Microbial-based Food Proteins Preparation and Food Safety Analysis

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Abstract. Microorganisms are widely present in life because of their large number and strong reproductive ability. Existing studies have proved that microorganisms can be used in many aspects, such as food safety, production, and follow-up processing mentioned in this paper. This paper consists of three parts. The first part is about the importance of food safety and microbiological testing in the food industry, the second part is about the application of microorganisms in single-cell proteins, and the third part is about the use of microbial methods to degrade antibiotics. This article mentioned that microorganisms play an important role in food safety, food preparation, and food subsequent handling. Microorganisms can not only be used to detect potentially harmful substances in food, to ensure food safety. The proper use of microbes can also improve the quality of food, such as the taste, taste and nutritional value of food. In fact, microbes can be used to degrade antibiotics in food, protecting both humans and the environment. There are many more uses for microbes. How to use microbes to improve the quality of life is always an area that needs more research, and researchers need to make more efforts and research to use microbes for the benefit of humans.

Keywords: Food Safety, Microorganism, Antibiotic, Detection.

1. Introduction

In contemporary society, there is an increasing concern with food safety among people from numerous countries, probably due to the worldwide seriousness of foodborne diseases. From the World health organization, global estimates in 2016, almost a tenth of people from the global population fell ill following the consumption of tainted food. Based on a survey investigating people who thought about food safety [1], including 4957 consumers in North American and European regions, 80 per cent of them laid emphasis on the critical role played by food companies in ensuring food safety and an ethical obligation to do so. In addition, more and more harmful substances are detected in food. For example, antibiotics are widely used in animal husbandry and agriculture, and are found in animals' bodies, feces and eggs. The use of antibiotics to treat diseases in domestic animals has been an extraordinary effort. China is a big country in livestock and poultry breeding, as well as in the production and use of veterinary antibiotics. The long-term and extensive use of antibiotics for animal use has caused environmental pollution, enhanced bacterial resistance, increased safety risks of livestock and poultry agricultural products, and jeopardized human health. Tetracycline was the main type of antibiotics used for veterinary use, and aureomycin and oxytetracycline were the main antibiotics used for growth promotion. At the same time, sulfa antibiotics are often used in animal husbandry. The abuse of antibiotics is very serious in China. Because of this, there are many studies based on the properties and degradation methods of antibiotics [2]. In laboratory environments, culture media tests, real-time polymerase chain reaction (RT-PCR) tests, and enzyme-linked immunosorbent assay (ELISA) assays are three typical test formats for food analysis. Nevertheless, during this decade, a cutting-edge food analysis method–nanotechnology was rapidly developed and applied in more and more areas.

In addition to food safety issues, the shortage of food proteins, especially proteins, is posing a threat to us humans. In this situation, it is very important to develop new food senior to us. And among the various foods we consume, animal and plant foods, microbial foods are also included. As a matter of fact, human have been consuming microorganisms since early times. For example, the mushrooms we consume, which have a beautiful taste, are colonies formed by the true south, and other food microorganisms such as fungus and ganoderma lucidum, which have great nutritional and medicinal values, are now widely cultivated and utilized by people.

As a result, this article will analyze the application performance of different detection methods in the detection of food hazards, and also analyze the important role that microorganisms play in the development of new functional foods. Different detection and analysis methods, such as media tests, PCR analysis and usage of nanosensors in food testing, will cover the theory, instruments and procedures used by each method.

