Recovery of difficult cultivable fungi from chicken through a new chicken based cultural medium

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Abstract—Although decades of the development of the molecular approach to investigate the microbial diversity in foods has shown a great promise, non-culturing method may not necessarily fully reflect the diversity of microbial community in all circumstances, and in extreme situation it shows even different diversity from culture-dependent methods. Accordingly, suitable growth medium and the corresponding protocols to permit the maximal growth of spoilage and pathogenic microorganism are in great need. In this study a near-native medium of chicken extract agar (CEA), prepared by modifying the conventional Rose Bengal medium (RBM) with the addition of sterile chicken extract, was developed to detect difficult cultivable fungi on raw chicken. Sterile chicken extract was obtained by homogenizing chick meat in sterile water, immersing overnight and filtering through 0.22 μm membrane. CEA of different concentration of chicken extract (1.5%, 2.0%, 2.5%, 3.0%, 3.5%, *W/V*) was prepared by adding chicken extract into RBM. Enumeration of fungi on CEA was used to evaluate the optimal concentration of chicken extract in CEA. Fungi colony recovered by CEA but absent on conventional RBM during standard cultivation period through *in situ* inoculation were subjected to identification based on fungal internal transcribed spacer (ITS) and 26S D1/D2 gene sequencing. A significantly greater colony form unit (cfu) ($P < 0.05$) and diversified fungi community were observed on CEA than on RBM. CEA containing 2.5% chicken extract gave the highest value of 62 cfu g^{-1}, twice that in RBM (30 cfu g^{-1}). By ITS analysis on six colonies recovered exclusively on CEA, five colonies with the similar morphological characteristics were disclosed to be the same species of *Aspergillus parasiticus* with similarity of 99%, and the sixth was *Debaryomyces hansenii* with similarity of 100%. The identification by 26S D1/D2 revealed the same information as that by ITS. The two species of *A. parasiticus* and *D. hansenii* identified in raw chicken by our novel near-native CEA medium are considered as either spoilage or pathogen in foods, thus to cause problems associated with food safety. The detection strategy utilized in this work may assist in the characterization of difficult cultivable spoilage and pathogenic microorganisms in other meats or meat products.

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Index Terms—**chicken, difficult cultivable, near-native medium, ITS, 26S D1/D2**

I. Introduction

Fungi are able to grow on almost all types of foods, such as cereals, meats, milk, fruits, vegetables, nuts, and fats. Their growth may result in food spoilage, thus giving off-flavors, toxins, discoloration, rotting and formation of pathogenic or allergenic vegetative cells [1][2]. The deterioration of sensory properties is often due to the production of exoenzymes during fungi growth. Moulds can produce a vast number of enzymes, including lipases, proteases, and carbohydrases [3]. Once getting into foods enzymes can continue exhibit their activities, being independent of destruction or removal of mycelium. Some moulds are even able to produce mycotoxins and antibiotics, which represent a potential health hazard to consumers [4].

Differences in nutrient composition and concentration between food system and the growth medium used to detect fungi may lead to a poor recovery of food borne microorganisms. For example, recovery of osmophilic yeasts from foods with high salt and sugar concentrations requires the use of non-conventional media [5]. Several authors have reported the unusual growth behavior of microbiota from dressing on various microbiological media, such as the requirement for extended incubation period (10 d), or the need for inclusion of additional ingredients like fructose to improve recovery [6][7]. And in a recent study by Waite et al, it was shown that ranch dressing based medium was helpful for the growth of fungi that was difficult to be cultivated in common PDA agar [8]. Therefore the characterization of difficult cultivable bacterial species present in chicken necessitates the use of a reliable culture media.

The objectives of this study were (1) to develop a near-native medium eligible to recover contaminating fungi in chicken carcasses by food-based growth media; and (2) to identify the contaminating microorganisms that can only recovered by the near-native media developed.

II. Materials and Methods

A. Formulation of chicken extract agar (CEA)

To prepare chicken extract, 50 g chicken meat was homogenized in 100 mL of sterile water after grinding using A11 basic Analytical mill (IKA Inc.), and immersed overnight at 4 ℃. After that, sterile supernatant was taken by filtering with gauze and 0.20 μm Millex-FG filter (Millipore Inc.). Then

3, 4, 5, 6, 7 mL chicken extract were diluted by sterile water till 10 mL respectively. The isolation medium was a modified chicken extract agar (CEA) containing 10% chicken extract dilution in a half strength standard RBM. The basal medium was prepared with agar (SoBiGel, Bie and Berntsen, Roedovre, Denmark) and autoclaved before cooling to 55°C. Medium of 18 mL was poured immediately into sterile Petri dishes (90 mm) in which 2 mL sterile chicken extract was added already.

