

A novel strategy to differentiate *Listeria* spp. and other gram-positive foodborne pathogens in the selective enrichment step using modified PALCAM broth

¹Sangadkit, W., ²Saranak, J. and ^{1,3*}Thipayarat, A.

¹Department of Food Engineering, Faculty of Engineering, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand

²Department of Physics, Syracuse University, Syracuse NY 13244-1130, USA

³Office of Education, Faculty of Engineering, Burapha University, Chonburi 20131, Thailand

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Abstract

This research explored alternative rapid screening strategies of *Listeria* spp. detection by the occurrence of blackened broth as a result of the esculin-ferric ammonium citrate reaction. The presence of *Enterococcus faecalis* in industrial food sample can mislead the presumptive results of *Listeria* spp.; hence, incorporation of sugar fermentability tests was exploited to distinguish *Listeria* spp. from other *Listeria* competitors. Various sugar alternatives were used as carbon sources and phenol red was added to indicate pH changes in the modified PALCAM broth (mPB). *Listeria monocytogenes* and other Gram-negative competitors (i.e., *Escherichia coli* and *Salmonella* Anatum) and Gram-positive non-*Listeria* bacteria (i.e., *Staphylococcus aureus* and *E. faecalis*) were cultured in these modified substrates and incubated at 37°C for 24 h. Viable cell count and broth color were monitored using the micro scale cultivation technique and spectrophotometer at discrete wavelengths from 340 to 650 nm, respectively. The results showed that the indicative signals of broth color changes were detected as early as 8-12 h after incubation. All tested strains grew very well in these mPBs maintaining high viable cell counts (10^6 - 10^7 CFU/ml). The esculin activity was very effective in differentiating Gram-positive from Gram-negative cultures at 550 nm. As Gram-positive bacteria, *E. faecalis* produced similar blackened broth similar to what *Listeria monocytogenes* did with the esculin-based broth. However, the use of other sugar supplements (i.e., sorbitol, sucrose, or xylose) improved the optical differentiation of *L. monocytogenes* from *E. faecalis*. The growth of all tested Gram-negative bacteria was inhibited by strong nature of mPB selective agents and other Gram-positive strains were void of esculin activity. The combined sugar fermentability and esculin activity were able to presumptively screen the presence of *L. monocytogenes* in industrial food samples and environmental swabs.

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Introduction

Listeria spp. is one of the most serious pathogenic contaminants present in ready-to-eat meals and food products derived from meat, milk and eggs. The outbreaks of foodborne listeriosis due to *L. monocytogenes* and the economic burdens necessitate a high throughput identification method for detection of *L. monocytogenes* (James *et al.*, 1985; Kvenberg, 1988). Exported food from Thailand to many countries has to be formally certified for no detectable level of *Listeria monocytogenes*. Frequent *Listeria* outbreaks from consumption of contaminated food products and several product recalls of major food manufacturers have also prompted food industry to improve their routine inspection and sanitary/hygiene policy for future prevention of *Listeria* contamination. The desirable industrial protocol for

Listeria identification should be rapid, simple, and cost effective (Anon, 1999).

PALCAM broth (PB), the conventional selective enrichment broth for *Listeria* spp. containing ferric ammonium citrate, esculin, and phenol red (PR) as combined indicators has been widely used to screen *Listeria* in food samples. Some Gram-positive bacteria including all *Listeria* strains hydrolyze esculin to α -D-6, 7-dihydroxycoumarin (esculetin) and glucose. Esculetin readily reacts with ferric ammonium citrate to blackening of the medium from the red broth (Netten *et al.*, 1989). For food industry, blackening of PB is a presumptive evidence of the presence of *Listeria* spp. The absence of blackened broth development indicates *Listeria*-free sample and eliminates the need for further biochemical tests (Capita *et al.*, 2000). The presumptive results are adequate to make a rational QC/QA decision for food

*Corresponding author.

Email: athipaya@yahoo.com

Tel: +66 2 4709246; Fax: +66 2 4709240

production.

