Bulletin of Health,
Science and Technology
BHST
ISSN 0858-7531
Volume 13, Number 2, 2015 : 01-07

COMPARATIVE EVALUATION OF LMX CULTURE MEDIUM AND MODIFIED LMX CULTURE MEDIUM FOR DETECTING *ESCHERICHIA COLI* IN WATER

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Abstract: *Escherichia coli* is used as a faecal indicator bacterium and biological parameter of water quality. Multiple tube fermentation has been widely used for determination of *E. coli* contamination in water. However, this standard technique has limitations particularly in the length of incubation time. An enzymatic assay using Lauryl sulphate MUG X-gal (LMX) culture media was developed as an alternative method for the rapid detection of *E. coli* contamination in water samples. This study proposed a modification of the traditional LMX culture medium, applied to the modified LMX culture medium for saving costs and improving the detection performance. Results determined that the modified LMX culture medium was feasibly preferable for practical water quality analysis.

Keywords: Enzymatic assay, *Escherichia coli*, LMX culture media, Water quality

บทคัดย่อ: เชื้อแบคทีเรีย *Escherichia coli* เป็นแบคทีเรียดัชนีที่ใช้ในการตรวจวิเคราะห์คุณภาพน้ำเชิงชีวภาพ วิธีตรวจวิเคราะห์เชื้อแบคทีเรีย *E. coli* ปนเปื้อนในน้ำที่นิยมใช้เป็นวิธีมาตรฐาน คือ วิธีวิเคราะห์แบบหลายหลอด (Multiple tube fermentation) ซึ่งวิธีวิเคราะห์นี้มีข้อด้อยด้านระยะเวลาที่ยาวนานในกระบวนการตรวจวิเคราะห์ จึงทำให้มีการพัฒนาวิธีตรวจวิเคราะห์อย่างรวดเร็วด้วยวิธีวิเคราะห์ทางเอนไซม์ซึ่งทำการตรวจวิเคราะห์ได้ด้วยอาหารเลี้ยงเชื้อแบคทีเรีย Lauryl sulphate MUG X-gal (LMX) การศึกษานี้มีวัตถุประสงค์เพื่อปรับปรุงอาหารเลี้ยงเชื้อแบคทีเรีย LMX สูตรมาตรฐานให้มีราคาถูกลงและมีประสิทธิภาพในการตรวจวิเคราะห์เพิ่มขึ้น ผลการศึกษาพบว่าอาหารเลี้ยงเชื้อแบคทีเรีย LMX สูตรดัดแปลงสามารถนำไปใช้ในการตรวจวัดคุณภาพน้ำได้

คำสำคัญ: วิธีวิเคราะห์ทางเอนไซม์, เชื้อแบคทีเรีย *Escherichia coli*, อาหารเลี้ยงเชื้อแบคทีเรีย LMX, คุณภาพน้ำ

INTRODUCTION

The availability of fresh and clean water is crucial to public health, economic development and social welfare (Odonkor and Ampofo, 2013). Contaminations by faecal indicator bacteria in water including total coliforms, faecal coliforms and *E. coli* have been linked to human gastrointestinal illness. *E. coli* was first suggested as a suitable indicator bacterium for faecal contamination in 1880s because it was found in high densities in faeces (Shash *et al*., 2010). The multiple tube fermentation (MTF) technique, developed in the early 20th century, became known as the standard assay for faecal contamination of water (Shash *et al*., 2010). One constraint of MTF however is the length of incubation time (24-96 h) required to complete the procedure (Nikaeen *et al*., 2009). Consequently, there is a need for more rapid analytical techniques to save time, labour and cost. The enzymatic assay using Lauryl sulphate MUG X-gal (LMX) broth was an alternative faster method, first described by Manafi and Kneifel (1989) and later modified by Ossmer (1993) to improve the sensitivity and reduce incubation time. The presence of coliform bacteria in water samples is indicated by a blue-green colour of the LMX broth due to the hydrolysis of the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), by the coliform enzyme called β-D-galactosidase. The presence of *E. coli* is confirmed by a red colour on the surface of the culture broth after the addition of Kovac's reagent. The red colour is produced by the chemical reaction of Kovac's reagent and indole, a product from the tryptophan breakdown process, by the bacterial enzyme called tryptophanase. This study proposed a modification of the LMX culture media to save costs and improve the detecting performance of *E. coli* in water. The comparison of standard LMX broth with the modified LMX broths for the qualitative detection of *E. coli* in water samples was investigated. In addition, the modified LMX culture broth was altered to a culture agar for quantitative enumeration of *E. coli* by the plate count technique.

