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Use of L-Glutamic Acid in a New Enrichment Broth (R-TATP Broth) for Detecting the Presence or Absence of Molds in Raw Ingredients/Personal Care Product Formulations by Using an ATP Bioluminescence Assay

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Synopsis

The present study reports the effects of adding L-glutamic acid to a new enrichment broth designated as R-TATP broth, to promote the growth of slow-growing mold microorganisms such as *Aspergillus brasiliensis* and *Aspergillus oryzae*, without interfering in the growth of other types of microorganisms. This L-glutamic acid containing enrichment broth would be particularly valuable in a rapid microbial detection assay such as an adenosine triphosphate (ATP) bioluminescence assay. By using this new enrichment broth, the amount of ATP (represented as relative light unit ratio after normalized with the negative test control) from mold growth was significantly increased by reducing the time of detection of microbial contamination in a raw ingredient or personal care product formulation from an incubation period of 48–18 h. By using L-glutamic acid in this enrichment broth, the lag phase of the mold growth cycle was shortened. In response to various concentrations of L-glutamic acid in R-TATP broth, there was an increased amount of ATP that had been produced by mold metabolism in an ATP bioluminescence assay. By using L-glutamic acid in R-TATP broth in an ATP bioluminescence assay, the presence of mold could be detected in 18 h as well as other types of microorganisms that may or may not be present in a test sample. By detecting the presence or absence of microbial contamination in 18 h, it is superior in comparison to a 48–96 h incubation period by using either a standard or rapid detection method.

INTRODUCTION

All living microorganism contain and use adenosine triphosphate (ATP) as a vital part of their energy and metabolic system. Energy is stored within the phosphate bonds of the ATP molecule. By using a luminometer in an ATP bioluminescence assay, the presence of microbial ATP assay is used to detect light energy when ATP is converted to adenosine monophosphate (AMP) by an enzymatic reaction (1,2). This converted energy is detected as light energy and the amount of light is measured by a luminometer that is reported as a value of relative light unit (RLU). A higher RLU value corresponds to a higher level of ATP that is present in a test sample. A positive detection for a microbial contamination

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in a test sample is represented by a value of an RLU ratio that is greater than 2 in comparison with an RLU for negative control without ATP. The calculated RLU ratio is based on the RLU of an incubated test sample versus the RLU of a non-incubated test sample (negative control). By using the ATP bioluminescence test kits from Charles River Laboratories, Inc. (Charleston, SC), an ATP bioluminescence assay is able to detect the presence or absence of microorganisms in either a nonsterile raw ingredient or a personal care finished product formulation that is susceptible to microbial contamination by using an incubation period of 24 h for most applications (1,3,4), 30 h for bacteria and fungi screening (5), or 48 h (6) However, the application of this technology may be limited for most test samples if they are contaminated with a slow-growing microorganism such as mold. The reason for this limitation in detection is that only low levels of ATP are released by growing mold after 24–30 h of incubation which may lead to a false-negative test result. This limitation becomes more significant if a higher nonmicrobial ATP level is also detected to be present in a test sample from a nonmicrobial source. Thus, the increased RLU background signal of the test sample will minimize the RLU ratio, especially when the RLU signal of the microbial ATP level is low from the growth of mold unlike that from bacteria.

Based on information from Machlis, it led us to study if the detection of mold microorganisms can be enhanced by adding L-glutamic acid to R-TAT broth (7). The efficacy of L-glutamic acid in shortening the lag phase of the fungal growth cycle was also studied and discussed by Griffin (8). The application of this information in a rapid microbial detection system has yet been studied or published. In the light of the observed limitation in using an ATP bioluminescence assay for detecting the presence or absence of microbial contamination in a test sample, our goal is to increase the detectable level of ATP that is produced by a slow-growing microorganisms, such as mold, by creating a new enrichment broth that can promote the growth of these microorganisms. This new enrichment broth should not affect the growth of bacteria and/or yeasts that may also be part of the microbial bioburden of a test sample. In addition, the new enrichment broth should have either no or very low levels of nonmicrobial ATP present.

Before an ATP bioluminescence assay is routinely implemented to screen for the presence or absence of microbial contamination in either a nonsterile raw ingredient or product formulation, the test method needs to be validated by inoculating 1% test suspensions in enrichment broth to demonstrate recovery. This validation testing involves the use of indicator test microorganisms. In our case, we used *Staphylococcus aureus* ATCC 6538 for demonstrating the recovery of Gram-positive cocci, *Bacillus subtilis* ATCC 6633 for demonstrating the recovery of spore-forming Gram-positive bacilli, *Escherichia coli* ATCC 8739 for demonstrating the recovery of Gram-negative bacilli, *Candida albicans* ATCC 10231 for demonstrating the recovery of yeasts, and *Aspergillus brasiliensis* ATCC 16404 for demonstrating the recovery of mold in a 1% test sample suspension of an ATP bioluminescence assay by using R-TATP broth as the enrichment broth.

