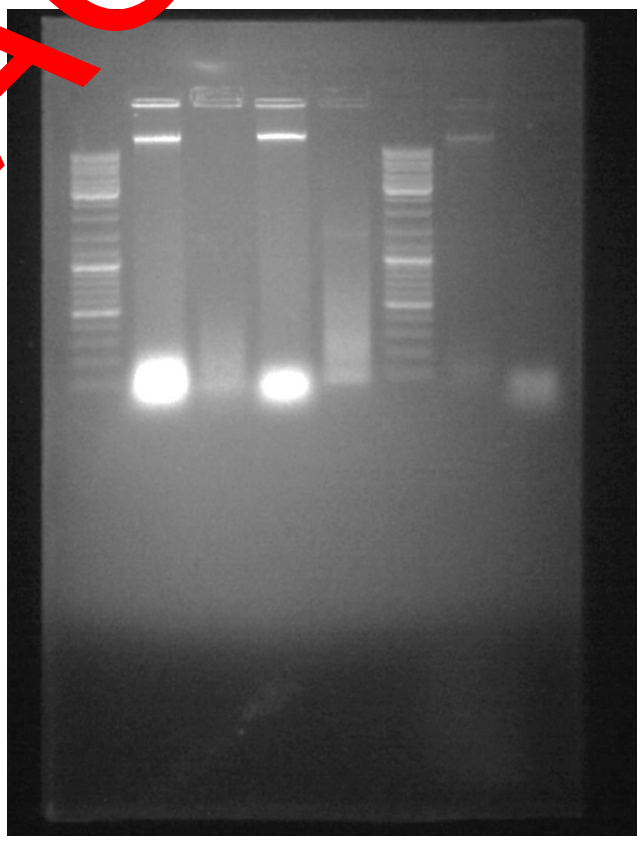
A novel method was carried out for the isolation of DNA from direct samples. DNA was isolated using modified CTAP method and the extracted DNA was confirmed by Agarose Gel Electrophoresis (Figure 7) showing the DNA bands at different incubation period (0h, 24h and 48h). Totally three different incubation time was used to determine the yield of DNA concentration. 0th hr shows the high yield and good quality of DNA (2370.7ng/ µl) (Table 2). Further the 0th hr incubated sample DNA was amplified in Universal-16S rRNA primers. The PCR products were separated on a 1% (wt/vol) agarose gel and visualized under UV-Trans illuminator for identification Figure 8. The band pattern at the 2nd lane determines the presence of bacteria in the batter.

DISCUSSION

DNA Barcoding is an emerging technique with broad range of applications in various fields. DNA barcoding can be used for species identification, biomonitoring of ecological assessment, detection of endangered, invasive and cryptic species. Barcoding are currently used for maintaining food safety and identification of pests infecting plants. The present research concentrates on identification of microbial communities present

M- DNA ladder (1kb); 1- 0th hr; 2- 24th hr; 3- 48th hr

Figure 7: Agarose Gel Electrophoresis image of genomic DNA from batter.



M- DNA ladder (1kb); 1- Genomic DNA; 2- 16s rRNA gene product

Figure 8: Agarose Gel Electrophoresis image of PCR amplified product of 16s rRNA gene product and genomic DNA.

Table 1: Total acidity and ethanol content in batter Sample 1 and 2.

S. No	Colour	Aroma	Total acidity (%)			Volatile acidity (%)			Ethanol concentration (%)		
			0h	24h	48h	0h	24h	48h	0h	24h	48h
1	White	Alcoholic	0.3 ± 0.02	0.82 ± 0.03	0.98 ± 0.023	0.26 ± 0.013	0.72 ± 0.012	0.84 ± 0.043	1 ± 0.2	7 ± 0.1	10.8 ± 0.2
2	Pale yellow	Alcoholic	0.38 ± 0.015	0.76 ± 0.04	0.94 ± 0.025	0.32 ± 0.06	0.65 ± 0.022	0.81 ± 0.038	0.7 ± 0.04	6.8 ± 0.2	9.6 ± 0.12

Table 2: Quality and quantity of DNA after different incubation time.

