Feasibility Analysis of Nanopore Sequencing Technology Applied to Food Microbiological Testing - Postprint

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Abstract

In recent years, DNA sequencing technology has developed rapidly, progressing from first-generation biochemical sequencing to third-generation single-molecule sequencing. As one of the third-generation sequencing technologies, nanopore sequencing is a physical method based on electrical signals, distinct from other currently popular sequencing technologies. Many researchers typically apply high-throughput sequencing technology to the study of food microorganisms. However, reports on the application of nanopore sequencing technology to the detection of microorganisms in food remain scarce. The MinION DNA sequencer, developed by Oxford Nanopore Technologies, is the world' s first commercial nanopore sequencer. Following continuous refinement, MinION has been widely applied in DNA sequencing in recent years. A single MinION sequencing run requires approximately 1 g of DNA, with a standard base-calling speed of 250 bases per second, an average read length of 13 kb to 20 kb, and a sequencing accuracy that can reach 98%. The high base-calling speed and high accuracy of nanopore sequencing fully satisfy the requirements for rapid detection, making its application to microorganism detection in food entirely feasible.

Full Text

The Feasibility of Applying Nanopore Sequencing Technology to Food Microbiological Detection

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Abstract

DNA sequencing technology has advanced rapidly in recent years, evolving from first-generation biochemical sequencing to third-generation single-molecule sequencing. As one of these third-generation technologies, nanopore sequencing represents a physical method based on electrical signals, fundamentally distinct from other currently popular sequencing approaches. While many researchers routinely apply high-throughput sequencing techniques to food microbiology studies, reports on using nanopore sequencing for detecting microorganisms in food remain scarce. The MinION DNA sequencer, developed by Oxford Nanopore Technologies, represents the world' s first commercially available nanopore sequencing instrument. Through continuous improvement, MinION has gained widespread application in DNA sequencing in recent years. Each MinION sequencing run requires approximately 1 g of DNA, with a standard recognition speed of 250 bases per second and average read lengths reaching 13-20 kb, achieving sequencing accuracy up to 98%. This high recognition speed and accuracy fully meet the requirements for rapid detection, making the application of nanopore sequencing to food microbiological detection entirely feasible.

Keywords: nanopore sequencing; DNA sequencing technology; food microorganism

1. Introduction

As living standards improve, consumers increasingly focus on food quality and safety. Microbial contamination represents a major source of foodborne hazards, posing significant risks to food quality and consumer health that necessitate effective monitoring methods. Current detection of microorganisms in food primarily relies on traditional culture methods. While effective, these approaches suffer from complex procedures and lengthy incubation times, making them ill-suited for rapid detection requirements. In recent years, researchers have applied molecular biology techniques to food microbiological detection, particularly for foodborne pathogens. Commonly used methods include PCR (polymerase chain reaction) [1,2], LAMP (loop-mediated isothermal amplification) [3-5], nucleic acid probe technology [6,7], gene chip technology [8], and DNA sequence analysis [9-11]. Among these, DNA sequence analysis enables analysis and identification of food microorganisms at the genetic level, effectively eliminating interference from false positives and false negatives during detection [10]. DNA sequencing constitutes an indispensable component of DNA sequence analysis, as subsequent analysis requires obtaining DNA sequences through sequencing. With continuous technological progress, DNA sequencing technology has evolved dramatically, advancing from first-generation chemical sequencing to third-generation single-molecule sequencing.

Nanopore sequencing technology represents a novel third-generation DNA se-

quencing approach [12]. This review summarizes the principles of nanopore DNA sequencing and its applications while exploring the feasibility of its application to food microbiological detection.

2. Historical Development of DNA Sequencing

In 1944, Avery et al. [13] demonstrated that DNA is the carrier of genetic information through pneumococcal transformation experiments. Watson and Crick [14] proposed the famous DNA double helix model in 1953, ushering in the era of DNA sequencing. Early reports on DNA sequencing include Whitfeld et al.' s [15] 1954 work using chemical degradation to determine polynucleotide sequences. In 1977, Sanger [16] invented the chain-termination method, while Maxam and Gilbert [17] developed chemical degradation sequencing. These two technologies marked the birth of first-generation sequencing. Compared to chemical degradation, Sanger sequencing offered advantages of speed, simplicity, and high accuracy, making it suitable for large-scale projects such as the Human Genome Project [18].