MATERIALS AND METHODS

Chemicals, culture media and microorganisms

Culture media and constituents for enzymatic assays were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (MO, USA). Kovac's reagent solution was purchased from HiMedia (Mumbai, India). *E. coli* strain TISTR073 isolated from human stools was obtained from the Thailand Institute of Scientific and Technological Research (Pathum Thani, Thailand). *Bacillus methylotrophicus* strain RYC01101 isolated from bovine stools (Chantarasiri, 2014) was provided by the Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok.

Preparation of water samples

The artificial water samples were prepared by re-suspending fresh colonies of *E. coli* strain TISTR073, the faecal coliform indicator bacterium with sterilised water and then diluting to obtain an optical density at 600 nm of 0.025. The water supply samples were aseptically collected from various faucets in Ban-Khai District, Rayong Province, Thailand.

Comparison of rapid enzymatic assays for detecting E. coli in artificial water samples

A comparison of rapid enzymatic assays for detecting *E. coli* in artificial water samples was investigated on standard LMX broth (commercial name: Fluorocult® LMX

Broth, Merck), modified LMX broth and modified LMX broth supplemented with 0.1% tryptophan. The standard LMX broth (0.5% tryptose, 0.1% tryptophan, 0.5% NaCl, 0.1% sorbitol, 0.27% K_2HPO_4, 0.2% KH_2PO_4, 0.01% lauryl sulphate sodium salt, 0.008% X-gal, 0.005% 4-methylumbelliferyl-β-D-glucuronide (MUG), 0.01% isopropyl β-D-1-thiogalactopyranoside (IPTG) and pH 6.8), modified LMX broth (0.5% peptone, 0.5% NaCl, 0.1% sorbitol, 0.27% K_2HPO_4, 0.2% KH_2PO_4, 0.01% sodium lauryl sulphate, 0.008% X-gal, 0.005% MUG, 0.01% IPTG and pH 6.8) and modified LMX broth supplemented with 0.1% tryptophan were prepared and sterilised. The artificial water samples containing *E. coli* were serially diluted with each testing broth to obtain 10^{-10} dilutions. All samples were incubated at 37 °C for 18 h. The presence of *E. coli* in the water samples was indicated by the development of a blue-green colour of the culture broth after incubation and a red colour resulted from indole reaction after addition of Kovac's reagent. The performance of the enzymatic assays for detecting *E. coli* in the water samples was determined by the highest dilution factor that yielded a positive result for *E. coli* testing. *B. methylotrophicus* strain RYC01101 was used as the negative control.

Application on the enumeration of E. coli in artificial water samples by plate count technique

The plate count technique on agar plates was used to quantitatively enumerate *E. coli* in the water samples for the modified LMX broth supplemented with 0.1% tryptophan and 1.5% agar. The artificial water samples were serially diluted to obtain 10^{-10} dilutions as previously described. One hundred microlitres of each dilution was spread plated on modified LMX agar and incubated at 37 °C for 18 h. The enumeration of *E. coli* was allegorized with the standard agar for the plate count technique, Plate Count Agar (0.5% casein enzymatic hydrolysate, 0.25% yeast extract, 0.1% dextrose, 1.5% agar and pH 7.0) under the same conditions.