MATERIALS AND METHODS

TEST MICROORGANISMS

The following test microorganisms were used in this study: EZ-colony-forming unit (CFU) cultures of *S. aureus* ATCC 6538 (Catalog number 0485C), *E. coli* ATCC 8739

(Catalog number 0483C), *B. subtilis* ATCC 6633 (Catalog number 0486C), and *C. albicans* ATCC 10231 (Catalog number 0443C) had been purchased from Microbiologic, Inc. (St Cloud, MN) Spore suspensions of *A. brasiliensis* ATCC 16404 and *Aspergillus oryzae* ATCC 10124 were prepared in-house by harvesting spores from potato dextrose agar slants.

ENRICHMENT BROTH COMPONENTS AND CHEMICALS

The following microbial growth media and enzymatic digest of protein were purchased from Becton Dickinson Company (Franklin Lakes, NJ): Difco™ TAT Broth Base, potato dextrose broth (PDB) (Difco), and Letheen broth (Difco), and Bacto™ Neopeptone. Sucrose, sodium thiosulfate, polysorbate 20, and sodium thiosulfate 1 N solution were purchased from Thermo Fisher Scientific. L-glutamic acid and antifoam (Sigman A 5757) were purchased from Sigma-Aldrich Products.

ATP BIOLUMINESCENCE REAGENTS AND GLASS BEADS

Sterile 0.5 millimeter (mm) glass beads (Catalog number 11079105; Biospec Products Inc., Bartlesville, OK) and Celsis® AKuScreen test kit (Celsis Catalog number AS1310 containin the Celsis LuminAMP and LuminEX reagents) were purchased from Charles River Laboratories, Inc.

TEST MICROORGANISM PREPARATION

For each of the EZ-CFU product microorganism, one pellet of a lyophilized bacteria and *C. albicans* culture was aseptically transferred to a separate 1.0 milliliter (ml) aliquot of pre-warmed 10 millimolar (mM) phosphate buffer pH 7.2 solution at 35.0°C ± 2.0°C. Each culture aliquot was then immediately incubated at 35.0°C ± 2.0°C for 30 min for complete hydration in which there is a 10^3 CFU/ml suspension. An in-house method was used to prepare spore suspensions of *A. brasiliensis* ATCC 16404 and *A. oryzae* ATCC 10124 in which each of the *Aspergillus* cultures were grown on several on potato dextrose agar slants until sporulation had occurred at a temperature of 20.0°–25.0°C for a minimum of 7 d. After sporulation, the spores for each culture were harvested from the potato dextrose agar slants by using sterile 0.85% saline solution (0.85% NaCl) with 0.05% polysorbate 80 to a level of 10^7 CFU/ml that was then diluted to a spore suspension level of 10^3 CFU/ml by using sterile 0.85% saline solution with 0.05% polysorbate 80.

PREPARATION OF ENRICHMENT R-TAT BROTH AND R-TATP BROTH

R-TAT broth was prepared in two parts. Part A starts by warming up 2 liters (L) of deionized water to 50.0°C. After warming to 50.0°C, 50.0 g of PDB is added to the deionized water and stirred until dissolved, and then heated to boiling for 1 min to completely dissolve the dehydrated PDB powder. Part B starts by warming 7.6 L of deionized water to a temperature of 50.0°C. After reaching a temperature of 50.0°C, the following ingredients

are separately added to the deionized water and dissolved by stirring: 250.0 g of Difco™ TAT Broth Base, 100.0 g of Bacto™ Neopeptone, 25.0 g of sodium thiosulfate, 50.0 g of sucrose, and 400 ml of polysorbate 20. After adding all of the ingredients, the mixture is stirred for at least 30 min or until all of the ingredients have been thoroughly dissolved. After dissolving, part B is added to part A and stirred for an additional 5 min. R-TATP broth was prepared the same way as R-TAT broth with the exception of 7.35 g of L-glutamic acid is added to part B (see Table I). After mixing part A and part B together, 100 ml aliquots of the R-TATP broth and/or R-TAT broth is dispensed into individual widemouthed square plastic bottles and sterilized at a temperature of 121.0°C ± 1.0°C at 15 pounds for 40 min. After sterilization, the caps on each container are tightened after cooling down to ambient room temperature. The final pH of R-TATP broth should be 6.5–6.6. Preparation of Letheen broth is performed by following the instructions from the manufacturer.