On the other hand, other Gram-positive organisms, mainly *E. faecalis*, hydrolyze esculin and produce the same black broth. The esculin activity from other Gram-positive competitors hampers the identification of *Listeria* contamination (Liamkaew and Thipayarat, 2011). Several researchers added selective inhibitors to the *Listeria* spp. enrichment substrates to suppress *E. faecalis* growth and enhance the selectivity to detect only *Listeria* in the food samples. Our previous works show that the typical selective inhibitors are too potent, and substantially discourage the multiplication of target *Listeria* spp. particularly the injured cells (Budu-Amoako *et al.*, 1992; Jacobsen, 1999; Vaz-Velho *et al.*, 2001; Nancy *et al.*, 2004).

Many conventional methods for the identification of *Listeria* species are based on the fermentation of sugar and haemolytic reactions (Seeliger and Jones, 1986). These biochemical tests are universally accepted and commonly used to confirm the target organisms in various pathogenic detection protocols. This work was to combine biochemical reactions, esculin hydrolysis and specific sugar fermentation, in the selective enrichment step. The optical density changes due to esculin activity and acid production from sugar fermentation were used as primary screening of *Listeria* spp. from non-*Listeria* competitors. Different sugar substrates that were preferentially used by the esculin positive non-*Listeria* were included in the modified PB (mPB) to improve the detectability and selectivity of the *Listeria* enrichment broth. Using our high throughput spectrophotometric method, the selective enrichment process was done in a 96-well microplate and quantified by a microplate reader as changes in the OD at 550 nm.

Materials and methods

Bacterial strains

All bacterial used in this study were from the culture collection of the Department of Medical Sciences, Bangkok, Thailand. *L. monocytogenes* DMST 17303 and *L. innocua* DMST 9011 represented *Listeria* contaminants. *E. coli* DMST 4609 and *S. Anatum* DMST 19600 were Gram-negative competitors. *S. aureus* DMST 8840 and *E. faecalis* ATCC 29212 were Gram-positive competitive bacteria. They were stored in slant agar tubes, cultivated in trypticase soy broth (TSB; Lab M, UK.) at 37°C for 24 h. An activated culture was in the range of 10⁶-10⁷ cells/ml. Serial dilutions of 10², 10⁴ and 10⁶ CFU/ml were inoculated in modified

PALCAM (mPB) to observe the effect of initial cell concentration on the OD change.

Media preparation

The standard PALCAM (PB, Lab M, UK.) was used to culture *Listeria* spp. and non-*Listeria* strains to study the development of black color due to esculin hydrolysis. Mannitol was replaced by dextrose, fructose, galactose, maltose, sorbitol, sucrose, or xylose (1% w/v) for mPB.

Cell cultivation

All selective enrichment for *Listeria* spp. were done in 96-well flat bottom microplates. Each well contained 180 µl of each medium and 20 µl of each bacterial strain. The micro plates were incubated at 37°C for 24 h and subjected to OD reading at 0, 2, 4, 6, 8, 10, 12, 18 and 24 h.

Viable cell count was performed using a micro scale cultivation technique on trypticase soy agar (TSA, Lab M, UK) at 0, 4, 8, 12, 18, and 24 h (Kim and Fung, 2005; Pavic *et al.*, 2010; Khueankhancharoen and Thipayarat, 2011; Chenu *et al.*, 2013). Each well of the 96-micro plate takes only 500 µl of TSA and 10 µl of diluted culture. The culture broth was diluted to the desired dilution with 0.85% NaCl solution in water. The micro plates were incubated at 37°C for 24 h. Colony counting by digital photomicrography was performed and calculated as log CFU/ml. Three replications were conducted in this experiment.

Optical density measurement

The broth color change was monitored using a digital camera. The optical density (OD) at 405, 450, 490, 550, 600, and 650 nm was measured by a microplate reader (M965, Metertech, Taiwan) at discrete intervals during 24 h of incubation. The values of OD responding to broth color change were plotted against incubation time to represent the time course of esculin/sugar biochemical metabolism.

Statistical analysis

Analysis of Variance (Duncan's test) was used to determine differences among the means of colony counts under different growth conditions. All statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Significant difference was designated at P<0.05. For the validation in food samples, Cohen KAPPA values were calculated and interpreted according to Petrie and Watson (2003).