Application on the qualitative determination of E. coli in water supply samples

The contamination of *E. coli* in the water supply samples was determined using the modified LMX broth supplemented with 0.1% tryptophan described above. Fifty millilitres of water supply was inoculated into a 250 mL-Duran® culture bottle (SCHOTT, Mainz, Germany) containing 100 mL triple-strength sterilised culture broths, resulting in the final concentration of culture broths at one-strength. After incubation at 37 °C for 18 h the results of enzymatic assay for detecting *E. coli* in the water supply samples were determined.

RESULTS AND DISCUSSION

The modified LMX broth was designed from standard LMX broth or Fluorocult® LMX Broth (Manafi and Kneifel, 1989; Ossmer, 1993) by modification of the protein constituent in the culture media to save cost. Tryptose supplemented in standard LMX broth or Fluorocult® LMX Broth and laboratory peptone in the modified LMX broth were the partial digested proteins used as the primary organic nitrogen source of bacterial growth. They may be different in form and some compositions depend on the protein sources such as milk, meat and soy, and the procedure of digestion such as acid hydrolysis and enzymatic hydrolysis. The qualitative determination of *E. coli* in artificial water samples by rapid enzymatic assay using culture media including standard LMX broth, modified LMX broth and modified LMX broth supplemented with 0.1% tryptophan was performed by thirty replications at different times between January and July 2015. Noticeably, the detection

performances on *E. coli* of all replications were reliable. The comparison of the results showed that the performance of all culture media for detecting *E. coli* was a dilution factor of 10^{-7} (Figure 1). The negative control, *B. methylotrophicus* strain RYC01101, did not develop the blue-green colour of the culture broth and the red colour from indole reaction.

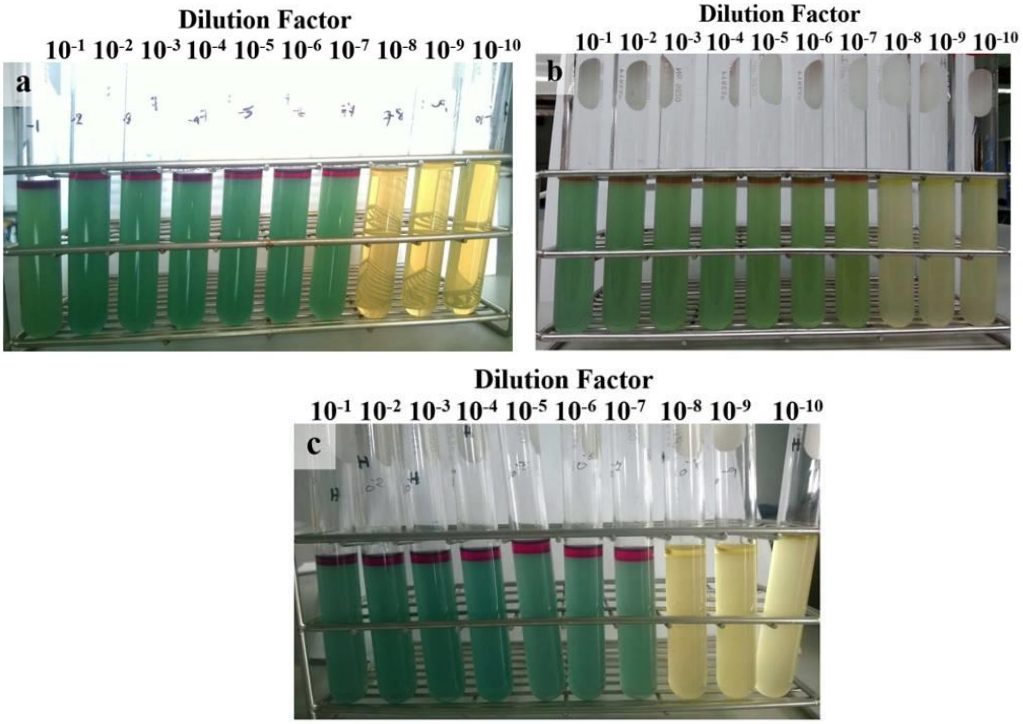


Figure 1. The presence of *E. coli* at various dilutions of artificial water samples after incubation for (a) standard LMX broth, (b) modified LMX broth and (c) modified LMX broth supplemented with 0.1% tryptophan.