TEST SAMPLE PREPARATION AND INCUBATION

A 1.0 g or ml aliquot of a raw ingredient or personal care product formulation is aseptically transferred to 100 ml aliquots of R-TATP broth to make a 1% test sample suspension. For each test microorganism, a 10 microliter (µl) aliquot of the 10^3 CFU/ml suspension (in test microorganism preparation) containing approximately 10 CFU is aseptically inoculated to a container with/without a 1% test sample suspension and incubated at a temperature of 30.0°C ± 1.0°C with agitation of 150 revolutions per minute (RPM). One uninoculated aliquot of R-TATP broth and one uninoculated aliquot of a 1% test sample suspension are also incubated as negative test controls. R-TAT broth containing L-glutamic acid concentrations of 1.0, 5, 10, 20, and 30 mM were prepared. *A. brasiliensis* ATCC 16404 inoculums containing 20 and 30 CFUs were aseptically added to each of the 100 ml aliquots of the enrichment broth.

ATP BIOLUMINESCENCE ASSAY

Incubated test samples are removed from the incubator shaker after 18 or 24 h of incubation at 30.0°C ± 1.0°C. For those aliquots of R-TATP broth or 1% test sample suspension that have been inoculated with *A. brasiliensis* and *C. albicans*, 15.0 g of sterile 0.5 mm glass beads are added to break up the mycelium and release the intracellular constituents

Table I
Ingredients in R-TAT Broth and R-TATP Broth

Ingredient	R-TAT broth (g/L)	R-TATP broth (g/L)
Difco™ TAT broth base	25.0	25.0
Potato dextrose broth	5.0	5.0
Difco™ neopeptone	10.0	10.0
Sodium thiosulfate	2.5	2.5
L-glutamic acid	—	735 mg (5 mM)
Sucrose	5.0	5.0
Polysorbate 20	4.0 ml	40.0 ml

including ATP. An additional 100 µl aliquot of antifoam is also added to these aliquots to prevent foaming during shaking. Each of these aliquots is then shaken side to side in a linear shaker for 30 min at an RPM of 200–300. After linear shaking, 2 × 50 µl aliquots are aseptically removed and transfer to two separate 12 × 55 mm cuvettes. For those enrichment broth containers that had been inoculated with bacteria only, 2 × 50 µl aliquots are aseptically removed and transferred to two separate 12 × 55 mm cuvettes.

DETECTION OF ATP

An ATP bioluminescence assay was conducted by using the Celsis AKuScreen test kit (Catalog number AS1310; Charles River Laboratories, Inc.) to determine the RLU from the ATP in each of the incubated test sample suspensions. To perform the assay, a rack of 12 × 55 mm cuvettes is prepared. In four for the 12 × 55 mm cuvettes, four separate 50 µl aliquots of R-TATP broth is pipetted separately into each cuvette. Two of the 50 µl aliquots of R-TATP broth will serve as a negative control. In the two remaining 50 µl aliquots of R-TATP broth cuvettes, 10 µl of ATP is aseptically added to each cuvette as a positive test control. After incubation, aseptically transfer 2 × 50 µl aliquots of the incubated 1% test sample suspension in enrichment broth into two separate 12 × 55 mm cuvettes. For the non-incubated 1% test sample suspension in enrichment broth, 2 × 50 µl aliquots is transfer into two separate 12 × 55 mm cuvettes. All of the cuvettes are placed into a Celsis Advance luminometer and the unit is started to process the cuvettes. The bioluminescence reagents are added to each cuvette by the luminometer and the amount of RLU detected in each cuvette is recorded by the luminometer. For each two cuvettes of a test sample, an average of the RLU is calculated and then the RLU ratio was calculated based on the RLU from an inoculated enrichment broth sample versus an uninoculated enrichment broth sample. Positive ATP bioluminescence test result for each of the test microorganisms is reported with an RLU ratio of greater than two.

RESULTS AND INTERPRETATION

NONMICROBIAL ATP CONTENT OF R-TATP BROTH

One of the key factors for using an enrichment broth in an ATP bioluminescence assay is that it must have a low nonmicrobial ATP background in order not to provide false-positive test reactions for the presence of microbial contamination in a test sample. In comparing the RLU values for the presence of nonmicrobial ATP in uninoculated 100 ml aliquots of R-TAT broth, R-TATP broth, and Letheen broth, R-TATP broth had the lowest mean RLU value of 78 with a standard deviation value of 15 (Figure 1). The mean RLU values and standard deviations for nonmicrobial ATP in R-TAT broth were found to be 91 ± 14. The mean RLU values and standard deviations for nonmicrobial ATP in Letheen broth were found to be 105 ± 28. These RLU test results confirm that R-TATP broth is an appropriate enrichment broth that can be used in an ATP bioluminescence assay for detecting the presence or absence of microbial contamination in nonsterile raw ingredients and personal care product formulations because of the fact that it contains a low level of nonmicrobial ATP.

