Chapter 7
Molecular Techniques for Microbial Community Profiling of Fermented Foods

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Abbreviations

CDNA	complementary DNA
DNA	deoxyribonucleic acid
FCM	fluorescence correlation microscopy
LAB	lactic acid bacteria
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RNA	ribonucleic acid
rRNA	ribosomal RNA
VNC	viable but nonculturable

7.1 Introduction

New developments such as increased consumer comprehension has compelled food processors to look into the microorganisms used in their processing to meet the demands of the consumers. Pathogenic microorganisms in food are observed, investigated and identified to ensure production of healthy and safe foods. Traditionally, bacteria causing food borne diseases were identified through analysis of cultures (Ferris et al. 2004). However, some bacterial cells are not culturable and advanced techniques have been developed to widen the range of bacteria that can be detected using molecular methods. Fermentation is a term used the biochemical processes carried out by organisms during their development, growth, reproduction or death. In relation to technologies used where the knowledge of fermentation is applied, organisms are used to manufacture food, alcoholic beverages, pharmaceuticals

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A. M. Elhadi Sulieman, A. A. Mariod (eds.), *African Fermented Food Products- New Trends*, https://doi.org/10.1007/978-3-030-82902-5_7

amongst other products on a large-scale basis. For fermentation to occur, it is important that organisms are cultured under favorable conditions and necessities such as vitamins, trace elements, salts and nitrogen. This type of metabolism produces end products of higher commercial value and that are utilized by human beings (Zheng et al. 2011). Such by products include; enzymes, proteins, hormones, cheese, vinegar, ethanol, cider, beer and wine. Fermentation process takes place in a fermenter or a bioreactor which can be used to carry out any other biochemical reaction. Studies have shown that there has been strong links between bread baking and beer brewing in history and around the fourteenth century, brewers' yeast was discovered to leaven bread. Brewers' yeast and sourdough cultures were the only leavening agents used for making bread up and until the ninetieth century when commercial yeast was invented.

Quality techniques like aerobic plate count is useful in identifying and counting bacteria present in a preprocessed food sample. The second technique comprises compound such as bacteriocin, growth nutrients which particularly hinder growth of selected microorganisms. The third method contains indicators fluorogenic or chromogenic stratum which distinguishes different types of bacteria by use of chemical reactions. The separation and sterilization of microorganism cultures enable phenotypic analysis and culture storage (Xia et al. 2020). Phenotypic methods include bio typing which focuses on the conditions and requirements for growth of bacteria, phage typing which looks into structural differences on the surface of bacteria and serotyping. Nonetheless, genotypic methods have been developed to improve on phenotypic methods. Polymerase chain reaction is formed by DNA specific portion amplification and ribosomal RNA (rRNA) gene is a suitable PCR since it consists adequate disparity amidst species & stains and commonly dispensed amid bacteria in DNA sequence. Several PCR structures have been evolved for distinction of various species which belong to one genus and varied pathogenic bacteria. Restriction enzymes have plasmids which not only use DNA but also involve other cellular properties such as metabolic activity to identification of bacteria. Pulsed-field gel electrophoresis allows large DNA fragments to become smaller and lead them towards the electron field using consecutive varying electric fields while amplified fragment length polymorphism technique is founded on the amplification of fragments from restricted digestion of the entire genomes.

Of all food types, fermented foods were among the first to be consumed by humans. These types of food are preferred for their nutritional value and also for their cultural importance amongst people of different backgrounds (Zhong et al. 2007). Microorganisms and enzymes were discovered about a hundred and fifty and two hundred years ago, were technically used by alchemists and philosophers. Biologists had little knowledge about the use of microbiota in fermentation processes. Since then, the use of molecular techniques in examining fermentation of food has become common. The changing chemical and physical state of food micro environments affects the biochemical activities, survival and growth of fermented food. Most of ecological cell activities that occur in situ occur in solid phase. Methods of microbial characterization, identification and enumeration are used to monitor