Evaluation of proposed mPB rapid *Listeria* detection protocol

Sample preparation

A total of 80 samples comprised of artificially- and naturally-contaminated food samples were tested. The artificial contamination was performed by mixing 1 ml of 1 log CFU/ml *L. monocytogenes* and *E. faecalis* culture and 25 g of sampling food matrix. The sample was enriched with 225 ml of buffered *Listeria* enrichment broth (Oxoid, Basingstoke, Hampshire, England) in a sterile bag. The sample was then homogenized using a stomacher for 2 min at 225 rpm. The aliquot of the BLEB culture was further evaluated by two different techniques.

International Organization for Standardization Method (ISO method)

The sample after enrichment step at 37°C for 24 h was then inoculated in original PB broth and incubated at 37°C for 24 h. After that the sample was diluted approximately (10^1 - 10^2 CFU/ml) and then 10 µl aliquot of PB was streaked onto each plate of Oxford and MOX selective agent to identify *Listeria* spp. and incubated for 48 h at 37°C. The presumptive colonies from each food sample appeared olive-green colonies surrounded by a black halo on Oxford and darkgrey/green colonies with sunken centers on MOX refer to the presence of *Listeria* spp. And then these colonies were subjected to confirmation test.

mPB technique for primary screening of *Listeria* spp.

A 20 µl aliquot of the BLEB culture from each food sample was cultured in mPB with xylose at 180 µl and was done in 96-well flat bottom microplate. The micro plate was incubated at 37°C for 24 h and subjected to OD₅₅₀ reading at 0, 2, 4, 6, 8, 10, 12, 18 and 24 h. Aliquots from samples that showed the blackened broths were diluted to the decimal dilutions of the original suspension (10^1 - 10^2 CFU/ml) and all positive samples were performed as described above.

Confirmatory methods for *Listeria*

The biochemical testing for example; gram positive, catalase positive, and oxidase negative (Becker *et al.*, 2006) were used to confirm the genus of typical *Listeria* colonies.

Moreover, the *Listeria* colonies were further identified by using chromogenic medium (Karpísková *et al.*, 2000). The target colonies were streaked on the medium and incubated at 37°C for 48 h. The typical *L. monocytogenes* forms characteristic blue colonies without a yellow halo and colonies formed by other species of *Listeria* are white, with or without

a yellow. The presence of *Listeria* spp. in a specified sample was recorded positive result.

Results and discussion

Optical density changes due to spectra esculin activity of bacteria in PB broth

To determine OD due to positive esculin activity and the pH color response due to sugar fermentation, *L. monocytogenes* and *S. aureus* inoculated in PB were compared spectrophotometrically at visible wavelength. Initial red color broth with *L. monocytogenes* was gradually transformed to darker red in 6 h as detected by an increase in OD by a microplate reader. The short detection time is probably due to high oxygen transfer rate in the shallow broth in each microwell (Hensler and Schedel, 1991; Duetz, 2007). The spectra of the OD (Figure 1) of esculin-positive, esculin-negative, and control (no bacteria) clearly distinguish the esculin-positive from esculin-negative and control. The optimal wavelength to detect the positive and negative esculin activity was determined at 550 nm which showed maximum difference between esculin-positive and esculin-negative (Figure 1). The OD₅₅₀ was used for the rest of this study.

The standard PB was applied to 6 common food-borne bacteria (*L. monocytogenes*, *L. innocua*, *E. faecalis*, *S. Anatum*, *E. coli*, and *S. aureus*) to determine the OD and pH color response by phenol red indicator in Figure 2. In the selected Gram-positive group, only *L. monocytogenes*, *L. innocua*, and *E. faecalis* convert esculin to esculetin which reacts with ferric ions to change the color of PB to brownish black. *S. aureus* does not possess esculin activity but metabolizes mannitol; hence, the PB broth becomes acidic and turns yellow (Roberson *et al.*, 1992). With lithium chloride, a broad spectrum inhibitor of Gram-negative bacteria (Curtis and Lee, 1995; Charlotte, 1999), in the PB, all tested Gram-negative strains (*S. Anatum* and *E. coli*) were selected out. The PB broth with Gram-negative bacteria remained red as control throughout the course of incubation. Therefore, this method does not require any powerful inhibitors such as acriflavine and nalidixic acid.