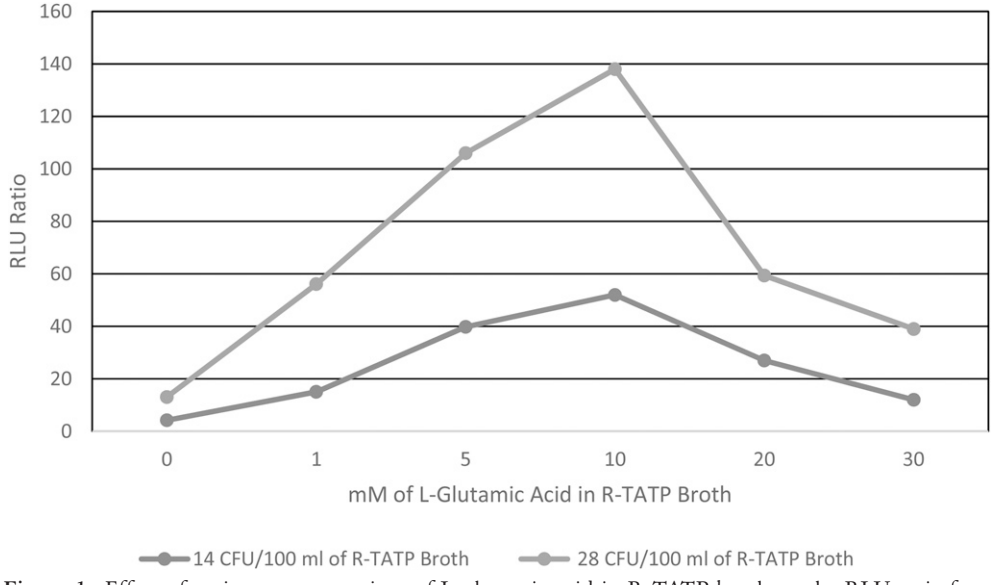

Figure 1. Effect of various concentrations of L-glutamic acid in R-TATP broth on the RLU ratio from *Aspergillus brasiliensis* growth after 24 h of incubation.

GROWTH PROMOTION OF L-GLUTAMIC ACID IN R-TATP ENRICHMENT BROTH FOR DETECTING THE GROWTH OF *ASPERGILLUS BRASILIENSIS*

By using R-TAT broth and the Celsis AKusScreen test kit, low levels (10–100 CFU/g/100 ml) of bacteria and yeast can be easily detected after using a 24 h incubation period by performing an ATP bioluminescence assay for most test samples. The detection of *A. brasilinesis* by using TAT broth varied with an RLU ratio of 2–5 which is comparable with that of Letheen broth which is recommended for use as an enrichment broth in an ATP bioluminescence assay by Charles River Laboratories, Inc.

R-TAT broth containing L-glutamic acid concentrations of 1, 5, 10, 20, and 30 mM that had been inoculated with an *A. brasiliensis* inoculums of 20 and 38 CFUs that were incubated at a temperature of 30.0°C ± 1.0°C for 24 h and then evaluated for the presence of ATP by using an ATP bioluminescence assay. The RLU ratio results of these samples demonstrated a clear dose–response relationship between the various concentrations of L-glutamic acid and the amount of ATP detected from the growth of *A. brasiliensis* in this enrichment broth. In the absence of L-glutamic acid in the enrichment broth, the RLU ratio for R-TAT broth was around 4.2 and 13 with *A. brasiliensis* inoculums of 14 and 28 CFU. By including L-glutamic acid in the R-TAT broth, the amount of ATP was significantly increased by having an RLU ratio of 40 and 106 with a 5 mM concentration, and there was a maximized RLU ratio of 100 and 140 when a 10 mM concentration of L-glutamic acid was used. By using 20 and 30 mM concentrations of L-glutamic acid in R-TAT broth, the RLU ratio started to decline (Figure 2). The test results indicated that the maximum enhancement for the production of ATP from the growth of mold is at a 10 mM concentration of L-glutamic acid. However, we chose to use a 5 mM concentration of L-glutamic acid in R-TAT broth to minimize in having a possible adverse effect of a higher concentration of L-glutamic acid on the growth of other microorganisms that may be present in a test sample.