Second-generation sequencing, also known as next-generation sequencing (NGS) [19], enables rapid sequencing with long read lengths and massive parallel sequencing, earning it the name high-throughput sequencing technology. In 2004, Roche launched the 454 platform, followed by Illumina' s Solexa platform in 2006 and ABI' s SOLiD platform in 2007 [20]. Unlike first-generation chemical methods, second-generation sequencing employs fluorescence-labeled deoxyribonucleotides (dNTPs) and captures fluorescent signals to determine DNA sequences.

As second-generation platforms matured, single-molecule DNA sequencing technologies began to emerge. In 2003, Braslavsky et al. [21] validated the feasibility of single-molecule sequencing. In 2008, Harris et al. [22] developed true single molecular sequencing (tSMS) technology, which Helicos Biosciences commercialized as the HeliScope platform—the first marketed third-generation sequencer. Also in 2008, Clarke et al. [23] combined -hemolysin with nucleases to sequentially cleave and translocate single-stranded DNA molecules through the pore under an electric field, demonstrating continuous base recognition and establishing the technical foundation for protein nanopore sequencing. In 2010, Stoddart et al. [24] modified amino acid sites in -hemolysin to enhance base recognition capability, making stable and reliable nanopore sequencers possible. Building on these advances, Oxford Nanopore Technologies successfully developed the MinION in 2014—the world' s first and only commercial nanopore DNA sequencer—and began sales in 2015 [25]. In 2009, Pacific Biosciences introduced single-molecule real-time (SMRT) sequencing [26]. Currently, Helicos' s tSMS, Pacific Biosciences' s SMRT, and Oxford Nanopore sequencing represent the mainstream third-generation sequencing technologies.

3. Principles of Nanopore Sequencing

A nanopore can be simply defined as a tiny hole with an inner diameter of 1–100 nm, where the pore diameter typically exceeds or is on the same order as the pore depth [27]. Nanopore sequencing differs fundamentally from other methods as it is based on electrical signals rather than chemical or biochemical approaches. DNA sequences consist of four bases—adenine (A), guanine (G), cytosine (C), and thymine (T)—with distinct molecular structures and sizes. When individual nucleotides are driven through a nanopore by an electric field, they cause characteristic changes in electrical signals. By monitoring these signal variations, the base sequence of single-stranded DNA fragments can be determined, ultimately revealing the genetic sequence [27].

Thus, nanopore sequencing represents a physical method based on electrical signals, distinguishing it markedly from first-generation chemical sequencing and second-generation biochemical sequencing. Chemical methods require reaction termination and sequence determination through gel electrophoresis and autoradiography, making the process relatively cumbersome [28]. Biochemical methods require library construction, PCR amplification, and fluorescence labeling [29], which are time-consuming and introduce PCR errors. In contrast, nanopore sequencing requires no enzymatic amplification, offering advantages of long read lengths, low cost, high throughput, and label-free operation [30].

4. Classification of Nanopore Sequencing

Based on nanopore origin, current DNA sequencing nanopores fall into two categories: biological and solid-state nanopores. Biological nanopores utilize naturally occurring channel proteins, while solid-state nanopores are artificially fabricated from silicon and its derivatives.

4.1 Biological Nanopores

The working principle of biological nanopores is illustrated in Figure 1 [Figure 1: see original paper]. When a high-salt solution is added to the detection chamber and an external electric field is applied across the nanopore, a constant current flows in the absence of single-stranded DNA. When a DNA molecule enters and translocates through the pore, it reduces the current, generating a blockade signal that is amplified and converted to digital data [31]. Since different bases produce distinct blockade signals, DNA sequences can be identified by analyzing these current signatures.