The OD₅₅₀ time course (Figure 2) differentiates the control, esculin-positive and esculin-negative in as early as 4 h after incubation. Initially, the OD₅₅₀ of red PB with approximately 10^6 CFU/ml cell inoculation varied from 1.0 and 1.2. Within 2 h, the OD₅₅₀ began to diverge reflecting the types of bacteria inoculated. After 10 h, the OD₅₅₀ was approaching asymptotic values. These numerical figures of OD₅₅₀ readings corresponded well with the development

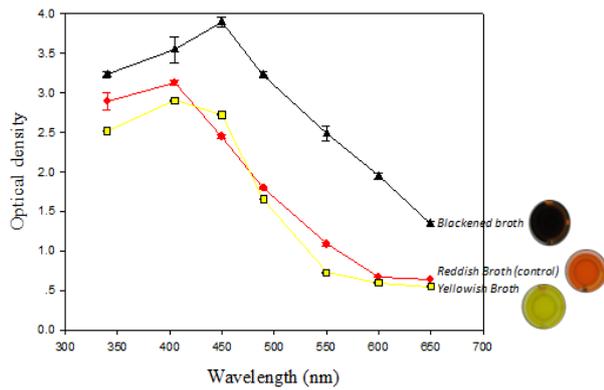


Figure 1. Optical density spectra of the no cell control, blackened broth due to esculin metabolism of *L. monocytogenes*, yellowish broth due to sugar fermentation of *S. aureus* using PB at 37°C for 12 h.

of broth color from red to black showing the esculin activity, or red to yellow indicating sugar fermentation without esculin activity (Figure 2). Several authors used flat bottom 96-well microplates containing 300 μ l of Stuart's broth (SB) to distinguish urease-positive from urease-negative bacteria based on optical density differentiation measured at 430 nm and 560 nm corresponding to the developed pink color in SB. (Stuart *et al.*, 1945; Koneman *et al.*, 2006). Spectrophotometric detection of *Listeria* spp. In environmental and food samples had been studied (Firstenberg-Eden and Shelef, 2000). Tugba and Debora (2013) reported the development of a rapid, high throughput, and quantitative colorimetric assay to determine urease activity. Bacteria from different environmental sources were successfully identified as urease-positive and urease-negative using a microtiter plate reader.

Since both *Listeria* and *E. faecalis* metabolize esculin and develop black broth, *E. faecalis* interfered the interpretation of esculin-positive results (Figure 2). Further modification of the standard PB system to improve differentiation between *Listeria* and *E. faecalis* would be very useful and provided decisive action towards possible *Listeria* contamination in food samples. Apart from being non-pathogenic, *E. faecalis* is physiologically similar to *Listeria*, and both are likely to present in the same food products. High numbers of *E. faecalis* without *L. monocytogenes* during enrichment could produce false-positive and lead to the needs for lengthy and expensive biochemical analysis.

Proposed sugar substitutes to differentiate L. monocytogenes from E. faecalis using PALCAM-based broths

The conventional PB contains esculin and D-mannitol as the indicative and alternative carbon

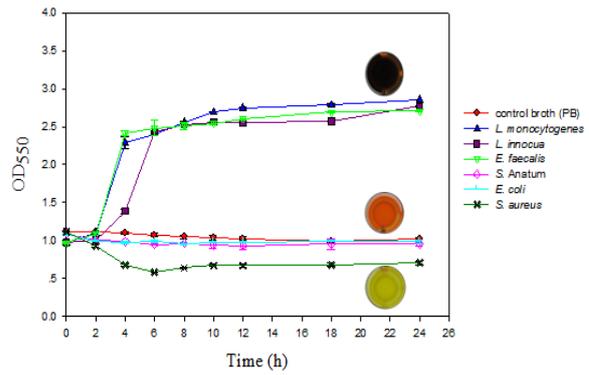
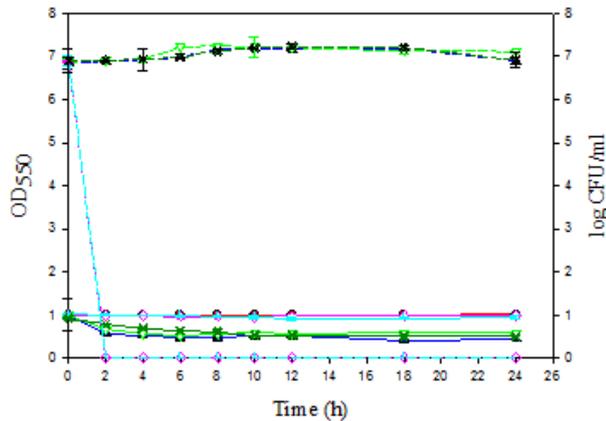