Previous authors have reported X-gal as an effective chromogenic substrate for detecting β-D-galactosidase activity of coliform bacteria (Manafi, 1996). The coliform bacteria including *E. coli* could hydrolyse X-gal to indigo dye which had no effect on the viability of the bacterial cells (Manafi and Rotter, 1991). Therefore, the potency of the enzymatic assay and the performances of all testing culture media for detecting *E. coli* were mainly influenced by X-gal quantity. The results indicated that standard LMX broth, modified LMX broth and modified LMX broth supplemented with 0.1% tryptophan showed similar performances for the qualitative detection of coliform bacteria and *E. coli* in the water samples. Similar findings have been previously reported for the determination of coliforms and *E. coli* in drinking water samples (Nikaeen *et al*., 2009) and marine water samples (Geissler *et al*., 2000) using standard LMX culture media and the MTF method.

A difference in the red colour appearance on the broth surfaces resulting from the indole reaction was observed. The resultant compound overlay on the surface of the modified LMX broth was pale red in colour (Figure 1b) and exhibited more slowly than the standard LMX broth for three minutes after the addition of Kovac's reagent. This could be caused by the lack of tryptophan in the modified medium, resulting in an inadequate effect of the indole reaction. The tryptophan source of the modified LMX broth was only from the peptone, whereas the LMX broth contained both tryptose and the ingredient tryptophan. To investigate the effect of adding tryptophan on the indole reaction, 0.1% tryptophan was supplemented with modified LMX broth. The result showed that the overlaying red-colour ring had a deeper

colour (Figure 1c) and exhibited faster than the modified LMX broth without supplementation of tryptophan. Moreover, the blue-green colour of the culture broth was also more deeply coloured because some indole compounds, the by-product of tryptophan from tryptophanase activity, could react with oxygen resulting in indigo dye. The supplementation of tryptophan by more than 0.1% was not significant in the colour level of the red compound (data not shown). This result indicated that exceeding the supplement of tryptophan to the LMX broth was unnecessary, particularly in the practical analysis for saving costs. For practical applications, the modified LMX broth supplemented with 0.1% tryptophan was recommended for improving performance. However, the modified LMX broth without supplementation of tryptophan was preferred for saving costs and economizing on culture medium constituents.

One constraint of standard LMX broth was the application on quantitative determination. The LMX broth only demonstrated the presence or absence of *E. coli* in the water samples. To convert the standard LMX broth to practical enumeration of *E. coli*, the modified LMX agar supplemented with 0.1% tryptophan and the plate count technique were employed. The results showed that the modified LMX agar showed the performance for *E. coli* determination by the dilution of artificial water at 10^{-5}, with the *E. coli* amount of $4.00 \times 10^6 \pm 1.41$ CFU/mL. This was similar to the performance for quantitative enumeration of *E. coli* by the standard Plate Count Agar (Table 1).

Table 1. Quantitative enumeration of *E. coli* in artificial water sample by the plate count technique

Media	**Quantitative enumeration data**	
	Dilution factor	**Amount of *E. coli* (CFU/mL)**
Modified LMX agar supplemented with 0.1% tryptophan	10^{-1}-10^{-3}	TNTC
	10^{-4}	4.85×10^6 ±0.21
	10^{-5}	4.00×10^6 ±1.41
	10^{-6}	0.00
Plate Count Agar	10^{-1}-10^{-3}	TNTC
	10^{-4}	4.85×10^6 ±0.07
	10^{-5}	4.50×10^6 ±0.71
	10^{-6}	0.00

TNTC denotes 'too numerous to count the colonies'.