B. Microbiological analysis

For culture-dependent analysis, chicken carcasses were obtained from local cold storage. Using aseptic techniques, 25 g of each sample (n = 3) were homogenized in 225 mL of sterile peptone saline (1 g of peptone and 9 g of NaCl per liter). After shaking at 230 rpm for 10 min with a stomacher, this suspension was serially diluted (1:10) in peptone saline. One milliliter dilutions were inoculated onto Rose Bengal Medium (RBM) and chicken extract agar (CEA) in triplicate to obtain the total aerobic count. Plates were then incubated for 120 h at 28 ℃. Colonies developing on plates were counted.

C. Isolation and characterization of contaminated bacteria from chicken extract agar (CEA)

Two new 90 mm Petri dishes were prepared, with one filled with RBM, and another CEA. The spaces of plates were divided and numbered correspondingly. All microorganisms recovered from CEA were inoculated into the two Petri dishes, one number corresponding to one bacterium. All plates were then incubated for 120 h at 28 ℃. Colonies only recovered in CEA were isolated, and further purified. Pure cultures were stored in 30% glycerol at -20 ℃.

D. Molecular identification

Molecular identification by sequencing the ITS and 26S D1/D2 regions of fungal DNA was performed for the isolates only obtained in CEA. DNA was extracted by Cetyl trimethylammonium bromide (CTAB) DNA extraction protocol [9].

The fungus-specific universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-GCATATCAATAAGCGGAGGA-3') were used to amplify genes encoding the ITS region, Primers NL1 5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used to sequence a 600 bp fragment of D1/D2 domain of 26S rRNA [10]. Similar PCR amplification and sequencing protocols described in Asefa *et al* [11] were used.

E. Sequence analysis

The ITS and 26S D1/D2 sequence obtained was aligned to sequences in GenBank using the BLAST program. The aligned sequences of the related species were retrieved from the NCBI nucleotide database. The program Clustal X (version 1.8) with default parameters was run for multiple sequence alignment. Phylogenetic and distance analysis of the aligned sequences was performed by MEGA (version 4.0). The resulted unrooted tree topologies were evaluated by bootstrap analysis of the neighbor-joining method based on 1000 replications.

F. Statistical analysis

Significant differences between averages of duplicate measurements were evaluated by performing one-way ANOVA at a confidence level of p = 0.05 with a posthoc Tukey test using SPSS 13.0 package.

III. RESULTS AND DISCUSSION

3. 1. Medium development and fungus enumeration

The changes of fungus enumeration were monitored by RBM and a series of CEA containing different concentration of chicken extract (Fig. 1). The mean colony form unit (cfu) on RBM were 30 cfu g^{-1}, while that on CEA medium increased with the concentration of chicken extract in general, with 2.5% giving the highest value of 62 cfu g^{-1} ($P < 0.05$). Further increase of chicken extract did not give the cfu raise.

Our results showed that fungus recovery was improved due to minimizing microbial stress associated with the transfer from chicken carcasses to conventional RBM, with 2.5% (*W/V*) of chicken extract giving the best recovery rate and diversity. The exact mechanism how our modified medium improves the growth of spoilage bacteria is unclear; however, it is reasonably inferential that some difficult-cultivable spoilage fungus can only utilize nutrients presenting in chicken carcass, thus they are able to grow on meats during storage but not on conventional medium by standard procedure.

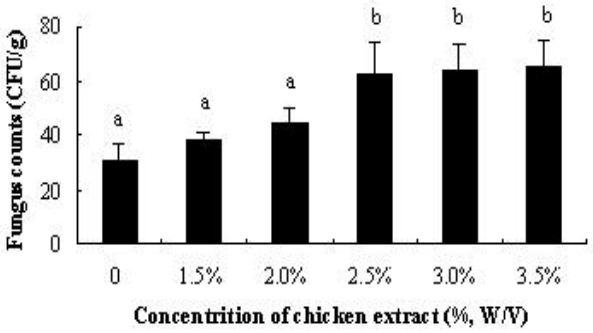

Fig. 1. Average microbial counts at medium containing different concentration of chicken extract dilution. a-b: differ ($P < 0.05$). Each point represents the mean value from three replicates. Vertical bars denote standard deviation.

