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Pei Ven Kam

*University of Nebraska - Lincoln*, peiven@bigred.unl.edu

Andreia Bianchini

*University of Nebraska - Lincoln*, abianchini2@unl.edu

Lloyd B. Bullerman

*University of Nebraska-Lincoln*, lbullerman1@unl.edu

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## Inhibition of Mold Growth by Sourdough Bread Cultures

Pei Ven Kam\*

Andreia Bianchini<sup>†</sup>

Lloyd B. Bullerman<sup>‡</sup>

\*University of Nebraska - Lincoln, peiven@bigred.unl.edu

<sup>†</sup>University of Nebraska - Lincoln, abianchini2@unl.edu

<sup>‡</sup>University of Nebraska - Lincoln, lbullerman1@unl.edu

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# Inhibition of Mold Growth by Sourdough Bread Cultures\*

Pei Ven Kam, Andreia Bianchini, and Lloyd B. Bullerman

## Abstract

Sourdough bread cultures are mixtures of wild yeasts and *Lactobacillus* bacteria living in flour and water, where they form an interesting symbiosis that makes the culture quite stable. The presence of lactic acid bacteria (LAB) in sourdough bread cultures increases the shelf life of the sourdough bread and other sweet baked goods made with these cultures, due to the inhibitory effect of organic acids on spoilage molds. In addition, it has been found that when sourdough LAB are cultivated they produce antifungal substances, such as organic acids (in particular, lactic acid and acetic acid), carbon dioxide, ethanol, and hydrogen peroxide, and other, as yet unidentified inhibitory substances, that prevent mold growth. The inhibitory effect of four American sourdough cultures were tested for antifungal activity against the common spoilage molds, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium expansum*, *Penicillium roqueforti* and *Cladosporium cladosporioides*. In these experiments, actively growing sourdough cultures were inoculated into modified deMan Rogosa and Sharpe (mMRS) broth and incubated. After incubation, the cultures were centrifuged and filtered through 0.2 $\mu$ m membrane filters, the supernatants were collected and mixed with Potato Dextrose Agar (PDA). This mixture was used as culture medium for the growth of the spoilage molds. The growth rates of molds growing in the presence of the sourdough culture supernatants were compared with controls, where the molds were cultivated only in PDA. The results showed that *Aspergillus flavus* and *Cladosporium cladosporioides* were inhibited the most by the sourdough cultures. Therefore, the use of sourdough cultures shows promise for preserving food products from spoilage, as they could be a source of natural antimicrobial and antimycotic agents for use in the food industry.

**KEYWORDS:** sourdough bread cultures, lactic acid bacteria, antifungal activity

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## 1. Introduction

Sourdough breads are known for long mold-free shelf life, unique flavor and texture (4, 8, 11). This type of bread is produced by the use of sourdough starter cultures that are composed mainly of yeasts and lactic acid bacteria (28, 29). Sourdough starters may prolong mold-free storage of baked goods because of antifungal substances that they produce, such as lactic acid, acetic acid, carbon dioxide, ethanol, hydrogen peroxide and as yet unidentified substances (6), which reduces the need for artificial additives and meets the consumer demands for natural, additive-free food products (24). The obligatory heterofermentative *Lactobacillus* spp. present in sourdough, which includes *Lactobacillus sanfranciscensis* CB1 (26), produce a mixture of organic acids that may play an important role in this inhibitory activity (5, 10, 16). According to Corsetti *et al.*, another organic acid that is important for mold inhibition is caproic acid (5). Moreover, Lavermicocca *et al.* working with *Lactobacillus plantarum* 21B, isolated from sourdough and identified phenyllactic and 4-hydroxyphenyllactic acids as active compounds with antifungal activity produced by this strain (12).

Mold spoilage of bakery products is a serious economic concern. Losses due to mold spoilage vary between 1 and 5% of products depending on season, type of product and method of processing (14). In addition to the economic losses another concern is the possibility that mycotoxins may be produced.

Since fungal spores are killed during baking, airborne molds recontaminate the baked goods during cooling, slicing, wrapping, and storage operations (13). The most common spoilage fungi isolated from bakery goods belong to the genera *Penicillium*, *Aspergillus*, *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium*, and *Rhizopus* (13, 18). Even under stringent conditions of production, baked products can be contaminated with molds, unless they receive some protective treatment, such as infrared, ultraviolet and microwave radiation post baking, use of fungal inhibitors (propionic, sorbic, benzoic acids) or the use of sourdough starter cultures (13, 23).