S.NO	Incubation period (Hours)	Quality of DND	Quantity of DNA (ng/µl)
1	0th	1.8	2370.7
2	24th	1.6	1261.4
3	48th	1.3	1099

in a fermented food (batter) using DNA Barcoding technique.[7]

The present study concentrates on identification of microbial communities on batter samples. Batter samples obtained from local stores in Coimbatore, Tamil Nadu. Serial dilution in nutrient agar and Potato dextrose agar was performed to separate bacteria and yeast. Sample 1 and Sample 2 were used to acquire bacterial and yeast colonies and the isolated pure colonies were subjected to gram staining and LPCB staining. The staining Gram Negative rod shaped bacterial cells were stained and observed by Gram staining technique and yeast cells were observed in Lacto Phenol Cotton Blue. Using PCR technique, the 16S rRNA and ITS genes from isolated bacterial and yeast colonies have been amplified. Products from PCR identified approx. Bacteria 1400 bp and yeast 750 bp. The amplified products were further sequenced and the obtained sequences were aligned using Codon and Codon software. The obtained organisms were found to be *Streptococcus lutetiensis*. and *Saccharomyces cerevisiae*.

Yeasts are a source of vitamins including B11 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B9 (folic acid) and H or B7 (biotin), in particular. Such vitamins play an essential role in the body's numerous metabolic processes such as breaking down carbohydrates, fats and proteins and supplying energy for the body. They also assist the nervous system; help sustain muscles used for digestion and all the yeast isolates tested have been found to be positive for development of Vit B_{12}.[9] Exopolysaccharides (EPSs) are exocellular polymers and are assumed to play a role in protecting against desiccation, toxic. Chemicals, bacteriophages, osmotic stress and adherence to solid surfaces and biofilm formation. Another physiological advantage is that the gastrointestinal tract maintains EPS longer, so that colonization by the probiotic micro-organisms may be improved. Thus, EPS containing probiotic crops can contribute to human health by having a beneficial impact on the gut microflora. *S. cerevisiae* strains, isolated from conventional fermented food products, containing polysaccharides have previously been identified. EPS has immunostimulatory and antitumor activity and EPS phosphate groups play an important role in activating macrophages and lymphocytes.[10-12]

After 24 hr and 48 hr of fermentation we also examined the color, scent, total acidity, volatile acidity, alcohol concentration. The batter sample concentration of total acidity, volatile acidity and ethanol was observed to increase for consecutive rising hours. The ethanol concentration of the Sample-1 at 48h was found to be 10.8±0.2% and Sample 2 showed 9.6±0.12%. Microbial population was found to be decreased in the batter and alcohol concentration increased due to fermentation. A novel method for isolating DNA from direct samples has been developed. The efficient isolation of DNA using modified process. Totally three separate incubation periods used for the DNA concentration yield test. At 0th hr, yield of DNA content was found to be 2370.7ng/µL. The content of the DNA was found to be decreasing by increasing time of incubation. Eventually, to save time this approach may be useful for isolating DNA. DNA from bacterial samples were successfully isolated and confirmed by the Agarose Gel Electrophoresis bands. This method will be useful for easy extraction of genomic DNA from various species.

CONCLUSION

Samples of batter were collected from local stores at Coimbatore, Tamil Nadu, in the present analysis. Bacterial and yeast colonies were isolated using serial dilution. Using PCR technique, the 16S rRNA and ITS genes from isolated bacterial and yeast colonies have been amplified and AGE analysis showed bands at 1400bp for bacteria and 750bp for yeast. The amplified

products were further sequenced and organisms were found to be *Streptococcus lutetiensis* and *Saccharomyces cf. Paradoxus*. The total acidity, volatile acidity and ethanol content of the batter samples were observed to increasing for consecutive hours. The ethanol concentration was found to be 10.8±0.2% and 9.6±0.12% for sample 1 and 2. This was found to be significantly higher. A novel approach for DNA isolation was determined and verified.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATIONS

PCR technique: Polymerase chain reaction technique; **ITS genes:** Internal transcribed spacer genes; **rRNA:** ribosomal RNA; **BLAST:** Basic Local Alignment Search Tool; **BOLD:** Barcode of Life Data System.

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