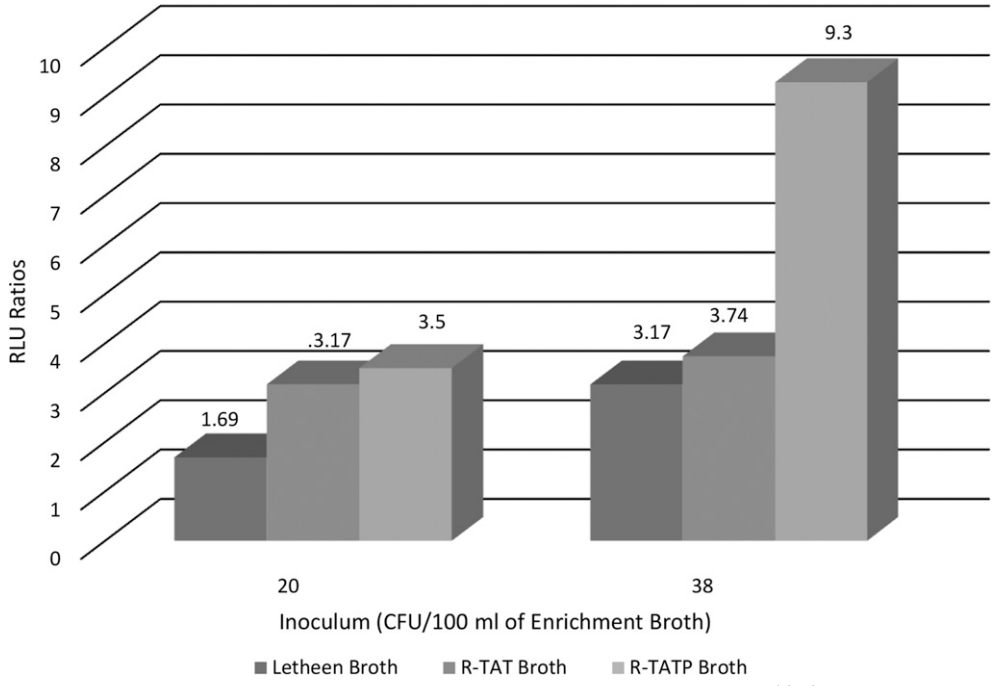

Figure 2. Eighteen-hour detection RLU ratios for *Aspergillus brasiliensis* ATCC 16404 growth in 100-ml aliquots of R-TAT broth, R-TATP broth, and Letheen broth.

POSITIVE MOLD DETECTION IN 18 HOURS BY USING AN ATP BIOLUMINESCENCE ASSAY

A comparative study was performed that included the use of R-TAT broth, R-TATP broth (R-TAT broth with a 5 mM concentration of L-glutamic acid), and Letheen broth to evaluate the efficacy of L-glutamic acid for detecting the presence of mold organisms by each of these enrichment broths. By using two different inoculum levels of *A. brasiliensis* ATCC 16404 for inoculating 100 ml aliquots each of the abovementioned enrichment broths, detection for the presence of microorganisms by using an ATP bioluminescence assay was conducted after 18 and 24 h of incubation at a temperature of 30.0°C ± 1.0°C. With a *A. brasiliensis* inoculum of 20 CFU and an incubation period of 18 h, the RLU ratios for R-TAT broth, R-TATP broth, and Letheen broth were found to be as follows: 2.1, 3.5, and 1.7 (Figure 3).

By using an *A. brasiliensis* inoculum of 38 CFU, the RLU ratios for each of the abovementioned enrichment broths were as follows: 2.5, 9.3, and 3.2 (Figure 3). By extending the time of the incubation period from 18–24 h, the RLU ratios had increased further with R-RATP broth showing the highest RLU ratio of 21 and 155 with an *A. brasiliensis* inoculum of 20 and 38 CFU (data not shown). The effect of adding a 5 mM concentration of L-glutamic acid to R-TAT broth on the growth of another *Aspergillus* species, *A. oryzae* ATCC 10124, was also observed with RLU ratios of 6.4 at 18 h of incubation and 64 at 24 h of incubation (Table II). These test results indicated that the new enrichment broth of R-TATP broth with a 5 mM concentration of L-glutamic acid is able to promote the growth of mold, including *A. brasiliensis* and *A. oryzae* to a detectable level only after using an 18 h incubation period at a temperature of 30.0°C ± 1.0°C by performing an ATP