2. Microbial-based analytical methods for food safety

2.1. The culture media tests

Culturing microorganisms from food samples with media is the most conventional microbial testing method in the food industry. Owing to its relatively low cost and environmental demand, media culture testing is still widely adopted in many countries' food quality detecting standards. The whole process needs to be carried under a lab environment, by a trained researcher. All containers and liquid-moving machines must have been done with antimicrobial disposition under standardized conditions. During the inoculation of samples on media, it needs to be generated with a clean bench or biological safety cabinet (differs with different target microbes). In terms of the overall procedures of media tests, the first stage is preparing a suitable cultural or selective medium for the target strain(s). Secondly, a serial dilution with an extract of the food sample needs to be done. Nutrient agar is one of the most common and simplest medium compositions, consisting of tryptone or peptone (a mixture of amino acids, vitamins, and many growth factors), meat extract (generally beef), yeast extract, sodium chloride and agar. Another usual medium is the nutrient broth which has the same ingredients as nutrient agar despite the agar. Therefore, the nutrient broth maintains its liquid state at room temperature. Different species of microorganisms could be cultured with different specific media due to their physiological features [3].

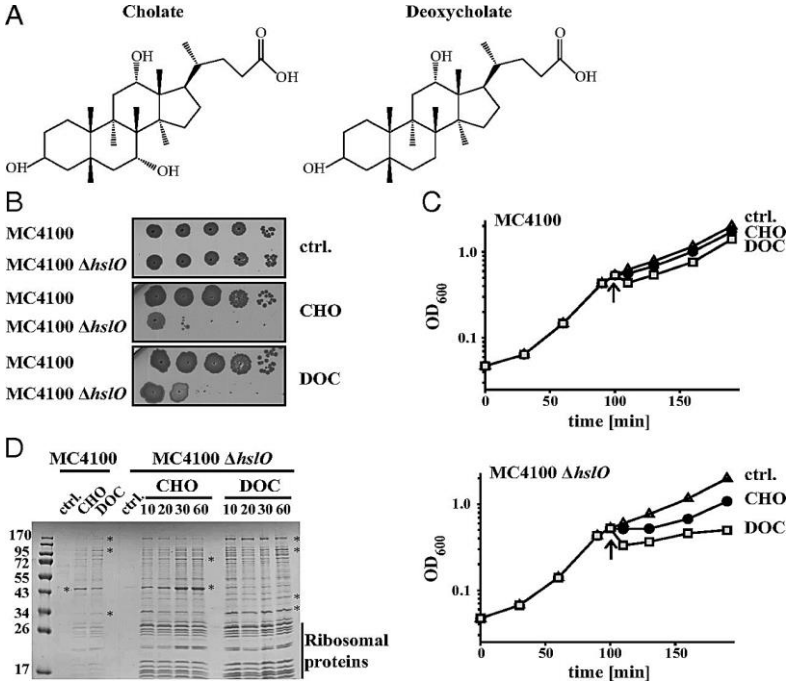

Figure 1. Bile salts cause protein aggregation [4]

For instance, MacConkey agar allows the colonization of only gram-negative bacterial species. Mannitol salt agar could select the staphylococcus genus. And xylose lysine deoxycholate (XLD) agar is used to isolate and distinguish enteric gram-negative pathogens, especially salmonella and shigella. Another popular medium for one of the most common targeting bacteria during food analysis-*Escherichia coli* (*E. coli*) is Violet Red Bile Agar (VRBA). The bile salts and crystal violet act as inhibitors to the growth of gram-positive bacteria. Bile salts could degrade the 3D structure of proteins by forming ionic bonds with the R-group of protein [4], as shown in Figure 1. They could also bind to the hydrophobic parts of membrane phospholipids and largely distort the membrane tension, which is conducive to bacteria lysis. The addition of crystal violet could easily enter the cytosol of gram-positive bacteria due to its thick and complicated fabric structure of peptidoglycan and increase the pH from 6 to 8 in order to decrease the number of the survival cell. What is more, as *E. coli* is facultatively anaerobic, lactose could be used during its fermentation to produce ATP. The breakdown of lactose to acid is detected by the colour shift of pH indicator neutral red and deposition of bile salts.

