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The performance of tofu-whey as a liquid medium in the propagation of mycobacterium tuberculosis strain H37Rv

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ABSTRACT

Objective: To investigate the performance of "tofu-whey liquid medium" for the propagation of *Mycobacterium tuberculosis* (MTB) strain H37Rv.

Method: Two hundred micro liters (200 μl) of 1 McFarland standard (1 mg/ml-bacillary suspension) were inoculated into different batches of tofu-whey liquid medium. Each series contained three trials of test (tofu-whey liquid medium) and control media (Middlebrook 7H9 medium). Turbidity was measured within three weeks of inoculation using a nephelometer. The combinations of various tofu-whey liquid culture media were as follows; T1 (tofu-whey + ADC + glycerol + Potassium sulfate + Magnesium citrate + Sodium glutamate); T2 (tofu-whey + ADC + glycerol + Potassium sulfate + Magnesium citrate); T3 (tofu-whey + ADC + glycerol + Potassium sulfate); T4 (tofu-whey + ADC + glycerol); T5 (tofu-whey + ADC); and T6 (tofu-whey only).

Results: In all test and control liquid culture media, the multiplication of *M. tuberculosis* was documented under light and fluorescence microscopy. Of various tofu-whey medium used, T1 demonstrated the most potential for MTB propagation. The increased turbidity reading represented by the value in "unit drop of % transmittance" was higher (25 scores) in the T1 tofu-whey medium, compared with the T6 tofu-whey medium (8 scores). The overall growth was significantly better in Middlebrook 7H9 culture media, although by the third day of incubation, the bacillary growth was superior in the T1 tofu-whey culture media. Sub-cultures in Lowenstein–Jensen (L–J) medium yielded between 87% (47 of 54) and 89% (48 of 54) recovery rate with between 7% and 13% contamination rate with coagulase-negative staphylococci.

Conclusion: Tofu-whey media can be used as an economical alternative to Middlebrook 7H9 in resource-limited settings.

Introduction

Diagnosis of tuberculosis in most developing and underdeveloped countries relies on the direct microscopic examination of a sputum specimen [1–3]. This technique has a low and variable sensitivity and cannot differentiate drug-resistant strains. As a result, laboratories have to use more reliable and more sensitive methods for diagnosis and identification of *Mycobacterium tuberculosis*. Generally, isolation of MTB is performed by inoculating the specimens into traditional solid

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culture medium which requires three to eight weeks to yield the result [4–8]. That is why the Stop TB Partnership Laboratory Strengthening Subgroup, partner organizations, laboratory experts, and the World Health Organization (WHO) Strategic and Technical Advisory Group for Tuberculosis have endorsed the WHO recommendation to use liquid culture media for culture and drug susceptibility testing. The use of liquid medium for the detection of MTB is mainly justified by increased sensitivity and detection rate from specimens with low bacilli count, along with reduced detection time as compared with solid culture media [9]. The United States Centers for Disease Control and Prevention (CDC) has recommended that every effort must be made for laboratories to use the most rapid methods available for diagnosis of Mycobacterium. These recommendations include the use of both liquid and solid medium. In previous years, the BACTEC 460 was used in many laboratories, but the high cost of disposing radioactive waste prompted the manufacturers to explore and develop alternative liquid culture medium [10,11]. Mycobacteria Growth Indicator Tubes (MGIT) utilize a modified Middlebrook 7H9 liquid medium with 0.25% glycerol (7 ml) with an oxygen-quenching fluorescent sensor embedded in silicon at the bottom to detect microbial growth directly from clinical specimens [12,13]. This system is simpler, more efficient, and non-radioactive in nature and does not need much space in the laboratory. However, in poor resource countries, MGIT is relatively expensive to acquire and sustain. In the present study, a liquid culture media that is cheap and might propagate a good in vitro growth of MTB is investigated. Tofu-whey liquid culture media is rich in isoflavones, oligosaccharides, peptides, and saponins. These components are similar to the carbohydrate, protein and nutrient content that is found in commercial laboratory media. Basically, tofu-whey liquid culture media is made from Soy products. Soy products (tofu and tofu-whey) contain plant proteins and nutrients [14]. The essential requirements that are needed by microorganisms to be propagated are all found in soy products like tofu-whey. The use of tofu-whey—a by-product of tofu production—has already been shown in *Lactobacillus plantarum* [15], and *Lactobacillus paracasei* ssp. *paracasei* [16]. However, their application in growing MTB was not highlighted. In the present investigation, the use of tofu-whey's potential for propagation of *M. tuberculosis* strain H37Rv is explored.