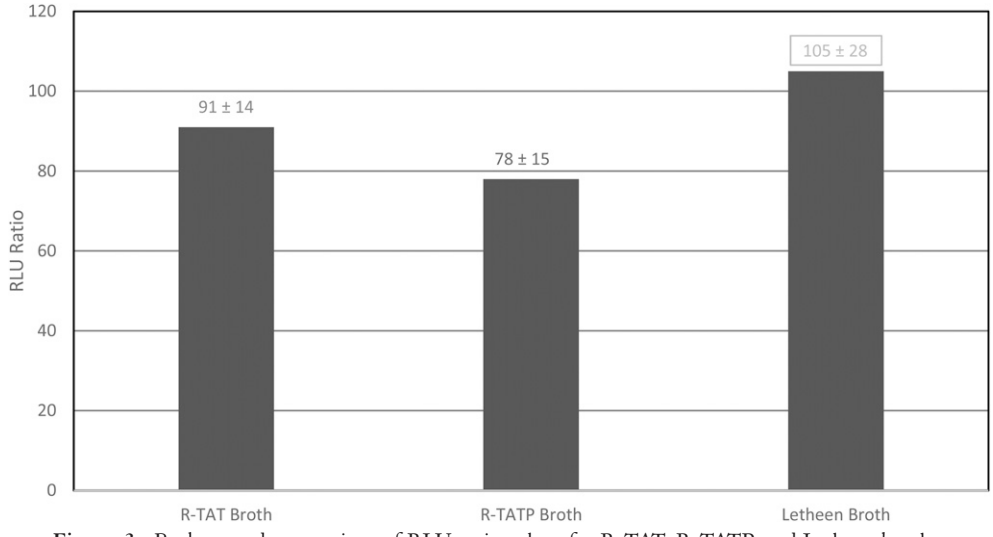

Figure 3. Background comparison of RLU ratio values for R-TAT, R-TATP, and Letheen broths.

bioluminescence assay. The result of an 18 h incubation period for detecting the presence or absence of mold in a test sample is significantly improved by having greater amounts of ATP being produced by mold in comparison with ATP bioluminescence assays using R-TAT broth and Letheen broth as the enrichment broth.

EFFECT OF AN 18 HOUR R-TATP BROTH INCUBATION PERIOD ON THE GROWTH OF OTHER TYPES OF MICROORGANISMS

Besides for the possibility that mold being present in a test sample of a nonsterile raw ingredient or personal care product formulation, other types of microorganisms may also be present. To determine whether R-TATP broth (with a 5 mM concentration of L-glutamic acid) could have an adverse effect in preventing the detection of other microorganisms by using an ATP bioluminescence assay, a study was performed to determine whether other representative microorganisms could also be detected. The following representative test microorganisms were used: *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231. Additional microbial inoculation studies with the abovementioned microbial species at inoculation levels of 18–38 CFU were conducted by either using 100 ml aliquots R-TATP broth alone or a 1% test suspension of a nonsterile raw ingredient or personal care product formulation in R-TATP broth.

Table II
RLU Ratios for Bacteria, Yeast, and Mold in Only R-TATP Broth after 18- and 24-h Incubation Periods

Test microorganism		*S. aureus* 6538	*E. coli* 8739	*B. subtilis* 6633	*C. albicans* 10231	A. brasiliensis 16404	A. oryzae 10124
Inoculum level	CFU/100 ml	18	38	35	29	24	32
R-TATP broth	Hours of incubation						
	18 h	1,871	>5,000	>5,000	21	3.7	6.4
	24 h	>5,000	>5,000	>5,000	1,229	21	4.2

Each of the inoculated R-TATP broth alone or the 1% test suspensions in R-TATP aliquots were incubated at a temperature of 30.0°C ± 1.0°C. After an incubation period of 18 h, an ATP bioluminescence assay was conducted on each of R-TATP broth alone or the 1% test suspensions of 17 personal care product formulations and 15 raw ingredients in R-TATP. At 18 h, positive ATP detections (RLU > 2) were observed for all tested aliquots of R-TATP broth by itself and the 1% test suspensions of tested raw ingredients and personal care product formulations in R-TATP broth that had been inoculated with the abovementioned selected bacterial species and *C. albicans*. For *A. brasiliensis* inoculated aliquots of R-TATP broth by itself and the 1% test suspensions of tested raw ingredients and personal care product formulations, positive ATP detections were detected for 15 of 17 personal care product formulations and 13 of 15 raw ingredients after 18 h of incubation at a temperature of 30.0°C ± 1.0°C (see Tables III and IV). After 24 h of incubation, 100% of the tested raw ingredients and personal care product formulations were found to have positive ATP detections for *A. brasiliensis*. By having positive ATP detections after 18 and 24 h of incubation, the test results confirm that low levels of *A. brasiliensis* can be detected in nonsterile raw ingredients and personal care product formulations that are susceptible to microbial contamination in which in R-TATP broth is used as an enrichment broth in an ATP bioluminescence assay.