The next step is distributing samples into the culture plates. As a matter of fact, two methods could be used for these procedures–the pour or spread plate method. Because organisms could grow in or on a medium through the pour plate method, advantages of the pour plate are that scientists could identify if bacteria are aerobes, anaerobes, or facultative aerobes according to the growing depth and also makes the growth of microaerophiles become possible. And merits of the spread plate are allowing an average distribution of microbial colonies. Based on these features, the main purpose of a pour plate is to determine the total quantity of colony-forming bacteria in a sample as well as give a rough identification of their overall metabolic pathway. The goal of a spread plate is to identify relatively aerobic colonies. Furthermore, another plate method-streaking technique is also widely used in microbial testing, which aims to isolate a pure strain from a population of a single microorganism species. For testing water samples, the membrane filtration method is more appropriate. The membrane filter in this system could capture large particles including most bacteria.

2.2. The PCR tests

Polymerase chain reaction (PCR) aims at inducing an amplification of a specific DNA sequence. In terms of its general procedures, four groups of components are demanded–at least a double-stranded DNA molecule or sequence which contains the target DNA piece, primers which are complementary to certain segments of the target sequence, a heat-resisting DNA polymerase and numbers of free deoxynucleotides. At the start of the first cycle, high temperatures (90~95 ºC) could denature all hydrogen bonds which connect two DNA strands. Then, after cooling down DNA templates to 60 ºC, primers begin to bind with the ends of the target sequence. After that, DNA polymerases add complementary nucleotides along the target sequence under 72 ºC. The next two cycles repeat the same steps in order to receive as many as enough target sequences and remove other unnecessary sequences.

Two general PCR detection types of methods are widely applied in food microbiology–endpoint and real-time PCR detection. The end-point PCR is a relatively older method which analyzes all DNA products through agarose gel electrophoresis after all cycles of PCR are finished. However, due to several disadvantages of the end-point PCR detection such as much more time consumption and higher risk of contamination during moving the sample for analysis, Real-time PCR has replaced this method in the food industry. Real-time PCR or qPCR amplification system is more widely applied in the food testing process. This system is carried by thermal cyclers where a fluorometer is built to identify the fluorescence inclined by binding fluorescent dye to specific or non-specific DNA sequences in PCR. The dye only emits fluorescence when it is attached to dsDNA and the increase in DNA product during PCR could contribute to an increase in emitting intensity assessed every cycle.

Besides, based on the fluorescent phenomenon, the detection methods could be non-specific by using SYBR Green or specific to a target sequence with three types of probe technologies including

5’ nuclease, molecular beacons, and FRET hybridization probes. A genetic probe means a single-stranded DNA or RNA sequence used to locate its corresponding sequence in a genomic sample.

The 5’ nuclease probe or TaqMan probe is a kind of DNA sequence that is constituted of a fluorophore at the 5’ end and a quencher at the 3’ end. The 5’ end is cleaved by Taq polymerase after the pairing of the complementary sequence with the target sequence. Subsequently, after cleavage of fluorescent dyes from the quencher, fluorescence is induced which arise more intensely with an increase in concentration of the target sequences.