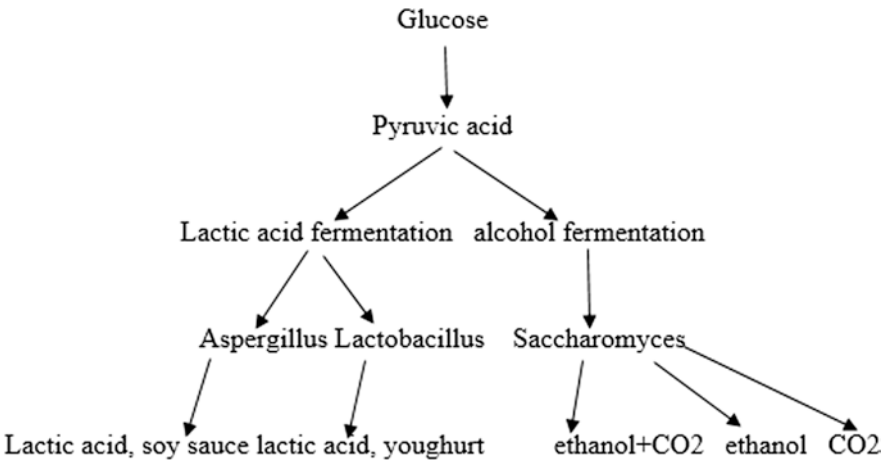


Fig. 7.1 Molecular techniques used to study microbial ecology of fermentation process

strains responsible for fermentation. There is a lot of diversity in ecosystems and molecular techniques that are culture oriented are used to study microbial communities. These studies could help to understand how microbiological processes in ripening and food processing that could improve safety when handling pathogenic bacteria. Below is a chart summarizing the fermentation process (Fig. 7.1).

7.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was first invented in 1953 by Watson and Crick when they discovered that DNA replication can be used to produce complementary DNA (cDNA) strands. This process can be used to amplify nucleic acid. Targeted areas of a bacterial gene can be amplified in an arbitrary sequence and can be a sequence of an oligonucleotide. It is easy for an oligonucleotide (a small sequence of nucleotides) to be detected. Various aspects of bacterial pathogens DNA can be tested, such as, multicopy ribosomal RNA (rRNA), cellular metabolites, toxins and virulence factors. Basic structure of DNA contains a deoxyribose sugar, a phosphate and either one of the four nucleotides. These nucleotides form complementary strands to combine with other nucleotides (Deishing and Thompson 2004). PCR is used to amplify a segment of DNA under the basis of DNA replication and can be used to screen for GMO (genetically modified organisms) in various types of food. The strand to be amplified is known as a template strand and while PCR can work the same way as gel electrophoresis, PCR can be developed further to discriminate bacteria, PCR steps was shown in (Fig. 7.2). Products can further be detected and various strains and species detected on micro wells and membranes. rRNA has shown limitation in terms of the number of strains that it can detect. These types of

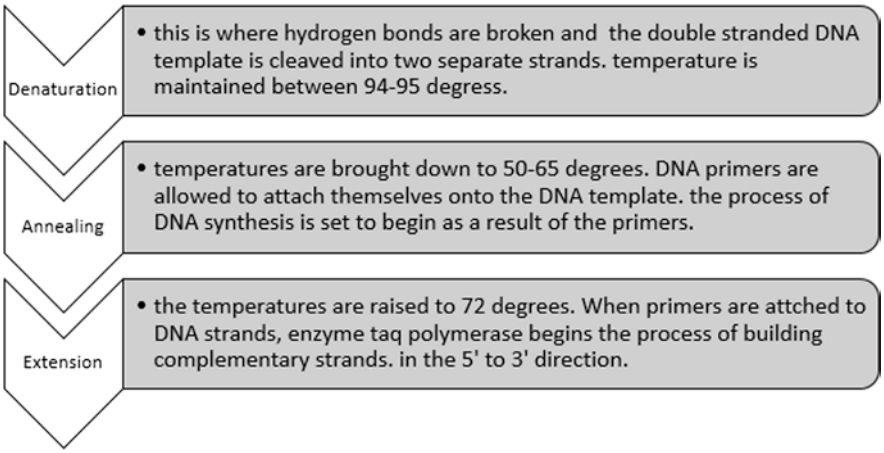


Fig. 7.2 Polymerase chain reaction steps

bacteria include uncultured as well as cultured organisms from food samples, clinical and environmental samples.