Materials and methods

Microbial culture

M. tuberculosis strain H37Rv was obtained from the National Tuberculosis Reference Laboratory. The control strain originated from The Research Institute of Japan, which is the Philippines' Supra-National authority in all its TB projects and activities.

Formulation of tofu-whey liquid test media

One thousand ml of tofu-whey was collected and filtered with sterile gauze. Test media was prepared according to the formulations listed below (T1 to T6). The media was autoclaved (121 °C, 15 PSI, 15 min) and allowed to cool in room temperature. For sterility check, the media were incubated at 37 °C for 24 h. The experiment was done in triplicate for 3 batches (6 test media × 3 trials each × 3 batches = 54 test media; 1 control media × 3 trials × 3 batches = 9 control liquid control media). Four ml of each formulation was dispensed in several 20 ml-capacity screw-cap glass test tubes. All liquid test media were sterilized in an autoclave.

Tofu-whey culture media that were used in the study:

- T1 Tofu-whey culture media 1

  22.5 ml Tofu-whey + 2.5 ml Albumin Dextrose Catalase (ADC)* + 0.05 ml glycerol + 0.05 g Potassium sulfate + 0.0625 g Magnesium citrate + 0.025 g Sodium glutamate

- T2 Tofu-whey culture media 2

  22.5 ml tofu-whey + 2.5 ml ADC* + 0.05 ml glycerol + 0.05 g Potassium sulfate + 0.0625 g Magnesium citrate

- T3 Tofu-whey culture media 3

  22.5 ml tofu-whey + 2.5 ml ADC* + 0.05 ml glycerol + 0.05 g Potassium sulfate

- T4 Tofu-whey culture media 4

  22.5 ml tofu-whey + 2.5 ml ADC* + 0.05 ml glycerol

- T5 Tofu-whey culture media 5

  22.5 ml tofu-whey + 2.5 ml ADC

- T6 Tofu-whey culture media 6

  25 ml tofu-whey only

Preparation of inoculums

1 mg/ml-bacillary suspension of *M. tuberculosis* strain H37Rv was prepared by harvesting 2 loops full of the microbial culture from L–J medium, suspended in a homogenizer tube (glass test tube with three to five glass beads inside) containing two drops of sterile distilled water. The harvested colonies were macerated by vortex mixer (Hi-Tech mixer M-90001. Hi-Tech Inc., ABC Labo Tokyo Japan) for two to three minutes; 7 ml of sterile distilled water were added to the macerated colonies. An aliquot of the bacterial suspension was adjusted to No. 1 McFarland turbidity by comparison with a turbidity standard equivalent to 1 mg/ml-bacillary suspension. This was used as the inoculum for the liquid control and test media [17,18].

Preparation of experimental test panels

Three test tubes of each formulation of tofu-whey containing 4 ml of tofu-whey liquid test media were inoculated with two drops (200 μl) of 1 mg/ml-bacillary suspension and incubated

at 37 °C for a period of three weeks. Initial checking for contamination was done 24 h after inoculation. A sudden increase in turbidity (sudden drop in % transmittance value) will be observed as the indicator of contamination if proven under microscopic examination and liquefaction or contamination of L–J solid media inoculated with sub-cultures.