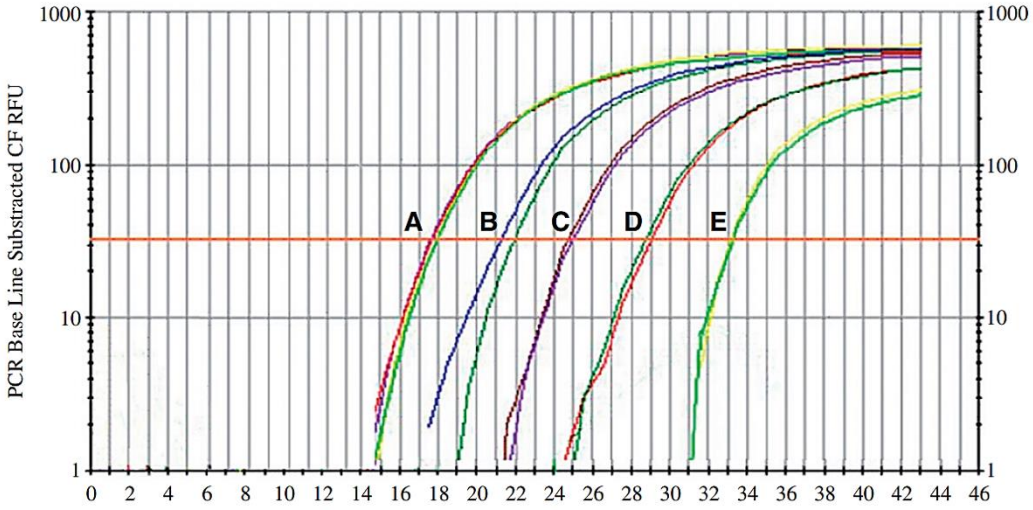

Figure 2. Detection limit of RT-PCR analysis [5]

As shown in Figure 2, the research carried by Montserrat Espiñeira and others in 2010 proved the specificity, sensitivity, repeatability, and timeliness of real-time PCR through three ways of determining the limit of detection (LOD) between endpoint and real-time PCR respectively [5], which included detections of minimal quantity of DNA for a clear figuration on an agarose gel or fluorescence signal and minimal proportion of soybean powder in two impure processed foods (cans of fish and flour powder).

2.3. Food safety analysis methods based on nanomaterials

Nanotechnology is about how to produce and apply nanomaterials, related gadgets, and systems. Three are two kinds of applications for nanoparticles in food categories–food processing and food packaging [6]. The food processing by nanomaterials contains an improvement for food testing and we are going to focus on the usage and compositions of nanosensors in this category.

The nanosensor is a means for detecting food safety by involving a series of attaching reactions with bacteria in food and transferring the amount and trail of bacteria through either infrared light or magnetic materials. One of the advantages of it is that it could be composed of several nanoparticles to increase the rapidity and accuracy of testing and enter some minute areas due to its tiny size, thus getting a very detailed and well-rounded result for the food sample. For example, nanocrystal quantum dots could be applied for PCR analysis and immunoassay.

From the research by Zurier et al. in 2020 [7], a new biosensor system, phage-based nanobots, has been generated for the detection of in situ bacteria, as shown in Figure 3. Compared with the PCR analysis which has a sensitivity of 100 CFU/L in spiked water samples for *E. coli* microbiomes, the method of using phage nanobots has much higher sensitivity which could even reach 1cfu/100ml in less than one litre of drinking water extraction. When it comes to the overall procedures of this method, the first step is modifying the small outer capsid (SOC) protein of wild-type Escherichia virus T4 (T4 phage) to make the binding of magnetic nanoparticles (MNPs) become possible through click chemistry. After a knockout of the original SOC on the T4 phage, it is alkynylated by adding click SOC which self-assembles on the T4 nanoluciferase (Nluc) cellulose-binding module. The MNPs are conjugated with click T4 phage by using click reaction which is carried with the form of one-pot

synthesis and produces little by-product. This nanobot showed high sensitivity and specificity to *E. coli*, which is mainly owing to the dipole properties of magnetosomes in bacteria [8].

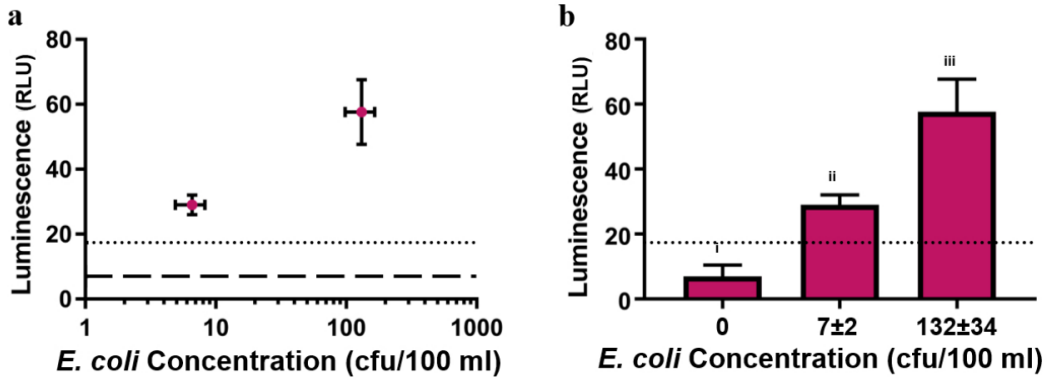

Figure 3. Performance of phage-based nanobots for detection of target bacteria [7]

