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Original Article

Adaptation of *Lactobacillus rhamnosus* riboflavin assay to microtiter plates

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Abstract

Riboflavin (vitamin B2) is essential to humans and must be obtained through the diet. It plays a significant role in the metabolism of carbohydrates, fatty acids and amino acids. The test microorganism, most commonly used to quantify riboflavin is *Lactobacillus rhamnosus* ATCC 7469 since this bacterium requires external B2 for growth. The objective of the current study was to reduce the time of the assay and volumes of assay media by adaptation to microtiter plates while still maintaining the repeatability of the original tube assay. A previously developed riboflavin tube assay was used as a guideline for adapting the method to a microtiter plate assay. The standard growth curve for the riboflavin assay was linear from 0 to 20 ng/mL ($R^2 = 0.99$) and from 0 to 10 ng/mL ($R^2 = 0.97$) when conducted in microtiter plates and tubes, respectively. The data showed no significant difference between the tube assay and microtiter plate assay ($P > 0.05$) for the commercial maize sample. Commercial cereal and grain samples were analyzed to confirm repeatability among multiple independent trials performed with the microtiter plates. The microtiter assay reduced the amount of time required for sufficient bacterial growth response to generate linear standard curves from 16.5 to 10 h.
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Keywords: *Lactobacillus rhamnosus*; Riboflavin; Vitamin B2; Microtiter plate assay

1. Introduction

Riboflavin is present in food or food-derived products in three forms, namely, free riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The coenzyme forms of riboflavin, FMN and FAD, participate as electron careers in various one- and two-electron redox reactions (McCormick, 1989). Therefore, inadequate amounts of riboflavin may lead to an imbalanced intermediate metabolism. According to Powers (2003), poor riboflavin status interferes with iron assimilation and circulating serum concentrations of homocysteine in humans, thus contributing significantly to anemia development and cardiovascular disease, respectively. Riboflavin deficiency is also considered a potential risk factor for cancer, although this has not been demonstrated in humans (Powers, 2003). Some eubacteria and archea organisms are able to synthesize riboflavin (Fischer et al., 2005). However, for humans, riboflavin is essential and must be supplemented with their diets (McCormick, 1989). Although B2 is found in several foods (liver, eggs, milk and green vegetables such as broccoli and asparagus), deficiencies are common in many parts of the world (Boisvert et al., 1993; Bailey et al., 1997; Benton et al., 1997; Madigan et al., 1998). In addition, a significant amount of riboflavin can be lost due to moisture, oxygen, pH and light through food processing (Ottaway, 2002). To meet the daily requirements for B2, many foods are now being fortified with this essential vitamin which provides a more balanced diet for those people who do not receive sufficient vitamins through a regular diet (McNulty et al., 1996). Therefore,

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improvements are needed to reduce the processing time and costs involved in the riboflavin quantification of large numbers of samples by the previously described microbiological method (Angyal, 1996). The method involves the use of *Lactobacillus rhamnosus* as a test microorganism based on its riboflavin auxotrophy and subsequent requirement for externally supplemented riboflavin. *L. rhamnosus* is nonpathogenic and requires minimal manipulations for growth (Sneath et al., 1986). The riboflavin determination using *L. rhamnosus* is based on its requirement for riboflavin while growing on a basal medium that provides all of the growth requirements for the organism except riboflavin (Voigt and Eitenmiller, 1985). The main disadvantages of the method, however, are the relatively long growth period (18–24 h) and the need for qualified staff with microbiological and analytical knowledge to perform the assay.

More rapid assays have already been developed for folic acid (Horne, 1997; Chun et al., 2006), folic acid derivates (Horne and Patterson, 1988) and methionine by scaling down the tube assay to microtiter plates (Zabala-Díaz et al., 2003). In addition to the decreased assay time, microtiter plates are cost efficient as more trials can be conducted simultaneously with less expense of space and media compared to tubes (Voigt and Eitenmiller, 1985; Newman and Tsai, 1986). The goal of the current research was to decrease the total amount of time required for riboflavin quantification and to facilitate the analyses of multiple samples simultaneously while retaining the accuracy and precision of the tube assay. This paper describes a microtiter plate-based assay for measuring riboflavin content in food samples using *L. rhamnosus*.