Biological nanopores are predominantly natural nanodevices with unique pore structures, biological activity, and functions. They are favored by scientists for their capacity for free molecular design and flexible chemical or biological modification [32]. Currently, the most commonly used biological nanopores are -hemolysin (-HL) and *Mycobacterium smegmatis* porin A (MspA) [33] (Figure 2 [Figure 2: see original paper]).

-Hemolysin is a heptameric pore protein comprising three domains: a cap region, rim region, and stem region. The stem forms a transmembrane channel through a -barrel structure inserted into the lipid bilayer, measuring approximately 5 nm in length with a 1.4 nm constriction diameter [34]. In 1996, Kasianowicz et al. [35] constructed the first biological nanopore sequencing model using -hemolysin, detecting current changes caused by single-stranded DNA and RNA translocation. While this model could detect nucleotide signals, the translocation speed was too fast (1–20 microseconds per nucleotide) [30] for accurate single-base identification. Building on Clarke [23] and Stoddart' s [24] work, Cherf et al. [36] combined -hemolysin with phi29 DNA polymerase to slow DNA translocation, enabling real-time sequencing (Figure 3 [Figure 3: see original paper]). In 2014, Oxford Nanopore Technologies successfully developed the MinION nanopore DNA sequencer using -hemolysin as the nanopore channel.

MspA from *Mycobacterium smegmatis* represents another suitable nanopore protein for DNA sequencing. MspA forms a conical octamer with a constriction zone approximately 1.2 nm wide and 0.5 nm long [37]. Compared to the 5 nm -hemolysin, MspA is more advantageous for single-base resolution. In 2008, Butler et al. used MspA nanopores to detect single-stranded DNA molecules, employing hairpin structures to control translocation speed and achieving good sequencing results [38]. Manrao et al. [39] combined MspA with phi29 DNA polymerase for DNA sequencing, controlling single-stranded DNA entry to approximately 28 milliseconds per base and obtaining highly resolvable blockade signals with up to 40 pA differences among the four deoxynucleotides (Figure 4 [Figure 4: see original paper]).

4.2 Solid-State Nanopores

Solid-state nanopores are fabricated from silicon and its derivatives, typically by drilling nanoscale holes in silicon or other material films using ion or electron beams, followed by pore shape and size refinement [40]. Solid-state nanopore-based DNA sequencing remains in the exploratory stage, with researchers investigating various sequencing methods to perfect the technology. Hall et al. linked DNA molecules to the -barrel of -hemolysin and electrophoretically loaded them onto solid-state nanopores to create a hybrid sequencing model, requiring pore diameters of 2.4–3.6 nm for optimal -hemolysin immobilization [41]. Garaj et al. studied graphene nanopores for single-molecule detection, finding that DNA translocation times were longer than other ions, suggesting DNA-graphene interactions that extended transfer times [42].

5. Applications and Feasibility Analysis

5.1 DNA Sequencing Technology in Food Microbiology Research

The impact of DNA sequencing on molecular biology has transformed food microbiology research, enabling culture-independent technologies such as functional genomics, transcriptomics, proteomics, and metabolomics [43]. High-

throughput sequencing (NGS) has been widely applied to food microbial strain sequencing and diversity studies, providing novel approaches for exploring functional mechanisms in foods, particularly fermented products [44].

Beyond genomics and diversity studies, DNA sequencing is applied to single-gene sequence analysis for microbial species identification in foods. Xu et al. [9] used PCR-pyrosequencing of specific genes to accurately identify 56 *Vibrio parahaemolyticus* strains. Cao et al. [11] employed the same method for rapid identification of *Salmonella enteritidis* in food, successfully identifying 16 strains. These studies used BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare obtained sequences against target microbial genomes for final identification.

BLAST analysis relies on microbial genome databases, and its applicability expands as more microbial genomes are sequenced. With rapid progress in food microbial genomics, the number of food microorganisms with completed or ongoing genome sequencing has increased substantially [45].

