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Original research article

Isolation and identification of sinapine-degrading bacteria from the intestinal tract of laying hens

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ABSTRACT

This study was aimed to isolate sinapine-degrading bacteria from the intestinal tract of laying hens and to identify the predominant bacteria. Thirty-week old healthy laying hens were killed, and the chyme in the digestive tract was inoculated into modified Czapek medium containing sinapin and cultivated at 37 °C for 10 days. The optical density (OD) values of the bacterial solutions at different cultivating times were detected by a spectrophotometric method. The predominant strains were identified by 16S rRNA gene analysis. We extracted the extracellular products of the predominant strains to determine the total protein using the Coomassie brilliant blue method, and to determine the activities of some extracellular enzymes using the agar plate diffusion method. Nine strains were isolated from the lower intestinal tract of laying hens. Among the 9 strains, 5 were from the ileum, 2 were from the ceca and 2 were from the jejunum. We could not isolate any strains from the upper intestinal tract, such as the stomach and duodenum. Eight of those 9 isolated strains were gram negative and one was gram positive. Strains YD-1 and YD-2 were better than other strains in their abilities to degrade sinapine. Strains YD-1 and YD-2 were identified as *Escherichia coli* and *Klebsiella* spp., respectively, by the 16S rRNA sequence analysis. The total protein level of the extracellular products was 1.213 g/L for YD-1 and 1.990 g/L for YD-2. Both extracellular products of YD-1 and YD-2 had the activities of protease, amylase and urease. This study confirmed that the primary site of sinapine degradation is in the lower intestinal tract of laying hens. The sinapine-degrading strains are mainly gram negative. Strains YD-1 and YD-2 are predominant in degrading sinapine and they belong to *E. coli* and *Klebsiella* spp., respectively. Both extracellular products of YD-1 and YD-2 contain protease, amylase and urease. Strain YD-2 is better than strain YD-1 in its ability to degrade sinapine.

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1. Introduction

Sinapine is one of the main anti-nutritional factors in the feed resources of Brassicaceae such as rapeseed meal. Sinapine tastes bad and can reduce the digestibility of protein. The most adverse effect of sinapine is to induce fishy taint in some animals (Niu et al., 2014), thus making meat, eggs, milk less acceptable to consumers (Wang et al., 2011). Fishy taint will seriously affect the quality of livestock products (Bao et al., 2013). The fishy factor is an irritant and can make eyes, nose, pharynx and respiratory tract discomfort. Therefore, understanding the metabolism mechanism by which sinapine induces fishy taint will help the use of Brassicaceae species as a feed resource in a variety of different feed formulations. Sinapine is one of the most important simple polyphenols and accounts for 70% to 85% of all phenols in rapeseed meals (Naczk and Shahidi, 1989). Despite a number of studies exploring the use of fungi or bacteria to degrade sinapine, there still lacks definite answers as to how it can be effectively de-activated. Thus, we isolated

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and characterized a number of sinapine degrading bacteria in the intestinal tract of laying hens and elucidated their ability to degrade sinapine in this study.

2. Materials and methods

2.1. Experimental materials

2.1.1. Experimental animals

Thirty-week old healthy laying hens were bought from Sichuan Dahen Poultry Breeding Co., LTD. The experimental protocol involved in the present study was approved by the Animal Care and Use Committee of Sichuan Animal Science Academy.

2.1.2. Reagents and instruments

Reagents mainly included sinapine thiocyanate standard substance (bought from Chengdu Inspection Institute of Food and Drug, Chengdu), sinapine thiocyanate extract (made in our laboratory), $NaNO_3$, $FeSO_4$, yeast extract, tryptone, NaCl, gelatin, skim milk, Tween 80, soluble starch, urea, phenol red, ammonium sulfate, Tris, etc. The genomic DNA extraction kit was bought from Qiangen company, Germany; HS Taq DNA Polymerase was bought from Takara Biotechnology (Dalian) CO., LTD; primer was synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Instruments mainly included electron balance, autoclave, water bath oscillator, clean bench, constant temperature oven, high speed refrigerated centrifuge, ultraviolet spectrophotometer.

2.2. Testing method

2.2.1. Isolation and culture of sinapine-degrading bacteria

1) Culture medium

Improved Czapek medium: $NaNO_3$, $FeSO_4$, KCl, sinapine thiocyanate were diluted with double-distilled water to 1,000 mL. Improved Czapek agar plate medium: Agar (2% to 3%) was added into the improved Czapek medium. The medium was autoclaved at 121°C for 30 min.

