RESEARCH ARTICLE

Optimization of proliferation conditions for detection of *Salmonella* in food

Yuanyuan Fan*, Limin Pan, Taixi He

Foshan Customs, Foshan, Guangdong, China.

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The current research about *Salmonella* detection mainly pays attention to the shortening of the detection time with many *Salmonella* rapid detection methods being emerged recently. This research investigated the comprehensive effect of various factors for the optimal proliferation conditions of *Salmonella* based on the method of GB 4789.4-2024. The proliferation quantity, growth characteristics, physiological and biochemical properties of the target bacteria were tested. The performance of culture medium was evaluated based on the test results. The culture medium with the best proliferation effect was selected as the optimized experimental medium. Using the number of *Salmonella* as the evaluation index, the optimal proliferation conditions of *Salmonella* in food were tested under different culture times, temperatures, and bacterial concentrations. The results showed that the optimal conditions of *Salmonella* proliferation were 10^8 CFU/mL at 42℃ for 24 h. To a large extent, the influence of various factors on the outcome varied greatly. The sensitivity and specificity of the culture medium selected in this research helped to improve the detection rate of *Salmonella* in samples, and the optimized culture conditions were also suitable for the proliferation of *Salmonella*.

Keywords: cultural conditions; foodborne pathogen; detection rate; *Salmonella*.

***Corresponding author:** Yuanyuan Fan, Foshan Customs, Foshan, Guangdong 528300, China. Email: yuanyuanfan2006@163.com.

Introduction

According to statistics from the China Ministry of Health, there are an average of 299 cases of food poisoning in China each year [1]. The World Health Organization (WHO) has clearly defined *Salmonella* as one of the mandatory items for food hygiene testing [2]. The transportation, processing, and production of raw food materials and feed can all be contaminated by *Salmonella*, and then, cause a serious threat to food safety.

The detection of pathogenic bacteria in food primarily relies on conventional plate culture method and auxiliary means. The recent research progress of fast detection methods for pathogenic microorganisms mainly focuses on the direction of rapid and automation and combines with molecular biology means to form technical fusion detection system [3]. Although the rapid detection time has been shortened, the higher sensitivity of those methods requires many additional validation experiments. Currently, there are three main detection methods including nucleic acid detection method, immunological detection method, and biosensor detection method. However, these methods are long-term, cumbersome, and prone to false positives, making them inadequate to meet the demands of modern food safety [4, 5].

Therefore, the combined effect of these factors is crucial.

Although some studies have compared the growth characteristics and applicable objects in different culture media [6], there are few studies on the optimal proliferation conditions for *Salmonella* in food. This research compared and analyzed the enrichment effects of three liquid culture media for *Salmonella* culture to select *Salmonella* isolation culture media suitable for different types of samples and optimize the proliferation conditions of *Salmonella* culture to find the best proliferation method and improve the accuracy and detection rate of *Salmonella*. This study would obtain the optimal conditions with combined effect of the cultural factors and provide both theoretical support and practical guidance for *Salmonella* detection methods.

Materials and methods

Sample collection

A total of 100 fresh poultry drumsticks, frozen saury, and frozen dumpling were purchased from the Nanjiang Market, Foshan, Guangdong, China. In addition, 100 human fecal samples from the first-grade students at junior high school (Desheng School, Foshan, Guangdong, China) and environmental samples through swabbing the classroom desk surface with a sterile cotton swab were collected.

Preparation of bacterial suspension and colony count

Salmonella typhimurium (ATCC 14028) and *Escherichia coli* (ATCC 25922) were purchased from Shanghai Hanni Biotechnology Co., Ltd (Shanghai, China). Both original bacteria cultures were diluted in sterile saline to make 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions, respectively. 1 mL of each bacterial dilution was then mixed with 15 - 20 mL of plate count agar medium in a sterile petri dish. After the agar solidified, the plate was incubated using Yiheng LRH-150 incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) at 36 ± 1°C for 48 ± 2 h before colony counting [7, 8].

Cultivation conditions

The method published in GB 4789.4 (National Health Commission of the People's Republic of China, State Administration for Market Regulation, Beijing, China) was used for *Salmonella* enrichment test [9-11]. Briefly, 0.1 mL of each *Salmonella typhimurium*, *Escherichia coli*, and the mixture of both bacteria suspension obtained from the dilutions of 10^{-8} were inoculated into selenite cystine broth (SC), Rappaport-Vassiliadis medium with soya (RVS), Rappaport Vassiliadis *Salmonella* enrichment broth (RV), and Rappaport-Vassiliadis medium (MM) liquid culture media (Guangdong Huankai Biotechnology Co., Ltd., Guangzhou, Guangdong, China), respectively, and incubated at 42 ± 1℃ for 18 - 24 hours, while 1 - 10 mL of SC were incubated at 36 ± 1℃ for 18 - 24 hours, simultaneously. To investigate the reasons behind the different effects of four different culture media on bacterial growth, and to better utilize their effects on bacterial growth, the components of these four different culture media were compared.