The *E. coli* colonies cultured on the modified LMX agar supplemented with 0.1% tryptophan were sharp blue (Figure 2a), because of the insolubility of the resultant indigo dye (Manafi, 2000) from the breakdown mechanism of X-gal. However, the colony colour of *E. coli* cultured on the Plate Count Agar was original white (Figure 2c). Interestingly, the indole reaction still occurred when adding Kovac's reagent on the surface of the agar (Figure 2b). For practical applications, the modified LMX agar supplemented with 0.1% tryptophan was preferred for water contamination and water pollution analysis, because of both the bacterial enumeration performance and preliminary distinguished efficacy of *E. coli*. The modified LMX agar without supplementation of tryptophan was unfavourable for practical use because of the poor performance of producing blue-colour colonies and red-colour overlaying on the agar surface (data not shown).

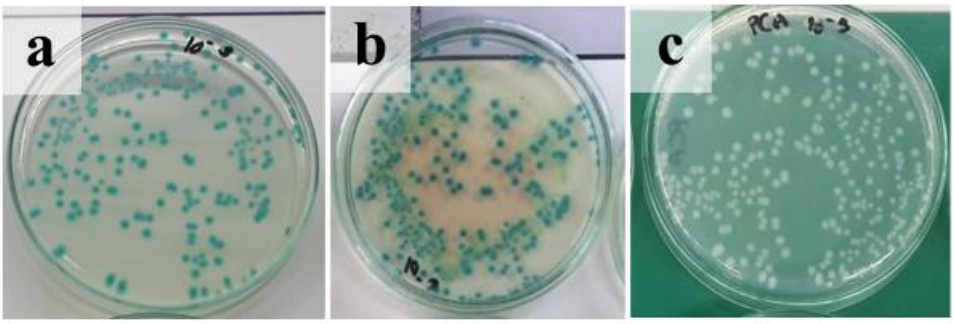


Figure 2. *E. coli* colonies for enumeration on agar plates. (a) Modified LMX agar supplemented with 0.1% tryptophan. (b) Modified LMX agar supplemented with 0.1% tryptophan after overlaying with Kovac's reagent. (c) Plate Count Agar.

Thirty water supply samples were aseptically obtained from different locations at different times during July 2015. The Metropolitan Waterworks Authority and the Provincial Waterworks Authority of Thailand provide guidance on water supply quality regarding biological parameters that *E. coli* must not be detected in 100 mL water samples. Therefore, the qualitative determination by the culture media was sufficient for the detection of *E. coli* in practical or routine uses. The contamination of *E. coli* in the water samples was qualitatively determined by all three culture media, standard LMX broth, modified LMX broth and modified LMX broth supplemented with 0.1% tryptophan. The results revealed that all the culture media used in this rapid enzymatic assay had similar performance for the qualitative determination of *E. coli* in the water supply samples. For the determination of *E. coli* in water supply samples, the modified LMX broth supplemented with 0.1% tryptophan was preferred for practical analysis and the modified LMX broth without supplementation of tryptophan was preferred for reducing the cost of analysis.

CONCLUSION

The experiments on artificial water samples for application on water supply samples determined that the rapid enzymatic assays by the modified LMX broth and the modified LMX broth supplemented with 0.1% tryptophan were practicable alternatives to the traditional LMX broth method for *E. coli* determination in water. The modified LMX broth supplemented with 0.1% tryptophan showed better performance than the modified LMX broth without supplementation with tryptophan, however the former was more expensive in terms of culture media constituents.

ACKNOWLEDGEMENTS

The authors would like to thank Samsen Water Treatment Plant, Metropolitan Waterworks Authority, Thailand for supporting the culture media and the guidance on this research.

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