Control strain growth determination

After inoculation of the tofu-whey liquid test media, initial turbidity reading (% transmittance) was read using a nephelometer (VITEK Model product No. 52–1210, Biomerieux Inc.); the next reading was taken on the third day after inoculation up to the third week. Reading of turbidity was performed to observe signs of bacterial growth. Tubes which showed increased turbidity were smeared and evaluated by Fluorescence and Acid Fast Microscopy for signs of contamination and consistency of characteristic bacterial morphology. All tubes were sub-cultured in L–J slant for colony count [19,20].

Results

Turbidity readings

Table 1 shows the summary of means of treatment values in all three batches of tofu-whey liquid test media and 7H9 liquid control medium. A downward trend was observed in all treatments and control after a three-week period indicating a decrease in % transmittance (increase in turbidity, increase in colonies of *M. tuberculosis*). The unit drop in % transmittance was computed by obtaining the difference between the inoculation and the third week values (inoculation – third^{t} week = unit drop % transmittance). The higher the value in the unit drop % transmittance, the more likely the liquid medium performance is to propagate *M. tuberculosis* which is observed by an increased turbidity. 7H9 control medium attained the highest unit drop % transmittance with a score of 57. Among the treatments, T1 was able to give the highest score (25), which is only half as good as the performance of 7H9 liquid control medium at the end of three weeks, but it exhibited an early growth spike during the third day reading. T6 obtained the lowest score (8) proving that tofu-whey alone supports only minimal growth.

As shown in Fig. 1, T1, T2, and T3 exhibited an increased turbidity within three days of inoculation .Observation of the first week values place T1, T2, and T3 performances running parallel with that of 7H9, while T4, T5, and T6 continue to show an increase in turbidity (increase in bacterial growth) at a slow pace. 7H9 liquid control media were only able to surpass the performances of T1, T2, and T3 during the first week of incubation and showed a further increase up until the third week, while T1, T2, and T3 started to show a decrease in

Table 1 – Comparison of the performance of T1 to T6 tofu-whey Test medium.

Medium	Blank	Inoculation	3rd day	1st week	2nd week	3rd week	Unit drop %T
T1	100.0	96.33	87.33	83.00	78.33	71.67	25
T2	95.00	91.67	85.33	81.00	79.00	77.67	14
T3	91.00	88.00	82.33	79.00	71.67	69.67	18
T4	51.67	48.33	41.33	37.00	32.67	30.00	18
T5	46.67	42.33	38.67	33.33	31.67	30.67	12
T6	42.00	36.0	33.67	30.67	29.00	28.00	8
7h9	100.00	98.00	94.00	82.67	60.00	41.33	57

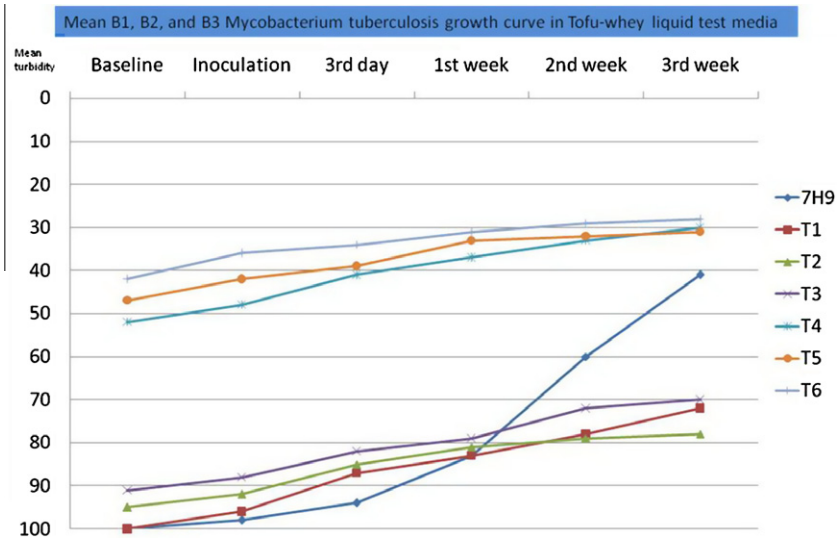

Fig. 1 – Growth curve of *M. tuberculosis* in Test (T1 to T6) and control media.