3. Microbial-based antibiotic analysis

Before performing an analytical test for antibiotics, it is impossible for us to get the degradation rate of Sulfonamide antibiotics by the engineering strain through traditional chemical process [9]. To this end, the high-performance liquid chromatography (HPLC) can be used for determining the degradation rate of sulfonamide antibiotics. There are two parts of this method, sample preparation and HPLC detection condition selection. Ecn-il and the wild control group were inoculated into the medium with 1% of the inoculation amount at 37℃ for overnight culture. The solid bacteria were centrifugated and prepared with a bacterial suspension with cell concentration of 1.0×10^9 CFU/mL. 10 mL was taken and 30, 50 and 100 mg/L SDZ sodium salt were added, respectively. After resting culture at room temperature for 3 h, the supernatant was centrifugated and put into the tube to complete the sample pretreatment. Select the VWD detector as the detection condition. ABTS was used as the substrate to determine the enzyme activity, and the absorption wavelength of 480 nm was used to calculate the enzyme activity. Cell suspension was obtained from sample pretreatment and determined. In order to test the proper pH, the above-mentioned cells were suspended in the buffer solution with different pH in the same volume and added with 50 mg/L sulfadiazine sodium salt. After reaction for 1 h, the precipitation was discarded to determine the optimal pH.

In order to obtain high copy constitutive plasmids, the recombinant plasmid PSB1A3-MRFP derived from IGEM tissue was modified in this study. The RFP gene sequence was successfully removed from the plasmid DNA sequence under the action of specific primers, and NdeI, BamHI and HindIII restriction sites were introduced. The PSB1A3-MRFP was first linearized through BKL kit, RFP sequence was removed, restriction sites were introduced, and then the plasmid was re-cycled under the action of blunting kination enzyme and named pSB18A. Finally, the recombinant plasmid pSB18A/INP-N-L was successfully transformed into the expressing strain EcN and named ECN-IL.

The efficient degradation strains of tetracycline, oxytetracycline and sulfadiazine were firstly isolated and screened. Through the separation and purification of the metabolites of the strain, the kinds and properties of the substances that can degrade antibiotics of the strain were analyzed [10]. The molecular structure of the degradation products was detected by HPLC analysis and ESI-MS method, and the degradation pathway was deduced accordingly, and the safety of the antibiotic degradation products was tested. In the process, Firstly, a strain named “N2-13” was isolated from the feces, sewage and soil samples by enrichment culture, plate initial screening and HPLC quantitative determination. The tetracycline degradation rate was 73.88% After rRNA gene detection. It was identified as Bacillus amyoliquefaciens. The result is that there are fourteen strains had a good ability to degrade tetracycline, among which strain N2-13 had the strongest ability to degrade tetracycline, with a degradation rate of 71.88%. Fourteen strains with good degradation effect on oxytetracycline were obtained, among which the N2-18 strain had the best degradation effect with a

degradation rate of 60.47%. Seven strains with good degradation effect on sulfadiazine were obtained, among which T-14 strain had the best degradation effect, and the degradation rate of sulfadiazine was 63.72%.