There are various methods in which PCR can be done. For example, multiplex PCR can use several primers of multiple samples of viable bacteria for the purposes of quantification and differentiation (Settanni and Corsetti 2007). Conventional PCR which utilizes the concept of reverse transcriptase is usually unable to detect dead cells and therefore, only viable cells can be detected using this method. Conventional PCR can also be used to detect VNC Cells since they cannot be detected using culture methods. The use of double stranded DNA dyes requires that one product is used for every reaction that is undertaken. It is important to note that many factors that play a part in the PCR process are difficult to regulate. These could be the equipment used, temperature, microbiological and chemical hygiene, and humidity (Stringer and Hall 2007). In addition, PCR detects the potential of an organism to produce toxins but does not detect the presence of toxic DNA in food. In addition, Enzyme of restriction, also known as endonuclease restriction, a protein developed through bacteria that cuts DNA at specific locations along the molecule (Fig. 7.3).

Apart from the fact that restriction enzymes are of different types, another benefit of using endonucleases is that the actual DNA sequence of an organism does not have to be known for a plasmid to be investigated. These enzymes split DNA at recognized sites to create a fingerprint (a fragment of DNA). Various fingerprints can be compared against one another to establish their identity. Even though plasmids do not encode housekeeping or survival genes, they encode other cell properties such as their metabolic activities, bacteriocin and antibiotic resistance. In addition, to plasmids being unstable, various strains of bacteria might lack plasmids and therefore the method of DNA fingerprinting becomes unreliable in some cases (Zhao et al. 2015). When a whole chromosome is used, there is a large number of fragments obtained and this might prove difficult to analyze. When cutting

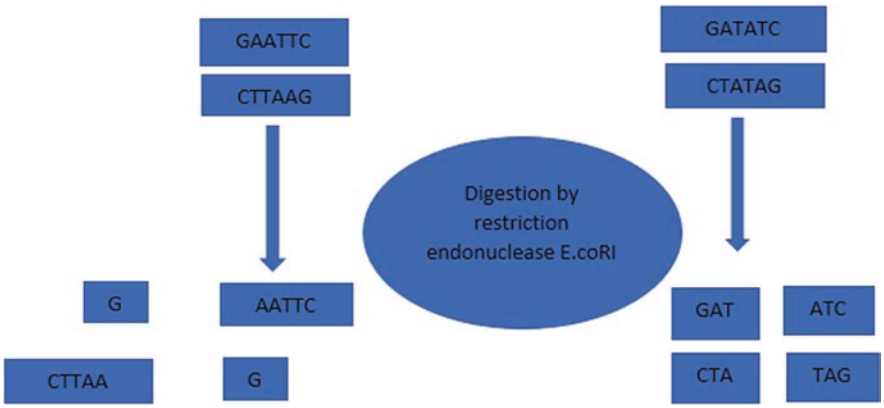


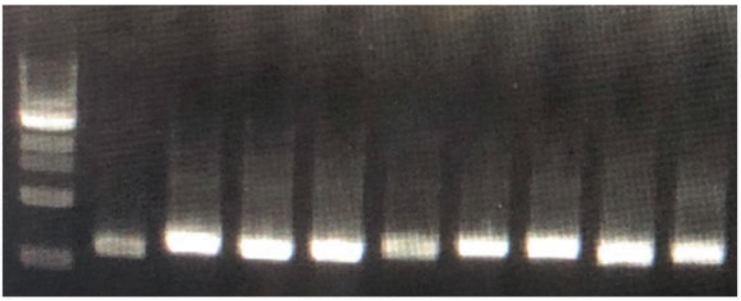
Fig. 7.3 Restriction endonuclease

techniques of low frequency are used, the number of fragments may reduce and the analysis becomes simpler.