DISCUSSION

Rapid microbial detection such as ATP bioluminescence assay for the has attracted many personal care and pharmaceutical companies for its ability to rapidly screen for the presence

Table III
RLU Ratios for Raw Ingredients Inoculated with Test Microorganisms Using an 18-h Incubation Period

Test microorganism:	*S. aureus* 6538	*E. coli* 8739	*B. subtilis* 6633	*C. albicans* 10231	*A. brasiliensis* 16404
Inoculum levels (CFU/100 ml of enrichment broth)	14–19	15–38	32–54	21–51	20–52
Name of raw ingredient	RLU Ratios				
Sorbitan monooleate	>5,000	>5,000	>5,000	63	7.8
Hydroxyethylcellulose	601	>5,000	>5,000	326	2.0
Sodium citrate	1.1	>5,000	70	8.3	1.5[a]
POP (2M) myristyl ether propionate	3,603	>5,000	>5,000	18	6.9
PPG-12-buteth-16	255	>5,000	439	23	4.5
POE (20M) sorbitan monopalmitate	790	>5,000	>5,000	79	11
C12-15 alkyl ethyhexanoate	1,832	>5,000	>5,000	62	2.2
Flamenco sparkle green #820J	1,997	>5,000	>5,000	52	2.2
Boron nitride	808	>5,000	>5,000	68	5.4
Chroma-lite dark blue #CL4501	4,198	>5,000	225	14	3.0
Timiron super silver	1,422	>5,000	>5,000	73	14
Cationic emulsion 35% APE free	>5,000	>5,000	>5,000	35	1.7[b]
Titanium dioxide coated grew-MP149	>5,000	>5,000	109	20	7.3
Diglyceryl diisosterate	>5,000	>5,000	>5,000	40	5.7
PPG 20 methylglucose ether	>5,000	>5,000	54	26	4.6

[a]With a 24-h incubation period for sodium citrate, the RLU ratio was 2.5.
[b]With a 24-h incubation period for cationic emulsion, the RLU ratio was 101.

Table IV
RLU Ratios for Personal Care Product Formulations Inoculated with Test Microorganisms Using an 18-h Incubation Period

Test microorganism	*S. aureus* 6538	*E. coli* 8739	*B. subtilis* 6633	*C. albicans* 10231	*A. brasiliensis* 16404
Inoculum levels (CFU/100 of enrichment broth)	14–19	15–38	32–54	21–51	20–52
RLU ratios					
R-TATP broth (positive control)	55–1,871	434 to >5,000	46 to >5,000	21–138	3.7–11
Type of personal care product formulation					
Body cream	1,474	>5,000	>5,000	15	3.7
Body lotion	2,341	>5,000	>5,000	17	1.8[a]
Body lotion	1,286	>5,000	>5,000	19	3.1
Skin toner	3,923	>5,000	511	75	5.0
Skin toner	5,314	>5,000	3,294	49	2.6
Body lotion	>5,000	>5,000	3,398	31	2.2
Body cream	3,739	>5,000	2,353	48	2.6
Mascara	727	>5,000	263	14	7.3
Night cream	>5,000	>5,000	>5,000	29	15
Mascara	543	>5,000	3.2	31	4.3
Eyeshadow	745	>5,000	867	22	6.2
Shampoo	168	>5,000	106	3.4	2.3
Foundation	1,941	434	232	7.7	3.6
Cleanser	1,479	>5,000	949	21	2.6
Hand cream	190	>5,000	646	32	4.2
Shampoo	15.1	>5,000	>5,000	20	6.5
Skin lotion	40.4	>5,000	>5,000	49	1.7[b]

[a]With a 24-h incubation period, the RLU ratio was 28.
[b]With a 24-h incubation period, the RLU ratio was 9.3.

or absence of microbial contamination in nonsterile raw ingredients before they are used in manufacturing and personal care product formulations before they distributed for sale to consumers. However, there are four main issues in using ATP bioluminescence technology. The first issue is that the detection of slow-growing microorganisms such as mold might be ambiguous because of the limited amount of ATP that is initially produced by a growing mold after 24 h of incubation. The second issue is the high RLU background from nonmicrobial ATP that may be present in either a raw ingredient or a personal care product formulation. This high level of nonmicrobial ATP of a test sample could minimize the RLU ratio between an incubated and non-incubated test sample that could cause a false-negative test result for the absence of contaminating microorganisms. The third issue is that the presence of high levels of nonmicrobial ATP in an enrichment broth might lead to having a false-positive test reaction for the presence of microbial ATP in a test sample that is not contaminated with microorganisms. The fourth issue is the dissatisfaction in detecting the presence or absence of mold in a test sample by using Letheen broth as the enrichment medium in which a longer incubation period has to be used in comparison for testing the presence or absence of bacteria in a test sample.