### 1.1 Objective

The objective of this research was to evaluate four different American sourdoughs for antifungal activity against a series of molds.

## 2. Materials and Methods

### 2.1 Sourdough Activation and Supernatant Preparation

The sourdoughs tested were identified as KOSD, KSDTCA, DPSD, and SFSDS. The origin of sourdoughs KOSD, DPSD, and SFSDS is unknown as they were obtained from different individuals that kept and propagated them in homes. The sourdough KSDTCA was the same as KOSD, however, it was propagated for at least 3 years in a different environment, and was included to evaluate if this change in environment affected its antifungal properties. The SFSDS was originally a commercial San Francisco type sourdough, but the origin is unknown and the culture tested in this study was obtained from an individual home environment. In the lab, the sourdoughs were kept in glass pint jars under refrigeration and were activated by transferring half of the original amount to new jars followed by addition of autoclaved flour and water in the ratio of 1:1.25. The mixture was incubated at 30°C for 24 hours. The next day, sourdough cultures were diluted using peptone water (0.1 %) in the ratio of 1:10 and 1 ml of each diluted sourdough was inoculated into 100 ml of modified deMan, Rogosa and Sharpe broth (mMRS) and incubated at 30°C for 24 hours (26). A control jar was also prepared. After incubation, each cultured broth was centrifuged for 10 minutes at 10,000 RPM to remove most of the bacteria and yeast cells. Then, the supernatant liquid was filtered through a 0.2 µm surfactant-free cellulose acetate membrane filter (NALGENE<sup>®</sup>, Rochester, New York) to remove any cells that were still present.

### 2.2 Mold Growth and Spore Suspension Preparation

The molds used as test organisms in the challenge studies were *Aspergillus flavus* NRRL 1290, *Aspergillus niger* WDC 31, *Penicillium expansum* NRRL 2304, *Penicillium roqueforti* NRRL 849, and *Cladosporium cladosporioides* NRRL 6078. For the experiment, all molds were transferred from pure stock cultures to new Potato Dextrose Agar (PDA) slants and incubated at room temperature (c.a. 25°C) for 7 days. After incubation, the spores were harvested by washing the slants and suspending in 10 mL Tween 80 (0.025 %) and counting the spores using a hemacytometer. The concentration of spores in the suspensions were determined and adjusted by dilution to achieve a final concentration of  $1 \times 10^5$  spores/mL.

### 2.3 Challenge Studies

Sourdoughs were tested for ability to inhibit all molds, and all combinations (mold x sourdough) were done in duplicate. The whole experiment was repeated in triplicate. Each *Petri* dish (plate) was prepared with 20 ml of a mixture of sourdough supernatant and double strength PDA in the ratio 1:1. Double strength PDA was prepared by using half the amount of water usually required, in order to achieve the desired agar concentration in the plates after combination with the sourdough supernatant. Control plates were prepared with uncultured mMRS broth combined with double strength PDA.

In this part of the experiment, each plate was inoculated with 10  $\mu\text{L}$  of the spore suspension prepared ( $1 \times 10^5$  spores/mL). The spore inocula were placed as a drop (10  $\mu\text{L}$ ,  $1 \times 10^3$  spores/mL) in the center of the agar surface in each plate. Then, all plates were incubated at room temperature (c.a. 25°C) for 7 days. The growth of the molds was determined by the size of each mold colony, determined daily, by measuring colony diameters (cm).

### 2.4 Statistical Analysis

The results of the experiment were analyzed statistically using The SAS<sup>®</sup> System 8.02. A factorial design with two factors (mold and sourdough) was used to analyze the results, and each factor had five levels. For the factor mold, each level was represented by one species of mold; while for the sourdough factor, each level was represented by one sourdough supernatant or the control.

## 3. Results and Discussion

Using the averages of daily measurements, growth curves for each mold in the presence of sourdough were obtained and compared to the controls (Figures 1-5). The growth curves of *A. flavus* and *C. cladosporioides* in the presence and absence (control) of the sourdough filtered supernatants are given in Figures 1 and 2, respectively. For both molds there was a statistically significant difference between their growth when the control was compared to the treatments. However, there was no statistical difference among the inhibitory activity of the sourdough bread cultures against *A. flavus* and *C. cladosporioides*.