5.2 Biological Nanopores in DNA Sequencing

Currently, only biological nanopores have been commercialized for DNA sequencing, while solid-state nanopores remain in the research phase. Oxford Nanopore Technologies' MinION is the first mass-produced nanopore sequencer, utilizing an -hemolysin-based platform that has been widely used in biomolecular research since its 2015 launch. Deschamps et al. [46] used MinION to sequence the *Agrobacterium tumefaciens* genome with high accuracy. In 2016, astronaut Kate Rubins successfully performed DNA sequencing aboard the International Space Station using MinION, marking the first successful DNA sequencing in microgravity [47]. Quick et al. developed a portable DNA sequencing system using MinION that was deployed in Guinea for Ebola patient sample analysis, enabling real-time monitoring of Ebola virus mutations and providing crucial technical support for outbreak control [48]. Menegon et al. [49] used MinION to sequence blood samples from wild rainforest frogs, rapidly identifying species. Given these broad applications in molecular biology, applying nanopore sequencing to food microbiology research is highly feasible.

5.3 Feasibility of Nanopore Sequencing for Food Microbial Detection

DNA sequencing technology continues to evolve, requiring less time and lower costs. The Human Genome Project using first-generation sequencing took 13 years and cost approximately $3 billion [50]. In 2008, sequencing Nobel laureate James Watson' s genome using second-generation technology required 2 months and about $1 million [51,52]. Nanopore sequencing has the potential to sequence mammalian genomes for $1,000 within 24 hours [30].

The MinION, as the first commercial nanopore sequencer, is slightly larger than a standard USB drive, weighs approximately 90 grams, and is highly portable [53] (Figure 5 [Figure 5: see original paper]). Priced at $1,000, it connects to

computers via USB for both power and data transfer [25]. Each sequencing run requires about 1 g of DNA in a 75 l sample volume [54]. Common DNA extraction methods from food microorganisms can readily meet these requirements. Wu et al. [55] extracted total microbial DNA from wheat surfaces using SDS and SDS/CTAB methods, obtaining concentrations of 3,317.5 g/ml and 3,820 g/ml, respectively. Qin Dan [56] extracted total microbial DNA from fermented meat products using five common methods, achieving concentrations of 260–410 g/ml.

Figure 5 [Figure 5: see original paper] MinION sequencing device [25]

MinION' s default runtime is 48 hours, though data generated within just 2 minutes can be used for analysis. Its standard recognition speed is 250 bases per second (250 bps) [54], with average read lengths of 13–20 kb [57]. Xu [9] and Cao [11] used sequences of only dozens or even a dozen bases to identify foodborne pathogens, while Menegon et al. [49] used approximately 600 bp to rapidly identify wild frog species. MinION's error rate of 5–10% [58] is lower than that of popular sequencers like Illumina MiSeq and HiSeq [59], and accuracy can reach 98% through increased sequencing depth [60].

Despite MinION' s advantages in speed and accuracy, the lipid bilayer used in biological nanopores has weak mechanical stability [61], and repeated washing reduces sequencing chip lifespan. Each MinION run costs $99, with flow cells lasting approximately 72 hours at a cost of $500–900 [54]. High operational costs may limit widespread application in food microbiology testing. In 2018, Oxford Nanopore Technologies planned to release the more portable SmidgION, a smartphone-powered sequencer using the same flow cells, though pricing was not disclosed [25].

Compared to biological nanopores, solid-state nanopores offer superior mechanical, chemical, and thermal stability [61,62], along with sub-nanometer adjustability of pore dimensions and shapes [63] and the potential for high-density nanopore arrays [64]. However, challenges including excessively fast DNA translocation and low recognition rates [61] currently limit their sequencing applications. Overcoming these limitations with solid-state nanopore chips would extend sequencing chip lifespan, reduce costs, and facilitate broader adoption in food microbiology detection.

Continued research efforts promise to develop more stable biological nanopores and overcome solid-state nanopore challenges, further refining nanopore sequencing technology to improve detection stability, reduce costs, and enable widespread application in food microbiological detection.

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