Determination of bacterial growth rate

Quantitative methods were used to evaluate the microbiological performance of the culture media. After culturing under the conditions specified in GB4789.4 standard, the selectively enriched cultures were mixed and streaked onto xylose lysine desoxycholate medium (XLD) agar plate using a 3 mm diameter sterile inoculation loop before counting the total number of colonies. If the colony with the size of the target bacteria on the selective plate was more than 10 CFU, the growth rate of the tested liquid culture medium was good. If the colony count of non-target bacteria on non-selective plates was less than 100 CFU, the selectivity of the tested liquid culture medium was good.

Biochemical and serological identification

API 20E identification system for Enterobacteriaceae and other non-fastidious

Gram-negative rods (BioMerieux, Marcy-l'Étoile, France) was employed for intestinal bacteria biochemical identification following manufacturer's instructions. The system consisted of 20 kinds of biochemical reactions plus a manual oxidation test to provide information about the bacteria's ability to metabolize certain substances, which helped in distinguishing between different species. The results of these tests were compared against a database of known biochemical profiles to identify the bacteria. Further, polyvalent O and H antigens were identified using *Salmonella* genus diagnostic serum (Ningbo Tianrun Biopharmaceutical Co., Ltd., Ningbo, Zhejiang, China) for serological identification. Based on the results of serological and biochemical typing, the serotype was determined according to the relevant *Salmonella* antigen table.

Single factor experiment

(1) The effects of cultivation time on the proliferation of *Salmonella*

1 mL of bacterial sample containing 10^4 CFU/mL of *Salmonella* was inoculated into XLD medium for culture counting at 42 ± 1℃ for 16 - 24 hours.

(2) The effect of cultivation temperature on the proliferation of *Salmonella*

1 mL of bacterial sample containing 10^4 CFU/mL of *Salmonella* was inoculated into XLD medium for culture counting at 41 ± 1℃ to 43 ± 1℃ for 24 hours.

(3) The effect of bacterial concentration on the proliferation of *Salmonella*

1 mL of bacterial samples containing 10^4 to 10^8 CFU/mL of *Salmonella* were inoculated into XLD media for culture counting at 42 ± 1℃ for 24 hours.

Statistical analysis

SPSS (IBM, Armonk, NY, USA) was employed for the statistical analysis of this research to determine the optimal conditions for *Salmonella* proliferation and the true relationship between various factors and response values. The optimal proliferation conditions for *Salmonella* and the order of the impact of various conditions on *Salmonella* proliferation were then determined [12-14].

Results and discussion

Growth of standard strains on selective culture medium

The growth of target and non-target bacteria reflected the properties of the culture medium such as the influence on the growth rate and selectivity of the target bacteria. The target bacterium *Salmonella typhimurium* grew well on all four types of culture media with a colony count greater than 10 CFU. In food samples, the growth rate of target bacteria cultured in RVS was slightly higher than other media, while non-target bacteria *Escherichia coli* were partially or completely inhibited after RVS cultivation with colony counts less than 100 CFU, which was far below the standard required concentration of 10^4 CFU/mL [14-17] (Table 1).

Comparison of culture medium components

RVS contains malachite green, which can inhibit the growth of non-*Salmonella* bacteria, while soy peptone is beneficial for the recovery of *Salmonella* bacteria. SC contains lactose as a fermentable sugar, while sodium hydrogen selenite inhibits Gram positive bacteria and most Gram negative intestinal bacteria, and has a good selective enrichment effect on *Salmonella typhi*. Generally, processed foods with severe bacterial contamination require a culture medium with good recovery ability, and RVS is more suitable. However, SC contains sodium hydrogen selenite, which has certain toxicity and poses safety risks. Further, the raw materials are unstable and greatly affected by temperature, making them prone to denaturation and causing the functional failure of the culture medium. SC medium has weak selectivity, but if it is used solely for the detection of *Salmonella typhi* and *Vibrio parahaemolyticus*, then SC medium is suitable as a selective enrichment broth [18]. The results indicated that RVS was more suitable for the enrichment of *Salmonella* in food and

Table 1. The growth of *Salmonella typhimurium* and *Escherichia coli* in four different media.

Medium	*Salmonella typhimurium* (cfu/mL)			*Escherichia coli* (cfu/mL)			Mixed culture (×10^7) (cfu/mL)		
	Food	Fecal	Environment	Food	Fecal	Environment	Food	Fecal	Environment
SC	34	51	50	2.0	3.0	4.0	30	45	49
RVS	35	45	52	1.5	4.0	3.0	39	36	41
MM	30	44	43	2.0	3.0	2.0	34	41	43
RV	35	46	45	2.0	3.0	3.0	34	32	39

environmental samples, while SC was more suitable for the enrichment of *Salmonella* in fecal samples. Therefore, based on the analysis of the results, RVS was selected as the optimized experimental medium to explore the optimal conditions for *Salmonella* proliferation.

Biochemical test results

The colonial characteristics of *Salmonella typhimurium* on XLD plates were pink with or without a black center. The colonies were typically pink, which was due to the fermentation of xylose and the subsequent color change of the neutral red indicator in the medium. This characteristic helped distinguish it from other bacteria that might grow on this selective and differential medium. The results showed that the test strain was *Salmonella typhimurium* without a black center.