7.3 Gel Electrophoresis

Figure 7.4 presented Gel electrophoresis, which is used to separate chromosomal DNA according to their sizes. These molecules of RNA, DNA and other proteins move through a gel filled matrix when an electric current is running through it. The ends of the gel are negatively and positively charged so molecules move from one end to the other, with the smaller molecules moving much quicker than the bigger molecules. Cantor and Schwartz were the first to replicate a karyotype of yeast *Saccharomyces cerevisiae* through gel electrophoresis in 1984. Since then, developments have been made to improve on resolutions of fragments on the screen (Taylor et al. 2005). Separation of molecules on the agarose gel is dependent on the size of DNA and independent from the sieving process. Separation of molecules also depends on different factors such as the voltage of electric current, concentration of gel, concentration of interpolating dye used, for example, ethidium bromide and buffer strength. Sometimes, the electric current may have to be increased to have a better separation and view of fragments. The analyzed fingerprints can be compared against bacterial fingerprints and their identity be established. Isolation of large DNA molecules can be done using pulse field gel electrophoresis (PFGE) which helps to produce a fingerprint of an isolate of a bacteria sample.

Bacterial isolates can be form contaminated food, sick people or food production areas (Sheikha 2021). Some of the benefits of PFGE is that it is has the ability to discriminate and is superior in differentiating strains and species and therefore used in epidemiological studies. Some of the bacterial strains that can be detected using PFGE include *Bacillus thuringiensis, B cereus, B. anthracis, S aureus, L*



A

B

Fig. 7.4 A and B. DNA separation of gel electrophoresis process

monocytogenes, C jejuni, and *Campylobacter coli.* Bacterial DNA isolation can be modified to improve enzyme digestion and lysis. Methods such as multilocus electrophoresis, amplified length polymorphism have proved inefficient when it comes to identification of these bacteria.

7.4 Amplified Fragment Length Polymorphism

This technique utilizes the knowledge of amplification of cleaved fragments that have been digested by restriction enzymes. The major steps involved in AFLP include digestion, ligation, amplification and electrophoresis. Two enzyme restrictions are responsible for digestion of DNA, producing two fragments with sticky ends (Melles et al. 2007). Ligation using adapters takes place and PCR templates are created. Matching fragments are then amplified, resulting in thirty-five to forty fragments. Some of the amplified fragments are strain specific while others are species specific. Detection of DNA is indicated by the absence or presence of DNA

bands. This method is highly polymorphic and reproduces highly compared to restriction fragment length polymorphism.

7.4.1 Restriction Fragment Length Polymorphism

RFLP 1

This technique of gene identification looks into the variations of homologous DNA and differentiates between species, populations, and individuals. The use of restriction enzyme sites can help to illustrate the differences between DNA. Using RFLP, restriction enzymes digest fragments and using gel electrophoresis, they are separated based on their size. The fragments are then passed through a membrane in a procedure known as Southern Blot. The actual length of the fragment is determined through hybridization to a DNA probe which is labelled. Although the emergence of modern and inexpensive methods of DNA fingerprinting has made this method obsolete, RFLP was an important tool for paternity testing, localization of diseases and genetic disorders and genome mapping (Drancourt et al. 2000).

When there is variation between fragments of homologous DNA or variation between individuals, then RFLP is said to have occurred. All fragments can be used in other genetic examinations and are considered as alleles regardless of whether they contain a coding region or not. When southern blot hybridization and restriction digestion are used together for the probe of rRNA, this is known as ribotyping. Ribotyping can be used to detect a wide variety of bacteria in fermentation than any other probe and it is more species and strain specific. In bacteria, ribosomal operons are arranged into 16S, 23S and 5S rRNA. This technique has proved successful in identifying strains like *Pseudomonas aeruginosa* and *Legionella spp*. These two species are difficult to identify using phenotypic methods (Van Belkum et al. 2007). Ribotyping may not be the best method to discriminate against different strains and species but has proven to be a rapid way of screening large bacteria isolates as shown by studies with *P. aeruginosa, L. monocytogenes* and *Salmonella*. In order to discriminate between various species and strains other methods such as serotyping and PFGE should be incorporated.

