Growth and Survival of *Flavobacterium aurantiacum* in Peanut Milk

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**ABSTRACT**

Tryptone-yeast extract-glucose (TYG) and trypticase soy broth (TSB) were evaluated for production and recovery of *Flavobacterium aurantiacum* stationary phase cells. In addition, growth of *F. aurantiacum* in peanut milk was tested. Trypticase soy broth was chosen as the best medium for producing stationary phase cells. Both non-defatted peanut milk (NDPM) and partially defatted peanut milk (PDPD) supported growth of *F. aurantiacum*. The growth of *F. aurantiacum* in both kinds of peanut milk was not inhibited by aflatoxin B$_1$ (1 mg/ml). About 10$^6$ stationary phase cells were inoculated in 0.067 M phosphate buffer (PB) at pH 5.0, 5.5, 6.0, 6.5, and 7.0, and in both peanut milks (pH 6.3 and 6.9). After a 24-h incubation period, the viable cell number decreased slightly in PB (pH 7.0, 30°C), but decreased 2-3 logs in other buffers. About 0.6-0.8 log decrease was observed in NDPM and PDPM. Phosphate buffer (0.067 M, pH 7.0), NDPM and PDPM were determined to be adequate for use in studies to investigate the removal of aflatoxin B$_1$ by *F. aurantiacum*.

In some countries, cow’s milk is in limited supply or expensive. Therefore, efforts to develop processes for the manufacture of dairy substitutes have focused on peanuts and other oilseeds as sources of inexpensive raw materials. Development of a milk-like beverage, cheese and other products (7). Detoxification or removal of aflatoxin has also been investigated. Some molds have been reported to detoxify aflatoxin was discovered by Ciegler and his colleagues (6), when they screened approximately 1000 microorganisms for their ability to either destroy or transform AFB$_1$, and aflatoxin G$_1$ (AFG$_1$). The irreversible uptake of AFB$_1$, AFG$_1$, and aflatoxin M$_1$ by growing and resting cells of this Gram-negative bacterium was reported (6,12,13,14). Stationary cells of *F. aurantiacum* completely detoxified toxin-contaminated milk, oil, peanut butter, peanuts, and corn, and it partially detoxified contaminated soybeans. *F. aurantiacum* cells became deformed after they were inoculated in media containing AFB$_1$, or AFG$_1$. It was suggested that the formation of deformed cells was due to the inhibition of cell wall synthesis by aflatoxin (12,13). The removal of aflatoxin by *F. aurantiacum* was reported to occur without new toxic products being formed.

The objective of this study was two-fold. The first objective was to identify a medium suitable for the harvest of stationary cells of *F. aurantiacum*. Secondly, we wished to determine growth characteristics of *F. aurantiacum* in broth and peanut milk. Some results presented in this paper were ultimately used to demonstrate removal of aflatoxin B$_1$ from peanut milk (11).

**MATERIALS AND METHODS**

**Culture**

*Flavobacterium aurantiacum* NRRL B-184 was used and the stock culture was maintained at 5°C on trypticase soy agar (TSA, Difco). Cells were activated by 2 successive transfers in trypticase soy broth (TSB, Difco) and incubated at 30°C for 48 h with no agitation.

**Preparation of non-defatted peanut milk (NDPM)**

Peanuts (cv. Florunner) were used throughout this study. The skins of peanut kernels were loosened by heating in hot water (79°C) for 90 s in a Groen steam kettle (Groen Mfg. Co., Chicago, Ill.). This was followed by hand removal of the seed testa from the samples (3). The peanuts were then dried for 24 h at 25°C and proc-
Preparation of partially defatted peanut milk (PDPM)

Peanuts were crushed in a Carver Laboratory Press (Model C, Fred S. Carver Inc., WI) using 9 to 11 metric tons of applied load for 45 min. This procedure removed approximately 50% of the oil from the peanut kernels. The skins of partially defatted peanut kernels were then removed by a peanut sheller. PDPM was then prepared by the same process used to make NDPM and was also stored at -20°C.

Growth of F. aurantiacum in tryptone-yeast extract-glucose broth

Modified TYG (referred to as TYG) contained the following (g/L): tryptone (Difco), 2.5; yeast extract (Difco), 2.5; glucose, 10.0; and KH₂PO₄, 1.0 (6). TYG was adjusted to pH 5.9 and 6.5 using 0.1 N NaOH or 0.1 N HCl.

The other medium used in this test was trypticase soy broth (TSB). Two-tenths ml of activated F. aurantiacum culture (about 10⁷ cells) was transferred into a 250 ml Erlenmeyer flask containing 100 ml of culture medium (TSB or TYG). Duplicate flasks were used for each medium. The inoculated flasks were incubated at 30°C in a gyratory shaker-incubator (New Brunswick Scientific, NJ) and continuously agitated at 200 rpm for 72 h. Populations of F. aurantiacum were determined periodically during 72-h incubation.

Growth of F. aurantiacum in non-defatted peanut milk and partially defatted peanut milk

One hundred-ml of NDPM and PDPM were placed into duplicate 250 ml Erlenmeyer flasks, and autoclaved at 121°C for 10 min. Flasks were inoculated with 0.2 ml of activated F. aurantiacum and incubated at 30°C with agitation (200 r.p.m.). Populations of the bacterium in NDPM and PDPM were determined periodically during a 72-h incubation period.