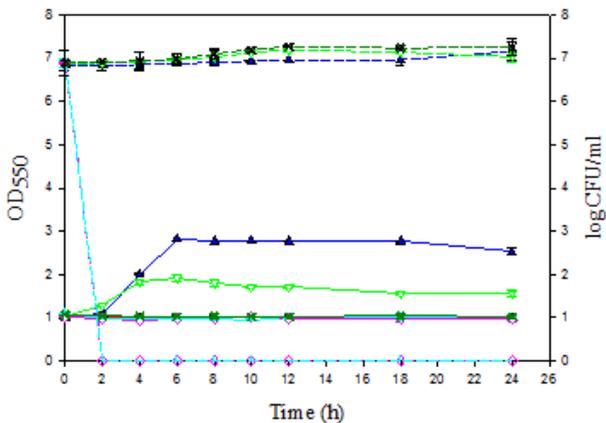
Figure 2. OD₅₅₀ time course of esculin-positive and esculin-negative bacteria (10^6 CFU/ml) cultured in the standard PB

sources. The latter was not preferable and assimilable by *L. monocytogenes* as evidenced by the development of black color and consequently high OD as the incubation progressed (Figure 2). Mannitol allowed easy distinction of *Listeria* spp. from *S. aureus* but not *E. faecalis* even at cell density of approximately 10^6 - 10^7 CFU/ml (Netten *et al.*, 1989). Therefore, our goal was to substitute mannitol with other kinds of sugar that only *E. faecalis* but not *Listeria* spp. preferred to metabolize more than esculin. Only metabolized esculin yields black broth, metabolized sugar yields yellow. The difference in OD of final broth color would provide a reliable signal to further differentiate *Listeria* from *E. faecalis*. Seven modified PB broths with 7 different sugar substitutes (i.e., dextrose, fructose, galactose, maltose, sorbitol, sucrose, or xylose) were inoculated with each bacteria tested. Colony counts of *L. monocytogenes*, *E. faecalis*, and *S. aureus* during the course of incubation time in all mPB broths showed no statistically difference among them (ANOVA, $P > 0.05$).

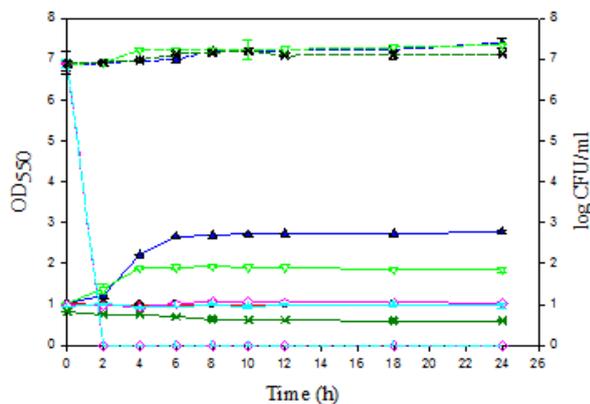
The 3 kinds of Gram-positive bacteria grew equally well in all mPB (Figure 3a, b, c, d and e). On the other hand, preferential metabolism of esculin and sugar was different among bacteria. Both *Listeria* and *E. faecalis* preferred to metabolize dextrose (Figure 3a) more than esculin (Petran *et al.*, 1989). The mPB with either strain turned from red to yellow within 6-12 h and the OD₅₅₀ decreased to lower that of the control. Perhaps the inability of esculin utilization was caused by the changes in the pH of the mPB and the accumulation of sugar fermentation by-products (Ziad *et al.*, 2002). In particular, the replacement of mannitol by maltose, a disaccharide composing of glucose and glucose, facilitated *Listeria* spp. to metabolize glucose for energy but the replacement of mannitol by fructose and maltose (data not shown) showed similar results to that by dextrose. Moreover