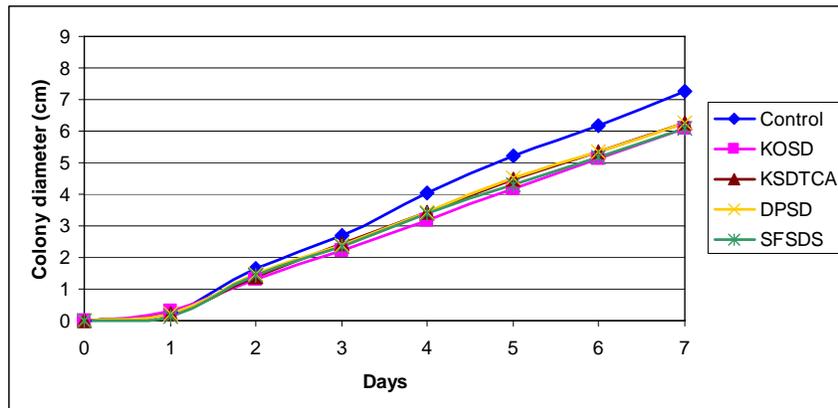


Figure 1: Growth curve for *Aspergillus flavus* in the presence and absence (control) of different sourdough supernatants during 7 days.

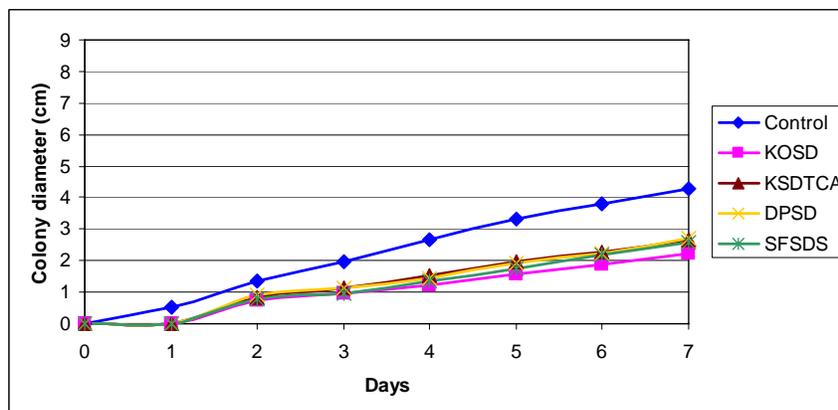


Figure 2: Growth curve for *Cladosporium cladosporioides* in the presence and absence (control) of different sourdough supernatants during 7 days.

The growth curves for *A. niger*, *P. expansum*, and *P. roqueforti* showed no significant differences between the controls and the treatments (Figure 3, 4, and 5). Among these three molds, *A. niger* seemed to be the least inhibited, while there were slight, but not significant, differences between the controls and sourdoughs for *P. expansum* and *P. roqueforti*.

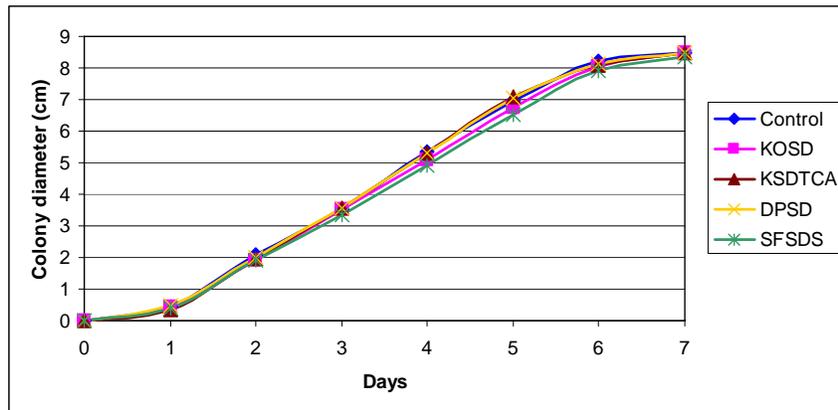


Figure 3: Growth curve for *Aspergillus niger* in the presence and absence (control) of different sourdough supernatants during 7 days.