Single factor test results

A bacterial suspension with a concentration of 10^6 CFU/mL was selected to test the effect of culture time on the proliferation of *Salmonella*. The results showed that the bacterial concentration reached a peak at about 24 h of culture, which could reach the order of magnitude of 10^8. The main reason was that the bacteria were in the adaptation stage in the early stage of culture, and some damaged bacteria or bacteria in a dormant state were beginning to repair and recover, so there was no obvious proliferation in the first 20 hours of growth. From 22 to 24 hours of culture, the growth of bacteria entered the logarithmic growth period, and the bacteria grew vigorously. With the increase of culture time, after 24 to 26 hours of culture, the growth of bacteria entered the stationary phase, and the number of colonies basically did not increase (Figure 1).

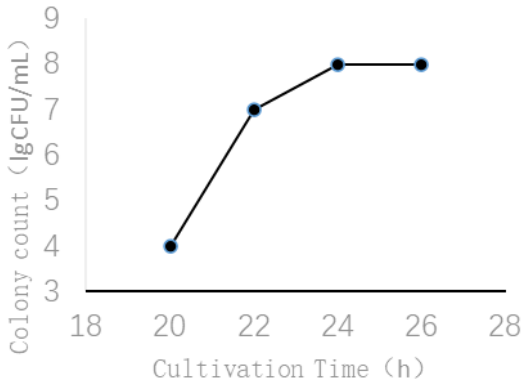

Figure 1. Effect of culture time on the proliferation of *Salmonella*.

The proliferation of *Salmonella* was optimal when the initial concentration of the bacterial solution was 10^6 CFU/mL. When the initial bacterial concentration was less than 10^6 CFU/mL, the proliferation degree of *Salmonella* was higher. However, when the initial bacterial concentration was greater than 10^6 CFU/mL, the number of *Salmonella* was higher, but its proliferation degree was lower, which might be due to the limited nutrients and energy in the culture medium. When the bacterial concentration was too high, competitive inhibition would occur between the bacteria, resulting in a balance or decrease in the number of bacteria, thus reducing the proliferation degree (Figure 2).

The bacterial culture reached its peak of 10^8 orders of magnitude at a temperature of about 42℃. When the temperature was 41℃ or 43℃,

the bacterial concentration did not differ much with all reaching 10^7 to 10^8 orders of magnitude, which was mainly because that, although temperature was one of the important factors affecting bacterial growth, the optimal growth temperature of different pathogens was different. In the appropriate temperature range, the bacteria would be the best growth state and have the strongest reproductive capacity. The culture temperature could promote the growth of bacteria, while too high or too low temperature could damage the bacteria and even lead to bacteria death (Figure 3).

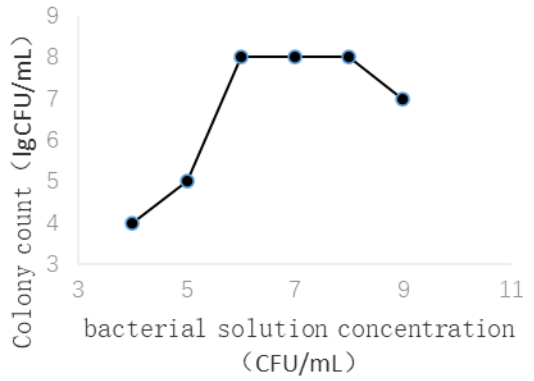

Figure 2. Effect of bacterial concentration on the proliferation of *Salmonella*.

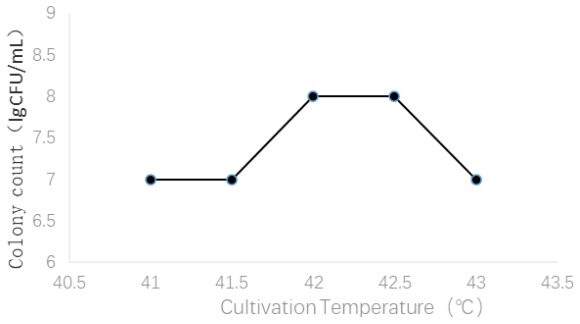

Figure 3. Effect of culture temperature on the proliferation of *Salmonella*.

Analysis of orthogonal experiment results

The results showed that the optimal factor combination for Salmonella culture was a cultivation time of 24 hours, a cultivation temperature of 42℃, and a bacterial concentration of 10^8 CFU/mL. All factors demonstrated significant impacts on the experimental indicators with the order of importance as cultivation time > bacterial concentration > cultivation temperature, indicating that the cultivation time had the greatest effect on the growth of *Salmonella*.

Selection principle

Any isolation medium has two basic characteristics of growth rate and selectivity. The microbial environment in which the target bacteria are located can also have a certain impact on the performance of the medium [19-22]. Therefore, whether it is isolating *Salmonella* from food or feces, to ensure high growth rate and selectivity of the enrichment culture medium, it is necessary to choose the appropriate culture medium according to the experimental sample, purpose, and needs, or to use both RVS and SC isolation culture media simultaneously to improve accuracy and detection rate.

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