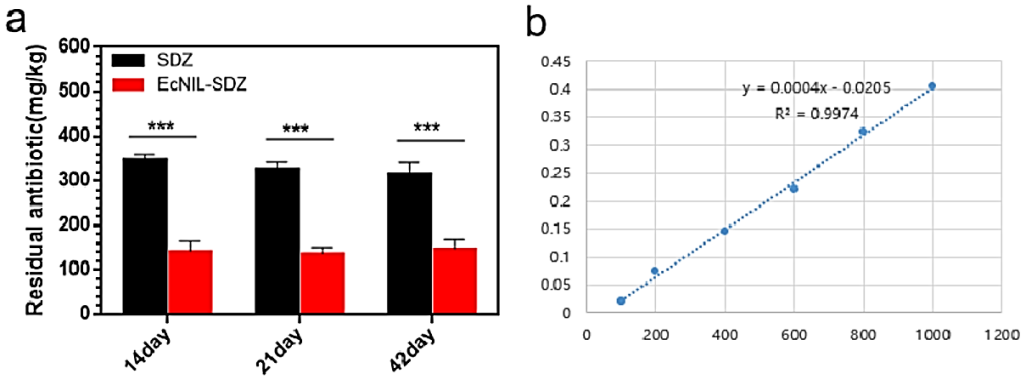

Figure 4. Degradation of sulfadiazine in chicken manure by engineered strains [11]

Figure 4 shows the degradation of sulfadiazine in chicken manure by engineered strains. The developed analysis method has a very high sensitivity in the detection of sulfonamides antibiotics. Because sulfonamide antibiotics contain amino groups, fluorescence detection (FLD) can be selected, and its sensitivity can reach 0.01 mg/L [11]. The second method is an immunoassay. There are many immunoassays for sulfonamides, mainly enzyme-linked immunoassays. There are many new methods in China in recent years. For example, synthetic antigen SMD-bas was prepared by coupling sulfamethoxine (SMD) with carrier protein bovine serum protein (BSA) by glutaraldehyde method as immunogen, and SMD-OVA was synthesized by the same method. Rabbits were immunized with the antiserum to obtain the antiserum, and indirect competitive ELISA method was established with the prepared antiserum. Below the international prescribed residue limit (100 μg/kg). The recovery rate of milk samples without basic background was 94.7%. In addition, by glutaraldehyde method will sulfanilamide methyl isopropyl well wow coupled with bovine serum albumin, with the method of ovalbumin and the drug coupling by immune sulfanilamide methyl isopropyl uh wow antibody, established the indirect ELISA method for the drug in the milk. The recoveries were 71.6%~106%. The third method is chromatography-mass spectrometry, which is divided into gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. In gas chromatograph-mass spectrometry (GC/MS), the drug is extracted from the sample and methylated by diazomethane before GC/MS analysis. Electron bombardment (EI) is generally used for mass spectrometry detection. For some samples with more impurities, silica gel column purification or liquid-liquid distribution purification can be further used. The second method was gas chromatography-mass spectrometry (GC-MS), which was used to detect 17 sulfonamides in chicken by liquid chromatography-electrospray tandem quadrupole mass spectrometry. The detection limit of the method was 0.0210 ug/kg.

4. Microbial-based food proteins

China is a large agricultural country, the food structure is mainly plant protein, the intake of animal protein is very different from European and American countries, in order to improve the physical fitness of the people, the development and production of single-cell proteins (SCP) opened up a new way to solve the problem of human food and feed. The five major problems in the contemporary world are population, food, resources, environment and energy, especially food and environment are attracting more and more attention from the society.

The SCP is bacteriophage proteins obtained by culturing single-cell organisms. The advantage of the SCP is rich in nutrients. The use of raw materials is wide, and can be local inexpensive large number of raw materials to solve the problem. And the production rate for the SCP is high, and the production is not subject to seasonal climate constraints, and easy to manually control. In addition, it