2) Inoculation and culture

Thirty-week old healthy Dahen laying hens that had been fed 8% rapeseed meal ration for 30 days and at peak production were killed by cervical dislocation. The chyme in the gizzard, glandular stomach, duodenum, jejunum, ileum and cecum was gathered and cultured for 10 days in the improved Czapek medium at 37 °C, then isolated bacteria were cultured in the same medium. The bacteria that grew the best were defined as the predominant bacteria. The common physical and chemical characteristics of the predominant bacteria were analyzed by referring to the method detailed in *Bergey's Manual of Determinative Bacteriology* and *Common bacteria identification manual.*

2.2.2. Study on the sinapine-degrading efficiency of the predominant bacteria

Bacterial solutions on the 1st, 2nd, 4th, 6th, 8th and 10th enrichment-culture-day were collected and filtered through microfiltration membrane (0.45 μm) (He, 2010). The optical density (OD) of sinapine in the filtrate was measured by spectrophotometry at 326 nm.

2.2.3. Gene sequencing and phylogenetic analysis of the predominant bacteria by 16S rRNA

The DNA of the predominant bacteria was extracted.

Upstream primer: 5′-AGAGTTTGATCCTGGCTCAG-3′, and downstream primers: 5′-TACGGCTACCTTGTTACGAC-3’. As template, the extractive DNA was amplified the 16S rRNA gene sequence of the isolated bacteria. The PCR reaction systems: Add 12.5 μL 2 × PCR Premix, 1 μL upstream primer (10 μmol/μL), 1 μL downstream primer (10 μmol/μL), and 1.0 μL template into a 0.2-mL reaction tube, then added 25 μL double-distilled water. The PCR reaction parameters were 94 °C 5 min, 94 °C 1 min, 55 °C 1 min, 72 °C 1.5 min, 30 cycles, extending 10 min at 72 °C. The PCR products were sequenced. The similarity of the sequences was analyzed by the Blast of National Center of Biotechnology Information (NCBI). The multi alignments of the sequences were analyzed by the program of Multiple Sequence Alignment of DNA star software. A phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis 5.0 (MEGA 5.0).

2.2.4. Study on the extracellular products of YD-1 and YD-2

Preparation of extracellular products: We inoculated two 1-L Lysogeny Broth (LB) broth media with two 10-mL bacterial suspensions of YD-1 and YD-2, respectively, shook 120 g culture for 24 h at 28 °C, and then centrifuged the bacteria solution 12,000 × *g* at 4 °C for 30 min. The supernate was filtered by vacuum, and then filtered again by 0.22 μm millipore. The filtered sediment was re-suspended and dialyzed in a buffer solution of 0.02 mol/L Tris-HCl (pH 7.5), then concentrated by polyethylene glycol 20,000. The concentrate was purified by 0.22 μm millipore filters to gain pure extracellular products.

Determination of the total protein of the extracellular products: the total proteins of the extracellular products in YD-1 and YD-2 were determined by Coomassie Brilliant Blue. The application solution of Coomassie Brilliant Blue was comprised of Coomassie Brilliant Blue stock solution and distilled water at the ratio of 1:4.3. The test tubes were labeled as blank tube, standard tube and testing tube. They were added 50 μL distilled water, 50 μL protein markers (0.563 g/L) and 50 μL extracellular products, respectively. Then 3 mL application solution of Coomassie Brilliant Blue was added in each tube, mixed and stood for 10 min at room temperature. The OD value of the reaction solutions was determined by the spectrophotometer at 595 nm. The total protein was calculated by the following formula.

$$\begin{aligned}\text{Total protein}(\text{g/L}) = (&\text{OD value of testing tube}\\ &- \text{OD value of blank tube})/\\ &\times (\text{OD value of standard tube}\\ &- \text{OD value of blank tube})\\ &\times \text{Concentration of standard tube}.\end{aligned}$$

We analyzed the activity of some enzymes in the extracellular products. The activity of the extracellular enzymes was analyzed by the agar diffusion method (Li et al., 1999). We drilled 2 holes in each of the 4 agar mediums containing 1% Tween 80, 8% skim milk, 2% urea and 4% soluble starch, respectively. Then, we injected 50 μL extracellular products of YD-1 or YD-2 into one of the two holes for each medium respectively, cultivated 24 h at 28 °C and judged whether they had the activities of protease, lipase, amylase and urease in extracellular products of YD-1 and YD-2 by the color reaction of the medium.

3. Results

3.1. Isolation and culture of sinapine-degrading bacteria

We successfully isolated 9 strains from laying hens' intestinal tracts and successfully cultivated them by an improved sinapine medium in this study (Table 1). The 9 strains were from the ileum,

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