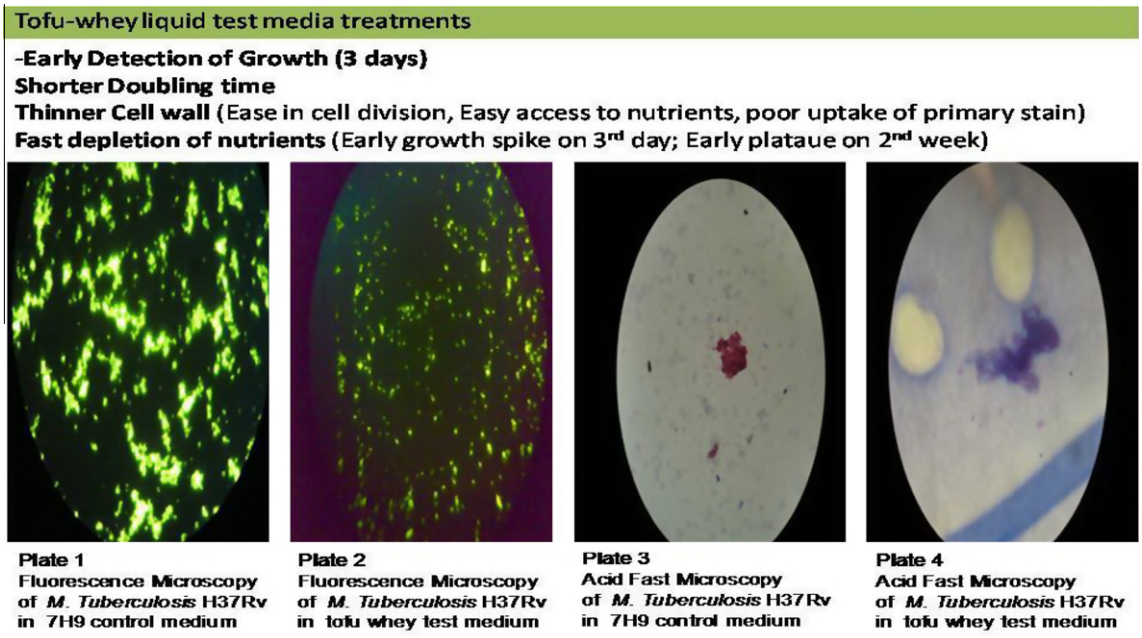

Fig. 2 – Under microscopic observation.

performance with an increase in turbidity observed at a slower pace.

Microscopic observation

As shown in Fig. 2, cultures of *M. tuberculosis* in Middlebrook 7H9 exhibit the characteristic thick mycolic acid cell wall (Plates 1 and 3). *M. tuberculosis* propagated in T1 to T6 tofu-whey liquid test medium were shorter and exhibited a thin mycolic acid cell wall (prompting shorter doubling time and early increase in turbidity during the third day) which had poor uptake of primary stain (Carbol fuchsin), making the tubercle Bacilli pale red in color (Plates 2 and 4). Gram-positive cocci in clusters were only observed contaminants in 7% to 13% of L–J medium inoculated with inoculums coming from tofu-whey test medium and are highly suspected biochemically of being *Staphylococcus epidermidis* by virtue of being coagulase-negative staphylococci that are susceptible to Novobiocin.

Subcultures in L–J medium

To verify that the increased turbidity in the test and control media were due to the increasing bacterial population of *Mycobacterium* tuberculosis, 200 μl of test and control media (third day, first week, and second week after inoculation) were subcultured into L–J medium, and the number of colonies were counted. Colony counts were proportional to the time when the inoculums were harvested from the test and liquid media. The results showed that the longest time from inoculation yielded the highest colony counts (second week subcultures produced colony counts of 1+ to 3+, equivalent to 100–500 CFU respectively), while the timing closest to the time of inoculation had the lowest colony forming units (CFU) (Table 2). Recovery rate from L–J medium ranged from 87% (47 of 54) to 89% (48 of 54) with 7% to 13% contamination rate with coagulase-negative staphylococci.