can be completely industrialized production of the SCP production than agricultural production that requires less labor, which can be carried out on a small equipment covering a limited area, not only in large quantities, and good quality, far more than the existing food varieties of protein. The single-cell organisms are easy to mutagenesis, easier to improve than plant and animal species, and it can be used physical, chemical and biological methods of directed glazing breeding to obtain good protein content of high quality and easy to extract the protein of good strains [12]. There are many types of microorganisms used for the production of the SCP, including bacteria actinomycetes, yeasts, molds and certain protozoa. These microorganisms usually have the following conditions: high content of nutrients such as proteins produced, no pathogenic effect on humans, good taste and easy digestion and absorption, simple culture conditions, rapid growth and reproduction, etc. The production process of the SCP is also simple. After the culture solution is prepared and sterilized, they and the strain are put into the fermenter and the fermentation conditions are controlled, and the strain will multiply rapidly. After the fermentation is finished, the bacterium is collected by centrifugation and sedimentation, and finally, the finished product is made into single-cell raw white after drying.

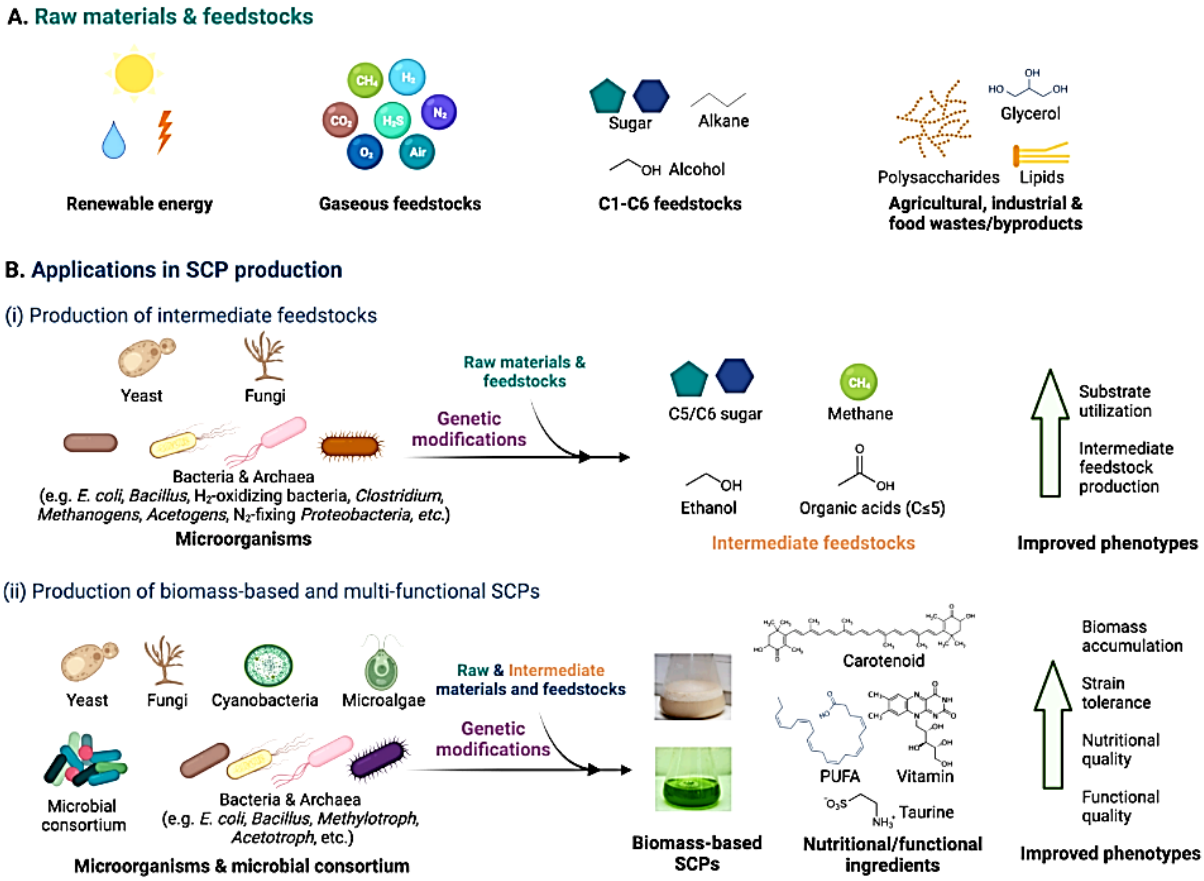

Figure 5. Potential use of genetic modification for developing SCP [13]