Effect of aflatoxin B₁ (AFB₁) on growth of F. aurantiacum in peanut milks

Aflatoxin B₁ (Sigma, St. Louis, MO) was prepared as a stock chloroform solution (5 mg/ml) and stored at -20°C. Aflatoxin B₁-contaminated peanut milk was prepared by adding 20 µl of AFB₁-chloroform stock solution to peanut milk (100 ml) to yield a final AFB₁ concentration 1 µg/ml. Peanut milks (100 ml) were then placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C for 10 min to sterilize the milks and remove the chloroform. We previously demonstrated that aflatoxin B₁ concentrations did not differ significantly before and after autoclaving (11). Peanut milks were then inoculated with about 10⁷ F. aurantiacum cells (0.2 ml inoculum) and were incubated at 30°C and continuously agitated at 200 r.p.m. The populations of F. aurantiacum were determined periodically during a 72-h incubation.

Harvest of stationary phase cells of F. aurantiacum

Approximately 10⁷ cells of F. aurantiacum were inoculated into duplicate 250 ml Erlenmeyer flasks containing 50 ml of TSB and incubated as previously described for TSB and TYG. After 48-h incubation, TSB containing F. aurantiacum was centrifuged at 5,000 x g for 15 min and the supernatant fluid was discarded. The pellet was washed with potassium phosphate buffer, (0.067 M; pH 6.7) and recentrifuged. Phosphate buffer (0.5 ml) was added to the final pellet and the resulting suspension was used in survival experiments.

Survival of F. aurantiacum in phosphate buffer (PB), non-defatted peanut milk and partially-defatted peanut milk

Phosphate buffer (0.067 M) was prepared to achieve the following pH values: 5.0, 5.5, 6.0, 6.5, and 7.0. A portion of pH 7.0 phosphate buffer was supplemented with 0.5% glucose. Each of these buffer solutions as well as NDPM and PDPM were used in survival experiments. Survival experiments were done by inoculating 0.2 ml of cell suspension into duplicate 250 ml Erlenmeyer flasks containing 50 ml of test medium, incubated at 30°C for 24 h with agitation (200 r.p.m.) and viable cells were determined after 1, 3, 6, 12, and 24 h.

Determination of microbial population

Populations of viable F. aurantiacum were determined by taking 1 ml samples of test solutions, making appropriate serial dilutions in potassium phosphate buffer (0.1M, pH 7.2), and pour plating 1.0 ml of each dilution on duplicate TSA plates. Colonies were counted after 72 h incubation at 30°C; populations were expressed as colony-forming units per milliliter (CFU/ml).

RESULTS

Growth of F. aurantiacum in tryptone-yeast extract-glucose broth

One of the initial objectives of this study was to identify a medium suitable for harvest of stationary phase cells. Such a medium should be easily prepared and support a good growth of F. aurantiacum. The tryptone-yeast extract-glucose broth (TYG medium) described by Haynes et al. (10) and later modified (pH 6.5) by Ciegler et al. (6) was described as being suitable for this purpose.

When growth of F. aurantiacum in TYG (pH 5.9 or 6.5) was compared to TSB (Fig. 1), no appreciable differences in the growth pattern during early stage of growth were noted. Viable cells in both TYG media decreased rapidly after 62 h, but not in TSB. The stationary phase of growth was reached after 36 h incubation in all media. During the incubation period, pH changed little in either TYG media, but increased about 1 unit in TSB. Trypticase soy broth was chosen as the best medium for producing cells because it resulted in the best growth and is a commercially-available product with no special preparation required.

Growth of F. aurantiacum in non-defatted peanut milk and partially defatted milk

Growth of F. aurantiacum in NDPM and PDPM differed slightly (Fig. 2). Growth of F. aurantiacum during the first 48 h was slower in NDPM as compared to PDPM. Thus, it took almost 12 h longer for the bacterium to reach the stationary phase when growing in NDPM than in PDPM. Changes in pH in NDPM and PDPM likewise differed. Partially defatted peanut milk maintained a similar pH throughout the experiment whereas the pH of NDPM initially increased slowly and then decreased abruptly after 48 h.
Effect of AFB₁ on growth of F. aurantiacum in peanut milks

Aflatoxin B₁ (1 μg/ml) did not affect the growth of F. aurantiacum in peanut milks (Fig. 3). This was not unexpected because this concentration was much less than the 10 mg/ml reported by Lillehoj et al. (12) to inhibit growth in TYG (pH 6.5).

Survival of F. aurantiacum in phosphate buffer, non-defatted peanut milk and partially defatted peanut milk

Survival of F. aurantiacum in PB at various pH values is illustrated in Fig. 4. The choice of buffer pH values tested was based on the study of Ciegler et al. (6), who reported that AFB₁ was degraded in solutions with pH > 7.5. Results from our survival test reveal that F. aurantiacum was most stable in buffer solution with pH 7.0 (with or without 0.5% glucose). After 24 h of incubation, the viable cell population decreased only slightly in PB at pH 7.0, with or without glucose 0.5%. In contrast, a 1-3 log₁₀ CFU/ml decrease occurred in buffer solutions at lower pH values. Flavobacterium aurantiacum populations decreased about 0.8 log₁₀ unit in NDPM, but increased about 0.6 log₁₀ in PDPM after 24 h (Fig. 5).
DISCUSSION

Both peanut milks supported growth of *F. aurantiacum*. Populations of viable stationary phase cells changed less than 1 log unit in both peanut milks during the survival study. Therefore, this microorganism was assumed to be stable in both these solutions. Moreover, *F. aurantiacum* was equally stable in pH 7.0 PB with or without glucose. Buffer (pH 7.0) without added glucose was deemed a suitable test solution for further studies for several reasons. First, the addition of an exogenous energy source does not affect aflatoxin removal (12). Secondly, addition of glucose did not improve the survival of *F. aurantiacum* in PB. Based on these results, NDPM, PDPM, and PB (pH 7.0) were selected for use in our studies demonstrating removal of aflatoxin from peanut milk (11). These data should also be helpful to others who might wish to investigate the ability of *F. aurantiacum* to remove aflatoxins from food.