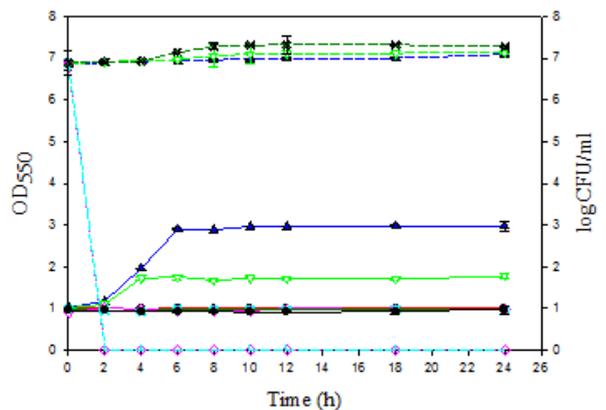
(a) Dextrose



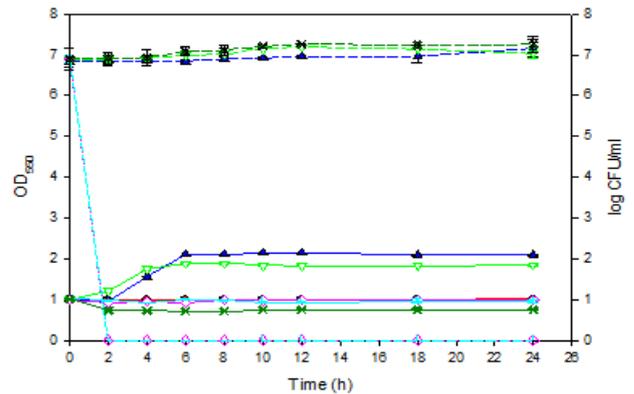
(b) Sorbitol



(c) Sucrose



(d) Xylose



(e) Galactose

Figure 3. OD_{550} and cell growth time course of *L. monocytogenes* and some competitors in mPB with sugar fermentable by *E. faecalis* not by *L. monocytogenes*. (OD_{550} , opaque line; \bullet , control broth (PB); \blacktriangle , *L. monocytogenes*; ∇ , *E. faecalis*; \diamond , *S. Anatum*; + , *E. coli* and \times , *S. aureus*). (cell growth, dashed line; \blacktriangle , *L. monocytogenes*, ∇ , *E. faecalis*; \diamond , *S. Anatum*; + , *E. coli* and \times , *S. aureus*).

the use of mPB was effective enough in suppressing the growth of Gram-negative competitors such as *E. coli* and *S. Anatum* (Figure 3a, b, c, d and e). In all mPB, the OD_{550} time course of both Gram-negative and Gram-positive competitors except *E. faecalis* was differentiable from *L. monocytogenes*. Four sugars (sorbitol, sucrose, xylose, and galactose), on the other hand, were preferentially metabolized only by *E. faecalis* not *Listeria* spp. and produced lighter color of final broth than *Listeria* did (Figure 3b, c, d and e). The different shades of black broths were detectable by eye and quantitatively by spectrophotometer. With equal cell growth, the difference in OD_{550} can be used to differentiate *Listeria* from *E. faecalis*.

The mPB broth with sorbitol or xylose showed the highest distinction of broth color difference as observed by eye. The OD_{550} readings of the sorbitol or xylose mPB reasonably agreed with the color seen by eye (Figure 3b and d). All four sugar replacements (sorbitol, sucrose, xylose, and galactose) reduced the black ferric ammonium citrate produced by *E. faecalis* which could utilize these substituting sugars and generated different yellowish shade. *L. monocytogenes* apparently had lesser preference towards sorbitol, sucrose, and xylose than esculin and turned the mPB black with as high OD_{550} as grown in original PB. Lighter tone of the black broth with *E. faecalis* was assumed as a result from the diauxic growth on the second sugar supplement in addition to esculin utilization. The supplement with fast-absorbing sugar provides a surplus of easy-accessible energy for fermentation and/or respiration.