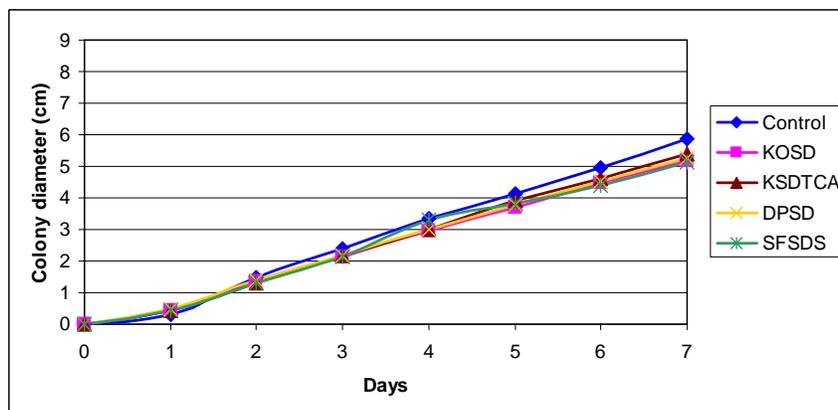


Figure 4: Growth curve for *Penicillium expansum* in the presence and absence (control) of different sourdough supernatants during 7 days.

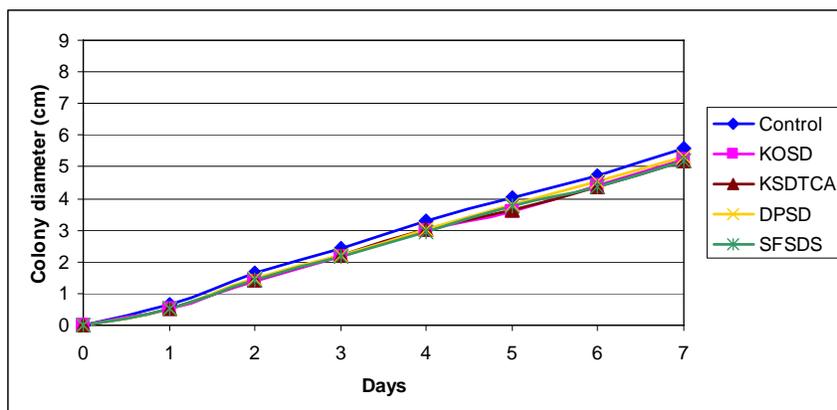


Figure 5: Growth curve for *Penicillium roqueforti* in the presence and absence (control) of different sourdough supernatants during 7 days.

Sourdough bread cultures consist of a mixture of mainly facultatively and obligately heterofermentative LAB and yeasts. LAB are predominantly responsible for dough acidification while yeasts are responsible for dough leavening through CO<sub>2</sub> production (11). The yeasts most frequently found in sourdoughs are *Saccharomyces cerevisiae*, *Saccharomyces exiguus* and *Candida holmii* (17). There are mainly 3 types of sourdoughs: type 1 sourdoughs are known as the traditional doughs sustained by continuous propagation at ambient temperature with a three-stage fermentation process (11). The LAB commonly found in type 1 sourdoughs are *Lactobacillus sanfranciscensis*, *Lb. pontis* (28), *Lb. fructivorans*, *Lb. fermentum* and *Lb. brevis* (15). Type 2 sourdoughs are mostly used in industrial processes with a one-stage fermentation process and the dominant strains in these doughs are *Lb. panis*, *Lb. pontis*, *Lb. reuteri* (11, 28), *Lb. johnsonii*, *Lb. sanfranciscensis* (11), *Lb. fermentum*, *Lb. delbrueckii*, *Lb. acidophilus*, *Lactococcus lactis*, *Lb. brevis* and *Lb. amylovorus* (28). Lastly, type 3 sourdoughs are dried preparations where water is evaporated from the culture by freeze-drying, roller drying, spray drying or drying in a fluidized bed reactor and the dough is then traditionally fermented after the culture is rehydrated (5, 11). The yeasts and LAB have a symbiotic relationship, where for example, maltose, one of the carbohydrates found in flour, is unused by the yeasts, but readily used by the bacteria, which by their growth produce lactic and acetic acid that lower the pH of the dough preventing it from becoming contaminated by growth of foreign microorganisms (25, 29).