2. Materials and methods

2.1. Bacterial strain

L. rhamnosus (American Type Culture Collection, ATCC 7469, Manassas, VA, USA) was used in the riboflavin assays. Stock cultures of *L. rhamnosus* were transferred monthly into stab cultures consisting of 10 mL of Micro Assay Culture Agar (Becton, Dickinson and Company, Franklin Lakes, NJ). Cultures were incubated at 37 °C for 24–48 h and transferred to a refrigerator at 4 °C for storage. A working culture was kept on a streak plate and was transferred weekly. The streak plate was stored at 4 °C.

To prepare the inoculum for the riboflavin assay, a subculture of the *L. rhamnosus* stock culture was made by inoculating 10 mL of Micro Inoculum Broth (Becton, Dickinson and Company, Franklin Lakes, NJ). The broth was incubated for 16–24 h at 37 °C. The culture was centrifuged using aseptic technique, and the liquid was decanted. The cells were washed three times with 10 mL of 0.85% sterile saline solution, and they were re-suspended in 10 mL of 0.85% sterile saline solution. Because the initial turbidity was too high for measurement on the spectrophotometer (600 nm) (Milton Roy Spectronic 20D, Rockford, IL), sterile saline solution was used to dilute the cell suspension and the absorbance was adjusted to 35–40% transmittance for tubes and 10–15% transmittance for 96-well round bottom sterile microtiter plates with lids (Corning Inc., Acton, MA). The resulting cell suspension, 20 μL for tubes and 4 μL for microtiter plates, was used to inoculate 10 mL and 200 μL broth solutions, respectively, for the assay. Both the tubes and the microtiter plates were kept at 37 °C.

2.2. Media preparations

The media was prepared by suspending 4.8 g of Bacto® Riboflavin Assay Medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in 100 mL of deionized water. The media was boiled 2–3 min to dissolve completely. It was subsequently dispensed in 5 mL amounts into tubes. The standard or test sample was added, and the volume was adjusted to 10 mL using deionized water. The tubes were autoclaved for 10 min at 121 °C.

2.3. Standard curve construction

The riboflavin stock solution was prepared by weighing 50 μg of riboflavin standard (Sigma, St. Louis, MO) and dissolving in 500 mL of deionized water in a volumetric flask. It was further diluted by adding 0.500–500 mL of deionized water in a volumetric flask. The diluted stock solution was distributed in the tubes using 0.0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 mL per tube. These volumes were adjusted to 10 mL with 5 mL of media and deionized water, which gave riboflavin concentrations of 0.0, 2.5, 5.0, 7.5, 10, 15, 20 and 30 ng/mL, respectively. The tubes were prepared in duplicate. The stock solution for the standard curve was prepared fresh daily to prevent the deterioration of riboflavin in the solution (Angyal, 1996).

2.4. Culture growth conditions

Cell growth was measured turbidimetrically at 600 nm (A600) on a spectrophotometer (Spectronic 20D) for the tubes and a Tecan Spectra Flour Plus (Research Triangle Park, NC) for the microtiter plates. The tube assays were allowed to grow for 18–24 h as recommended by the commercial supplier (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), while the microtiter plates were allowed to incubate for 14–22 h. The cells exhibited a lag phase which was defined by the time period between the inoculation of the bacterial cells and the beginning of the exponential phase of the growth curve.

2.5. Sample preparation

Samples were weighed out to 0.500 ± 0.01 g and transferred to an anaerobic culture tube (Belco Glass Inc., Vinelands, NJ). To each sample, 12.5 mL of 0.1 M sulfuric

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