All living microorganisms must rely on the uptake of microbial nutrients from the environment to sustain energy, metabolism, and growth (6,9,10). By creating a more nutritious enrichment broth that has a low level of nonmicrobial ATP, the best possible growth

condition can be provided for detecting the presence or absence of mold contamination and allow the expression of other non-mold microorganisms that may present in an incubated test sample. By adding PDB (Difco), Becton™ Neopeptone, Sucrose to Difco™ TAT Broth Base, the purpose of these components is to provide additional microbial nutrients to support the growth of mold. By adding polysorbate 20 and sodium thiosulfate to the Difco™ TAT Broth Base, the purpose of these two components is to neutralize the antimicrobial activity of preservative systems that may be present in a test sample. Furthermore, the addition of L-glutamic acid to this new enrichment broth mixture is used to accelerate mold growth by shortening the lag phase of the mold growth cycle in which mold can be detected faster by using an ATP bioluminescence assay. The effects of amino acids including glutamic acid on fungi growth had been previously studied on *Saccharomyces cerevisiae* for transaminase activity (11), as a carbon source for the growth of *Cryptococcus albidus* (12) and on *Streptomyces viridoochromogenes* for spore germination (13). However, none of these articles had reported on the effects of glutamic acid in shortening the lag phase of the microbial growth cycle, and there had been no studies conducted that involved the usage of a rapid microbial detection method such as an ATP bioluminescence assay.

Microbial growth or proliferation is defined as an orderly increase of the components of an organism that is followed by cell multiplication. The microbial growth cycle consists of three phases. The first phase of the microbial growth cycle is the lag phase in which a microorganism adapts to their new environment by forming proteins and metabolites for multiplication. For mold, the lag phase involves the preparation of hyphal elongation and branching. The second phase of the mold growth cycle is the exponential phase when new cell materials are synthesized at a constant rate and the amount of cell mass increases in an exponential manner. For mold, this is the germination period. The third phase of the mold growth cycle is the stationary phase where there is an exhaustion of microbial nutrients or the accumulation of toxic byproducts from mold metabolism that causes growth or proliferation to cease completely (10,14). The length of the lag phase of the growth cycle for a microorganism will depend on the microorganism and as to whether microbial nutrients are available for metabolism. The lag phase of the mold growth cycle such as *A. brasiliensis* is 0–15 h in length in comparison with bacteria which is 0–6 h in length (10,15,16). For a yeast such as *C. albicans*, the length of the lag phase is between 0 and 3 h (17). The length of the lag phase will vary between different types of mold species (18).

Without direct biochemical evidence, we are proposing that the significant increase in the RLU by the ATP production by *A. brasiliensis* is a result of a shorter lag phase in the fungal growth cycle allowing the advancing and enhancing of the exponential phase for mold growth. This is reflected by the increased production of ATP from *A. brasiliensis* that directly corresponds to the increased concentrations of 1 and 10 mM L-glutamic acid. Furthermore, the RLU signal up at least 40-fold in R-TATP broth alone after 24 h incubation period (Figure 1). After 18 h of incubation, the RLU signal was greater than a 20-fold in the 1% test suspensions in R-TATP broth for 15 of 17 personal care product formulations and 13 of 15 raw ingredients (Tables III and IV). However, this rate of detection in a test sample at 18 h is not a concern because this type of situation will be detected during the validation testing of an ATP bioluminescence assay for a nonsterile raw ingredient or product formulation by a personal care or pharmaceutical company. If mold could not be detected after using an 18 h incubation period, it looks like that the presence of mold in a 1% test sample suspension in R-TATP broth would eventually be detected instead with a 24 h incubation period based on our test results.

To date, the use of an 18 h incubation period for detecting the presence or absence of microbial contamination in a raw ingredient or personal care product formulation raw ingredient or personal care product formulation test sample by using an ATP bioluminescence assay has not been reported elsewhere. There is no prior art to the best of our knowledge in using L-glutamic acid in an enrichment broth to promote the growth of mold in a rapid microbial detection method such as an ATP bioluminescence assay.

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