Timing of subcultures in Lowenstein–Jensen medium

A. Inoculation

Total Tubes	Positive tubes	Reading	Colonies	Contamination (C)	NG
1. 18	16	1+	50-80	2	0
2. 18	16	1+	50-80	1	1
3. 18	16	1+	50-80	1	1
	89%			7%	4%

B. Third day

Total Tubes	Positive tubes	Reading	Colonies	Contamination (C)	NG
1. 18	16	1+	50-100	2	0
2. 18	16	1+	50-100	2	0
3. 18	16	1+	50-100	2	0
	89%			11%	0%

C. First week

Total Tubes	Positive tubes	Reading	Colonies	Contamination (C)	NG
1. 18	15	1+	50-100	3	0
2. 18	16	1+	50-100	2	0
3. 18	16	1+, 2+, 3+	50-500	2	0
	87%			13%	0%

D. Second week

Total Tubes	Positive tubes	Reading	Colonies	Contamination (C)	NG
1. 18	15	1+	50-100	3	0
2. 18	16	1+	50-100	2	0
3. 18	16	1+, 2+, 3+	50-500	2	0
	87%			13%	0%

Table 2 – Sub-cultures in Lowenstein–Jensen medium.

Colony count	Inoculation	3rd day	1st week	2nd week
NG (no growth)	0	0	0	0
+n (1 to 50)	0	0	0	0
1+ (50 to 100) if < 100, indicate number of colonies	1+ 89% recovery 7% contam 4% no growth	1+ 89% recovery 11% contam	1+ 2+ 3+ 87% recovery	1+ 2+ 3+ 87% recovery
2+ (100 to 200)	0	0	13% contam	13% contam
3+ (200 to 500)	0	0		
4+ (>500)	0	0	0	0

Discussion

This study was conducted to explore not only an alternative medium for MTB propagation, but also for a more economical means of addressing this challenge in resource-limited settings [4,25]. The best result was observed using T1 tofu-whey liquid culture medium running almost parallel to that of 7H9 liquid control medium. T1 liquid culture media could achieve significant turbidity (increase in colony forming units) in as early as three days. Using Middlebrook 7H9, the growth spike took place within the first week after inoculation. This might be as a result of the shortened doubling time of *M. tuberculosis* in tofu-whey liquid test media, which is attributed to the missing nutrients in tofu-whey liquid test media. Generally, the nutrients available in Middlebrook 7H9 might allow for the construction of a thicker mycolic acid cell wall which in turn increases the time needed for the TB bacilli to propagate [6–8]. For example, biotin is a coenzyme that is used in fatty acids and leucine metabolism [21–23]. Biotin is an essential component which sustains the increase in the number of bacteria in Middlebrook 7H9 until the second week of incubation. This was proven by sub-culturing of inoculums from test and control media in L–J medium. The lower pH of tofu-whey test media (6.1–6.2) compared with the pH of Middlebrook 7H9 (7.0) may also play a critical role in falling short of performance starting from the first week of incubation [24,25] and may require higher concentrations of calcium and magnesium to sustain growth of MTB.

Conclusion

Tofu-whey media can support the growth of *M. tuberculosis* and may be explored as a cost-effective and economical substitute for Middlebrook 7H9 in resource-limited settings. Similarly, experiments should be conducted on tofu-whey prepared with other coagulants (Magnesium sulfate, sea water, weak acids, and enzymes) in its performance in the propagation of *M. tuberculosis*. Last, but not the least, tofu-whey liquid test medium should be tested in clinical specimens containing *M. tuberculosis* to assess its actual laboratory performance.

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