7.5 Immunological Methods

These techniques of genetic identification are used to study the immune system of an organism and develop reagents that can be used as experimental apparatuses. Examples of surface structures include extracellular organelles, membrane proteins, capsular polysaccharides and lipopolysaccharides (Swain et al. 2014). Serotyping which is also known as serology and refers to the use of antibodies that create a bacterial or viral infection. By the fact that this method uses antibodies in detection

of bacteria, serotyping is considered a molecular method of identifying bacteria in fermented foods. Gram negative bacteria such as *E. coli, Campylobacter* and *Salmonella* is best detected by serotyping. Gram positive bacteria such as *Listeria* can also be identified using serotyping (Aarnisalo et al. 2003). Immunoassays are methods used for the preciseness of antigen-antibody reactions to identify molecules of biological samples. This method can be used for the identification of *B cereus an C perfringens, staphylococcal toxins, cholera* and *botulinum*. This method can be used for both metabolites such as toxins and surface antigens. More recent methods of immunological techniques have been developed to the advantage of processing samples in less effort and time. Food can now be used to identify the present bacteria instead of pure and isolated samples of bacteria. Currently, enzyme linked immunosorbent assay is the best immunological technique to use for whole antigen products or targets. Such bacteria include *Salmonella, Campylobacter, B. Cereus,* and *E. coli.*

7.6 Future Methods in Bacteria Identification

Although these methods are modern, they are based on the genotypic and phenotypic identification of DNA in bacteria, the difference is in the set up or instrumentation of sample analysis (Fig. 7.5). They include biosensors, microarrays (Bachand et al. 2006) and flow cytometry.

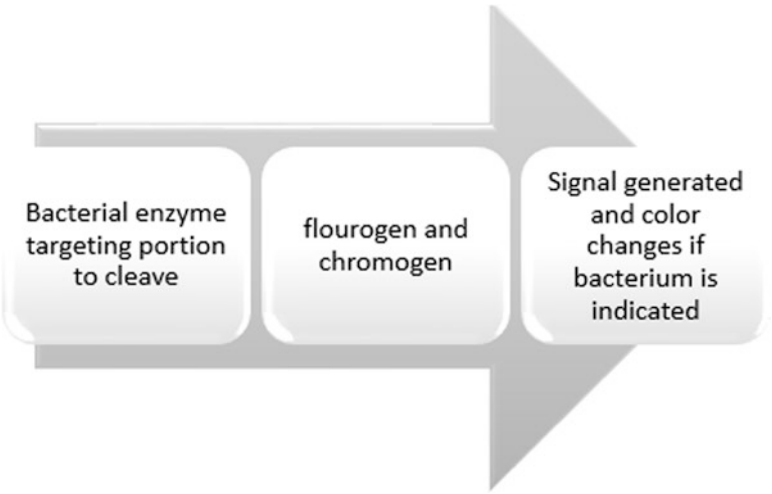


Fig. 7.5 Future of bacteria identification

7.7 Flow Cytometry

Commonly applied in chromosomes and mammalian cells, this method is common in medical diagnosis and cycle analysis. The instrumentation of this method involves using a fluorescent microscope with a focus and cells flowing through it. The main elements include a computer, an electric network, controls, fluid lines and a light source. Samples are illuminated one at a time as they pass through the focused light (Rahman et al. 2006). Protein amounts in bacterial cells were first studied using flow cytometry and have become common ever since. This technique is preferred due to its ability to integrate aspects of both homogenous and heterogeneous samples like cellular structure analysis, cell counting and cell detection. Yeast from bacterial cells will be scattered by the light according to the granularity and size of cells. Algae can easily be identified using the photosynthetic pigments due to its auto fluorescent characteristics. FCM is commonly used in the serological distinction between parasites, viruses, fungi and bacteria (Ferris et al. 2004). One major advantage of flow cytometry is that it is used to differentiate between VNC, dead and viable cells using fluorescent dyes indicating enzyme activity, intracellular pH, membrane potential, membrane integrity and respiration.

7.8 Biosensors

In comparison to gel electrophoresis, culture methods, immunology, and PCR, this is the fastest growing technology in identification of bacteria. The equipment consists of a biological material that is integrated into a transducer that senses chemical or biological change and converts the change into a signal. Drugs, carcinogens, pesticides, pollutants and pathogens from food, water and soil are examples of analyses that can be detected using biosensors based on phage display peptides, immunology and DNA. Biota such as animal tissues, nematodes, algae and plant tissue have been used as biosensor detectors (Rahman et al. 2006). Acids, ammonia and carbohydrates are examples of metabolites that can be produced by viable microbes. A microbe such as *Vibrio fischeri* becomes bioluminescent and can be used to detect viability of fluorescent bacteria (expresses luminescent proteins like luciferase protein and green fluorescent protein). For the detection of pathogens in food, there are various types of biosensors such as antibody and receptor-oriented biosensors, enzyme based and DNA based biosensors.