3. 2. Isolation of chicken carcass microbes

A total of 87 colonies was isolated from CEA medium. They were transferred to fresh RBM and CEA plates respectively to detect whether there were difficult cultivable fungi and only recovered by our modified CEA medium. Finally 6 colonies easily growing in CEA but not on RBM, named as CMA24, CMA25, CMB25, CMB29, CMB30, CMC22 were obtained.

CMA24, CMA25, CMB25, CMB29 and CMC22 were

similar in their colony morphological features (CMB30 and CMA25 shown in Fig. 2). The colonies obtained by CEA could also be recovered at low amount on RBM with extent incubation time. CEA was formulated to mimic the chicken environment to improve yeast recovery by minimizing microbial stress associated with the transfer from meat to conventional agar-based media, thus leading to a high recovery of fungus from the chilled chicken.

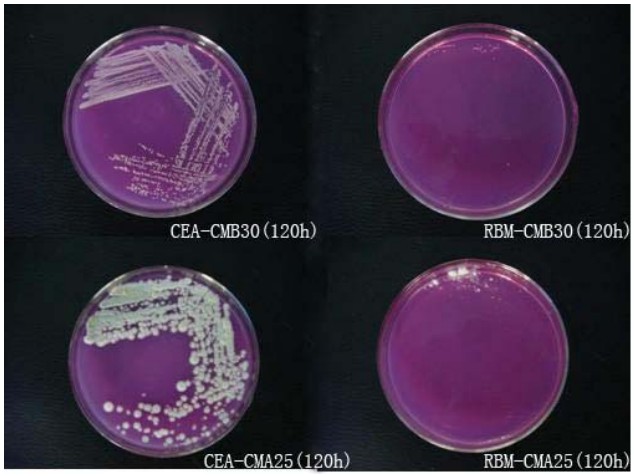

Fig. 2. Colonies of CMB30 and CMA25 growing on CEA medium

It was clear that besides significantly increasing the colony form unit, the CEA medium developed in our study also could detect the fungi that hardly grow on conventional RBM. Accordingly, the modified CEA medium containing 2.5% of chicken extract was chosen as the optimal near-native medium for the detection of bacteria on chicken carcass during storage.

3. 3. Identification of strains by molecular analysis

After DNA sequences alignment in GenBank, it was disclosed that the CMAB30 had a high degree of similarity (100%) to *Debaryomyces hansenii*, and the other 5 strains similar to *Aspergillus parasiticus* (99%). The ITS sequences of the 6 bacteria were further aligned with their corresponding sequences. A phylogenetic tree based on all known representatives of validly described related species was constructed. Genera *Aspergillus* and *Debaryomyces* were included as reference strains, and similar results were obtained by the phylogenetic tree (data not shown).

Table 1 ITS and 26S D1/D2 sequences analysis of 6 difficult cultivable isolates

Colony	Accession No	Closest relatives	Similarity (%)
A24 A25 B25 B29 C22	DQ026005	*Aspergillus parasiticus*	99
B30	EU149790	*Debaryomyces hansenii*	100

Aflatoxins are highly toxic, mutagenic and carcinogenic secondary metabolites predominantly produced by *A. flavus* and *A. parasiticus*. These two species infect several agricultural products like cereals, corn, oily seeds, tree nuts, drained fruits and spices. Thus the contamination of food and feed by aflatoxin producing fungi is a serious problem, not only due to the economic losses resulting from significant yield reduction and low quality of food, but mainly due to the serious worldwide health hazard to both human and livestock [12][13].

Yeasts are best known for their positive contributions in the fermentation of bread, alcoholic beverages, cheeses and other products. However, yeasts can also cause spoilage in a wide range of foods, particularly in processed, preserved, and refrigerated food [14][15]. *D. hansenii*, is one of the most prevalent yeast species isolated from dairy products. It is able to grow in the presence of salt at low temperature and to metabolize lactic and citric acids. *D. hansenii* is particularly strong users of lactic acid, especially at the surface of the curd, causing an increase in pH which enables the growth of less acid tolerant species, such as micrococci and coryneform bacteria [16].

IV. Conclusion

Compared with the conventional culture methods and culture independent approaches, the improved near-native medium developed in current study may at times give better representation of the different fungus species, especially those difficult cultivable spoilage microorganisms, present in meats. Further studies are needed to examine the eukaryotes existing in meat and meat products by this strategy.

Acknowledgement

Financial support for this study was provided by the Chinese Ministry of Science and Technology (2007BAD70B00).

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