Under ideal conditions, the strain is easy to multiply the yield of single cell protein, and the time required is 500 times faster than the time required to multiply the amount of crop protein, and 1000-5000 times faster than the time required to multiply the yield of other general breeders. The production of the SCP is not limited by season, space and sunlight. As shown in Figure 5, different methods have been used for developing SCP. Microorganisms can produce a large number of microbial proteins, not only for direct human consumption, but also as a high-protein feed for livestock and poultry, to catch for us to provide high quality and high price of meat protein. Its fat content is only 1 0% of lean beef, popular among consumers. On the one hand, the development of microbial protein food can reduce the contradiction of reduced arable land and food shortage, and on the other hand, the development of microbial protein food with high protein is also conducive to improving the food structure of people.

The production process of SCP can be generally outlined as follows [13]: strain-strain expansion culture-fermenter culture-culture broth-separation-bacteria-washing or hydrolysis-drying. On the one hand, the production of SCP can make use of various kinds of waste materials, waste liquids and waste residues as raw material sources. On the other hand, a large amount of protein, vitamins and other nutrients can be obtained for animal feed and food, thus alleviating the food crisis. If the protein content is calculated, 1 kg of SCP is equivalent to 1-1.5 kg of soybeans, the establishment of a plant with five 100-ton fermenters can produce 5,000 tons of SCP per year, equivalent to 0.3 million hm^2 of soybean production on arable land. Therefore, the development and production of SCP has a broad prospect in worldwide.

For the application, the SCP is rich in protein and many kinds of vitamins and inorganic salts, which is an ideal source of protein with comprehensive nutrition, and mixing it into cookies, beverages and dairy products can improve the nutritional value of these traditional foods. The protein tissue-forming properties of the SCP can be used to make new foods such as “artificial meat”. The SCP is also often used as a food additive to supplement protein or vitamins, minerals, etc. And it can be used in the production of meat products and baked goods to maintain the moisture, taste and flavor of food.

5. Conclusions

This article analyzed the role of microorganisms in food safety and the development of new functional foods. As for the content about the SCP, this research has mentioned its low cost, high productivity, relatively strong adaption to the environment, low difficulties for controlling, low access for extraction, guaranteed safety for human intake and wide application, compared with the traditional raw material of food. For the degradation of antibiotics, different analysis methods for testing the sulfonamide residues were discussed, such as HPLC. When it comes to food testing methods, this research analyzed synoptically the procedures for preparing culture media and how to culture strains on them including the pour, spread plate methods and membrane filtration. The simple theories of selective mediums were mentioned as well as the examples of MacConkey agar, mannitol salt agar, XLD agar and VRBA. Then, we explained the principle of PCR analysis and made a comparison between the efficiency of end-point and real-time PCR.

Although we have discovered many species of microorganisms which are beneficial or detrimental to human health and developed lots of detailed methods to detect and prevent them from taking in with multiple ways, especially through the alimentary pathway, there is still a huge range of demerits with our testing method. To cite a notable example, real-time PCR amplification is relatively more advantageous than many other conventional testing methods due to its direct testing pattern and the convenience of probe techniques, but it is difficult to frequently examine the environment in the machine so the intactness of the target DNA sequences or the DNA probe may be influenced. Consequently, the uncertainties of experiment results could increase. In addition, even though some advanced testing methods such as the phage-based nanobots mentioned has described perfectly in recent years, there has not been any research which measured the risk of this modified virus to human health and leakage out from the experimental environment. On account of the increasingly crucial impacts acted by microorganisms and consideration from people about them, it is urgent for us to study more deeply in the domain of microbiology and enhance food safety in a global range through using manifold ways to guarantee the quality of foods.

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