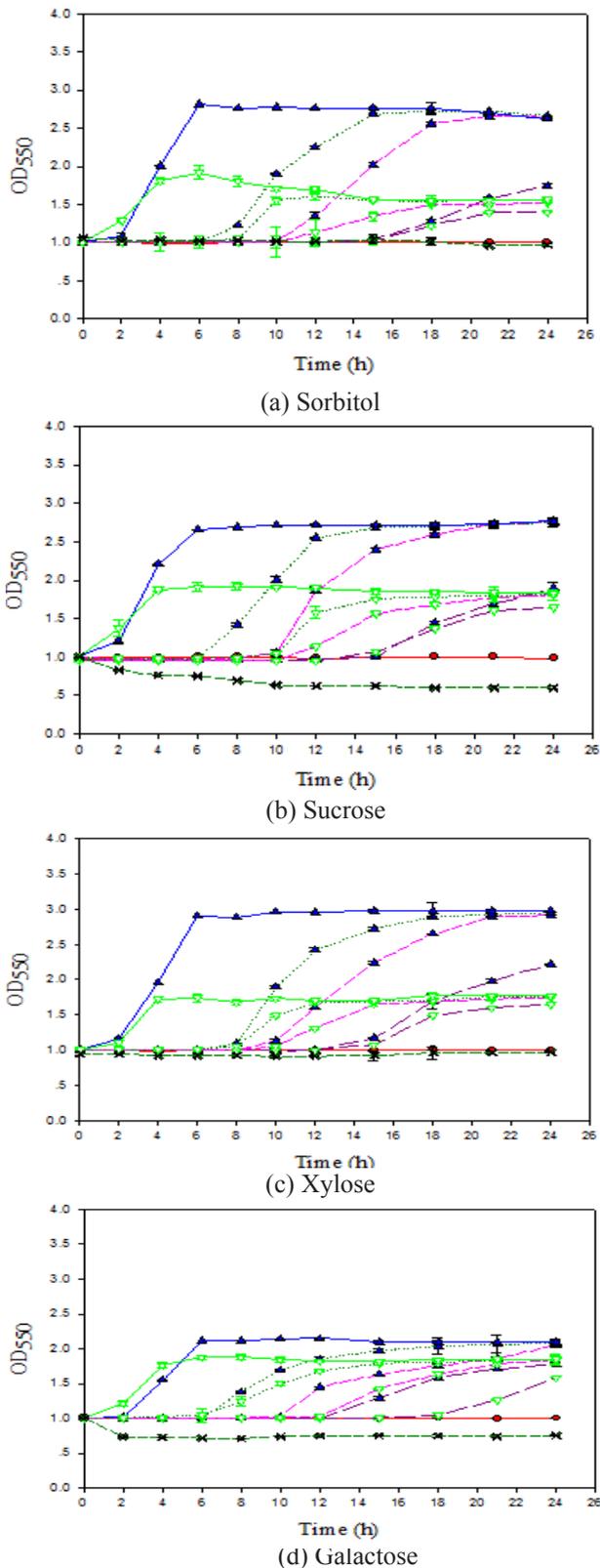


Figure 4. Time course of esculin hydrolysis (increased OD_{550}) by *L. monocytogenes*, *E. faecalis* and *S. aureus* at various initial cell densities (●, control broth (PB); *L. monocytogenes*: ▲ (10), ▲ (10²), ▲ (10⁴), ▲ (10⁶); *E. faecalis*: ▽ (10), ▽ (10²), ▽ (10⁴), ▽ (10⁶); *S. aureus*: × (10⁶) CFU/ml) in mPB with different sugar substitutes.

If one sugar is more favorable than the other, it will be metabolized faster producing more intracellular energy molecules and activity (Romana and Conway, 1996).

Effect of initial cell concentration on the color change of the proposed mPB

To evaluate the sensitivity of the proposed mPB (substitution of mannitol by sorbitol, sucrose, xylose, and galactose), *L. monocytogenes* and *E. faecalis* at 10, 10², 10⁴, 10⁶ CFU/ml and *S. aureus* at 10⁶ CFU/ml initial cell concentrations were inoculated in the microwell plates and OD_{550} was monitored for 24 h (Figure 4). *S. aureus* inoculation was included as a negative control and showed coherent results in both fermentable (sucrose and galactose) and non-fermentable (sorbitol and xylose) sugar sources. Figure 4 shows that the discrimination of *L. monocytogenes* from *E. faecalis* by this spectrophotometric method was highly dependent on the sugar sources. By varying the initial cell densities, galactose showed the poorest OD_{550} differences between *L. monocytogenes* and *E. faecalis* cultivation. Sorbitol, sucrose and xylose substitutions, on the other hand, produced the largest difference of OD_{550} readings.