The antifungal activity of sourdough LAB varies and it is mainly associated with obligatory heterofermentative *Lactobacillus* spp. Many researchers have reported antifungal properties of LAB, due to their production of acids and antimicrobial substances. These fermented products have also been reported to have therapeutic and preservative properties (9). Some LAB have been shown to affect mold growth and mycotoxin production. El Gendy and Marth investigated interaction between *A. parasiticus* and *Lb. casei*, and found that when the fungus was added to a 3-day culture of *Lb. casei* the growth of the mold was reduced (7). The authors suggested that the reduction was caused by some nutritional changes in the medium after the growth of the bacteria. Later, Batish *et al.* screened 19 lactic acid bacteria strains for their antifungal activity against *A. parasiticus*, *A. fumigatus*, *Rhizopus stolonifer*, and *Rhizopus* sp. They found *Lactococcus lactis* subsp. *diacetylactis* DRC1 and *Streptococcus thermophilus* 489 to be the most inhibitory strains to all fungal cultures (1). Subsequent work and findings by Batish *et al.* and other researchers have shown that several factors influence the effectiveness of the antifungal substances produced by LAB, or may cause inactivation of these compounds. Factors such as incubation temperature, pH, extended incubation time and medium ingredients have impacts on the activity of the antifungal substances (2, 3, 19, 20, 21, 22).

Within the heterofermentative group of LAB, *Lb. sanfranciscensis* CB1 (27), displays a high degree of antifungal activity due to its production of a mixture of organic acids including acetic, caproic, formic, butyric and *n*-valeric acids, with caproic acid a key factor. These acids were reported to have synergistic inhibitory effects on species of *Fusarium*, *Penicillium*, *Aspergillus* and *Monilia* (5). Lavermicocca *et al.* (12) working with *Lb. plantarum* 21B showed that the culture filtrate was active against bacteria originally isolated from beer, *Pantoea agglomerans* (known previously as *Enterobacter agglomerans* and *Herwinia herbicola*) and bakery spoilage fungi (12, 16). The compounds present in the active fraction of bacterial culture filtrate were identified as phenyllactic acid, 4-hydroxy derivative (*p*-hydroxyphenyllactic acid) and palmitic acid by gas chromatography/mass spectrometry (12). Yet, there was no synergistic effect in the acid mixture and phenyllactic acid played a key role in inhibiting fungal growth (12). While working with another strain of *L. plantarum*, Niku *et al.* identified benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one, and 3-(2-methylpropyl)-2,5-piperazinedione in the culture filtrate and associated these compounds with the inhibitory effect on *Pantoea agglomerans* and *Fusarium avenaceum* (16).

From the available literature on the effect of LAB on mold growth and mycotoxin production, it appears that LAB have the potential to be used as biological control agents in foods to prevent mold growth in general, and have specific antifungal activity against fungi isolated from bakery products. Moreover, the inhibition of spoilage and mycotoxigenic fungi by fermenting LAB could improve the shelf life of fermented products and reduce the risk of exposure to mycotoxins (9).

Currently, most of the research reported describes the inhibitory activity of specific LAB isolated from sourdough. However, more research could be done to determine if the use of whole sourdough cultures or their filtrates, as antifungal agents, are more effective than the use of isolated bacterial strains. The whole sourdough culture may work as a complex community, producing stronger inhibitory activity against molds than when isolates are used separately. Furthermore, in the future, natural inhibitory compounds from sourdough could be isolated or extracted and added to a variety of foods to prolong shelf life.

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## Author Biographies

**Pei Ven Kam**, B.S. Food Science and Technology at the University of Nebraska-Lincoln, is originally from Petaling Jaya, Malaysia. She is currently pursuing her Master of Science degree in Food Science and Technology at UNL under supervision of Dr. Lloyd B. Bullerman.

**Andreia Bianchini**, M.S. Environmental and Agricultural Microbiology from the Federal University of Rio Grande do Sul, Brazil, is the Laboratory Supervisor of the Mycology and Mycotoxins Laboratory in the Food Science and Technology Department at the University of Nebraska – Lincoln. She is also pursuing her Ph.D. in Food Science and Technology at UNL under supervision of Dr. Lloyd B. Bullerman. Her main area of research is food safety, including the biological control of mold growth by lactic acid bacteria and their metabolites and the detoxification of mycotoxin-contaminated grains by extrusion and other physical/thermal processes.

**Lloyd B. Bullerman**, B.S., M.S., Ph.D, is Professor of Food Science & Technology at the University of Nebraska-Lincoln. His research interests include food safety, food microbiology, food toxicology, food mycology, mycotoxins and the effects of processing on survival of molds and stability of mycotoxins in foods. Of current interest are Fusarium toxins, particularly fumonisins, deoxynivalenol, zearalenone and moniliformin in cereal grains and cereal based food products, and the effects of food processing on the stability of these toxins, in foods. Also of current interest are possible applications of antifungal and antimycotoxigenic effects of lactic acid bacteria and other microorganisms.