In fermentation processes, microorganisms produce various metabolites like carbohydrates, enzymes, alcohols and acids. Microbes that help in carrying out the fermentation process include yeasts, molds and lactic acid bacteria (LAB) (Yilmaz and Velioglu 2009). LAB is particularly involved in the fermentation of sourdough fermentation, vegetable fermentation, and dairy products. Pediococci and lactobacilli are starter cultures that are involved in the fermentation of meat. In monitoring microorganisms, it is important to; identify the bacterial flora of foods and starter

cultures, establish the count of bacteria present in foods and lastly, identify the specific biotype and strains in foods. It is also important to detect hazardous microorganisms such as molds, yeasts, viruses and various bacteria. Below are some examples of fermented foods and their ecology. Some of the applications of a biosensor include;

- Prosthetic devices
- Water quality management
- Toxins of defense interest
- Food quality monitoring
- Soil quality monitoring
- Environmental monitoring
- Disease detection
- Drug testing

7.9 Conclusion

At a systems level, in situ visualization, targeted profiling of food, community profiling modern molecular tools have the capacity to provide a high resolution evaluation of fermentation processes. We are able to provide a groundbreaking realization of the selection of targets, analysis tools and techniques for experimental purposes. Researchers still face many challenges in respect to careful selection of these analysis tools, molecular targets and techniques. PCR is a process that can be used to amplify nucleic acid (Singh et al. 2012). Targeted areas of a bacterial gene can be amplified in an arbitrary sequence and can be a sequence of an oligonucleotide. It is easy for an oligonucleotide (a small sequence of nucleotides) to be detected. Various aspects of bacterial pathogens DNA can be tested, such as, multicopy ribosomal RNA (rRNA), cellular metabolites, toxins and virulence factors. Basic structure of DNA contains a deoxyribose sugar, a phosphate and either one of the four nucleotides. In gel electrophoresis, separation of molecules on the agarose gel is dependent on the size of DNA and independent from the sieving process. Separation of molecules also depends on different factors such as the voltage of electric current, concentration of gel, concentration of interpolating dye used, for example, ethidium bromide and buffer strength. Sometimes, the electric current may have to be increased to have a better separation and view of fragments. The analyzed fingerprints can be compared against bacterial fingerprints and their identity be established. Isolation of large DNA molecules can be done using pulse field gel electrophoresis (PFGE) which helps to produce a fingerprint of an isolate of a bacteria sample. Restriction Fragment Length Polymorphism is a technique of gene identification that looks into the variations of homologous DNA and differentiates between species, populations, and individuals. The use of restriction enzyme sites can help to illustrate the differences between DNA. Using RFLP, restriction enzymes digest fragments and using gel electrophoresis, they are separated based on their

size. The fragments are then passed through a membrane in a procedure known as Southern Blot (Van Belkum et al. 2007). The actual length of the fragment is determined through hybridization to a DNA probe which is labelled. Although the emergence of modern and inexpensive methods of DNA fingerprinting has made this method obsolete, RFLP was an important tool for paternity testing, localization of diseases and genetic disorders and genome mapping. Apart from the fact that restriction enzymes are of different types, another benefit of using endonucleases is that the actual DNA sequence of an organism does not have to be known for a plasmid to be investigated. These enzymes split DNA at recognized sites to create a fingerprint (a fragment of DNA). Various fingerprints can be compared against one another to establish their identity. Even though plasmids do not encode housekeeping or survival genes, they encode other cell properties such as their metabolic activities, bacteriocin and antibiotic resistance. In addition, to plasmids being unstable, various strains of bacteria might lack plasmids and therefore the method of DNA fingerprinting becomes unreliable in some cases. These methods have made it easier and increased the effectiveness of microbial food profiling.

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