Different initial cell densities affected the onset of broth color development (Figure 4). Higher initial cell concentrations in the mPB, especially with sorbitol and xylose substitutions resulted in faster detection of increased OD_{550} . At 10⁶ CFU/ml of both bacteria, the divergence of broth color was detected as fast as 6 h. Similar results were observed by Firstenberg-Eden and Shelef (2000) who could detect esculin activity within 3 h of incubation of *L. monocytogenes* 10⁷ CFU/ml. At low initial cell concentrations (approximately 10²-10⁴ CFU/ml), there were noticeable delays of esculin hydrolysis and of *L. monocytogenes* and *E. faecalis* differentiation (up to 16-22 h after incubation). When the initial cell concentration was 10 CFU/ml, poor signals of the optical density differences led to erratic results. So the minimum cell densities required to detect the diauxic growth on the sugar supplements in addition to esculin utilization within 24 h was 10² CFU/ml. The initial cell concentration at 10⁶ CFU/ml was more effective in differentiating *L. monocytogenes* from *E. faecalis* than lower initial cell concentration. These results support the standard detection protocol of *Listeria* (Curtis and Lee, 1995; ISO 11290:98, 1998; BAM, 1998; USDA/FSIS, 2005). In final food products, *Listeria* came from raw materials and processing environment in low numbers and as injured forms. To protect the false negative results, most standard *Listeria* detection protocols require

Table 1. Detection of *Listeria* by a standard method and mPB with xylose protocol in food samples

Samples		Number of samples	Number of <i>Listeria</i> positive samples	
			Standard (ISO, 1998)	mPB
Artificial contamination	Sterilized milk	5	5 (100%)	5 (100%)
	Chicken meat	10	8 (80%)	10 (100%)
	Pork meat	10	9 (90%)	10 (100%)
Natural contamination	Sterilized milk	5	0 (100%)	0 (100%)
	Chicken meat	10	7 (70%)	10 (100%)
	Pork meat	10	9 (90%)	10 (100%)
Industrial samples		30	8 (26.66%)	10 (33.33%)
Total		80	46 (57.50%)	55 (68.75%)

pre-enrichment step by using a non-selective enrichment broth (i.e., buffered *Listeria* enrichment broth, Trypticase Soya Broth) to recover injured cells and to multiply target bacteria to up to 10^6 - 10^8 CFU/ml before selective enrichment.

Evaluation of a mPB for the isolation of Listeria in food samples

The mPB with xylose in Figure 3d showed the highest distinction of broth color for differentiating *L. monocytogenes* from *E. faecalis*. So the mPB with xylose was used as the model for testing the efficacy of the proposed protocol with commercial and industrial samples (i.e., milk, chicken meat, pork meat, and industrial samples). The proposed protocol was compared with the conventional method (ISO, 1998). Of the 80 samples examined, 55 (68.75%) were found to be positive for *L. monocytogenes* using the mPB with xylose protocol and 46 (57.50%) by the ISO standard method (Table 1). The results of artificially and naturally contaminated samples by testing with mPB showed high accuracy results than that of the standard method and both detection methods were assessed for the agreement between the two methods using 2×2 contingency table. Statistical analysis showed the value of Cohen KAPPA was 0.95 indicating a very good agreement (Cohen KAPPA > 0.8).

Conclusion

The mPB with sorbitol, sucrose, or xylose substitution for mannitol provided a good selective broth system to differentiate *Listeria* from *E. faecalis* by optical density measurement. The combination of high throughput microscale technique and

spectrophotometric detection of esculin metabolism in PALCAM based broth are promising as an effective and fast check for *Listeria* contamination in food samples. OD_{550} was conveniently used to differentiate between blackened (positive-esculin) and yellow (negative-esculin) broths. Substitution of mannitol in the conventional PB by sorbitol or xylose provided conclusive identification for *L. monocytogenes* or *E. faecalis* as detected by eye or spectrophotometer. The minimum cell densities for differentiating *Listeria* from *E. faecalis* was 10^2 CFU/ml within 24 h incubation. This technique can be conveniently applied to detect the presence of *L. monocytogenes* in industrial food samples and environmental swabs making this method a good alternative for rapid quantification and screening of *Listeria* and *E. faecalis*.

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