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Fluorogenic Selective and Differential Medium for Isolation of *Enterobacter sakazakii*

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4-Methylumbelliferyl-α-D-glucoside, the fluorogenic substrate of α-glucosidase, was used as a selective marker to develop a differential medium for *Enterobacter sakazakii*. This bacterium showed strong fluorogenic characteristics clearly distinguishable from other microorganisms. On the basis of reducing background noise, an optimum basal medium and nitrogen source were selected. Incubation conditions were optimized.

Enterobacter sakazakii has been implicated in severe forms of neonatal infections such as meningitis and sepsis (1, 2, 3, 4, 6, 9, 10, 14, 16, 22). Although the natural habitat of *E. sakazakii* is unknown, dried infant formula has been epidemiologically identified as the source of *E. sakazakii* infections (7, 18, 20).

Violet red bile glucose (VRBG) agar is one of the media currently used for isolation and enumeration (19), but it is not sufficiently selective, and the other medium, tryptic soy agar (TSA), requires a long (48- to 72-h) incubation time to produce characteristic yellow-pigmented colonies. Furthermore, yellow-pigmented *Enterobacteriaceae* can be isolated from clinical specimens and fresh water (11).

Muytjens et al. (15) described enzymatic profiles of *E. sakazakii* and related species with specific reference to the α-glucosidase reaction. Farmer et al. (5) found 53 of 57 strains of *E. sakazakii* to be positive for α-glucosidase activity. Therefore, detection of α-glucosidase activity would be a powerful tool to use in developing a differential medium.

4-Methylumbelliferyl-α-D-glucoside is a substrate for α-glucosidase and becomes fluorogenic by cleavage of the free 4-methylumbelliferyl moiety when exposed to long-wave UV radiation. To date, no studies have been performed on *E. sakazakii* for differentiation and selection using α-glucosidase activity with a fluorogenic substrate. This study was undertaken to develop a selective and differential medium for *E. sakazakii* using α-glucosidase activity.

Bacterial strains used in this study are listed in Table 1. All bacteria were cultured in tryptic soy broth and combined to construct culture cocktails. *E. sakazakii* (four strains) culture mixed cocktail was used in the basal medium selection. The background culture mixed cocktail (16 strains excluding *E. sakazakii*) was used for basal medium selection and nitrogen source optimization. The total culture mixed cocktail (20 strains including *E. sakazakii*) was used to determine optimal growth conditions and was used in the verification test. Data for each treatment were analyzed statistically by the *t* test (factor equals media) using the SAS general linear models procedure (17).

Among the α-glucosidase substrates, 4-nitrophenyl-α-D-glucopyranoside and 4-methylumbelliferyl-α-D-glucoside were tested as possible markers. 4-Nitrophenyl-α-D-glucopyranoside forms yellow-colored colonies and 4-methylumbelliferyl-α-D-glucoside produces fluorescent colonies under UV irradiation (365 nm). However, 4-nitrophenyl-α-D-glucopyranoside has limitations because the yellow breakdown product, 4-nitrophenol, is easily diffusible on agar, making it difficult to read (8, 13). But 4-methylumbelliferyl-α-D-glucoside produces distinct, not easily diffusible, and extraordinarily brilliant fluorescent *E. sakazakii* colonies. The tested *Escherichia coli*, *E. coli* O157:H7, and *Enterobacter cloacae* did not produce fluorescent colonies like those of *E. sakazakii*.

In the selection of basal medium, three kinds of media were tested, including (i) selective and differential media (VRBG agar), (ii) selective media without a differential substrate (tryptone bile agar), and (iii) nonselective media without a differ-

TABLE 1. Bacterial strains used to evaluate selective and differential media

Strain	Source[a]
Enterobacter sakazakii ATCC 51329	ATCC
Enterobacter sakazakii ATCC 29544	ATCC
Enterobacter sakazakii ATCC 29004	ATCC
Enterobacter sakazakii ATCC 12868	ATCC
Escherichia coli O157:H7 ATCC 35150	ATCC
Escherichia coli O157:H7 ATCC 43889	ATCC
Escherichia coli O157:H7 ATCC 43890	ATCC
Escherichia coli ATCC 25922	ATCC
Escherichia coli B E4a	WSU
Escherichia coli K-12 2B	WSU
Klebsiella pneumoniae K1a	WSU
Klebsiella pneumoniae Revco 41	WSU
Klebsiella pneumoniae Revco 55	WSU
Pseudomonas aeruginosa ATCC 15442	ATCC
Salmonella enterica 6170	WSU
Salmonella enterica serovar Typhimurium ATCC 19585	ATCC
Salmonella enterica 4509	WSU
Enterobacter aerogenes ATCC 13048	ATCC
Enterobacter cloacae Rev 1210 Case 00–5395	WSU
Enterobacter cloacae Rev 1343 Case 00–12286	WSU

[a] ATCC, American Type Culture Collection (Manassas, Va.); WSU, Food Science and Human Nutrition bacteria collection at Washington State University (Pullman, Wash.).

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TABLE 2. Selectivity of 4-methylumbelliferyl-α-D-glucoside solid media and their background noise production after incubation at 30°C for 24 h

Media	Recovery of microbial flora (%)[a]		Background noise (%)[b] with 16-strain cocktail
	E. sakazakii cocktail	16-strain cocktail	
VRBG agar	69.7	72.2	52.4[b]
Tryptone bile agar	92.2	91.3	1.0[a]
TSA	100.0	100.0	43.5[b]

[a] Colonies recovered on VRBG and tryptone bile agar expressed as percentage of CFU ml^{-1} recovered on TSA.
[b] Occurrence of nontarget fluorescent colonies expressed as percentage of total CFU ml^{-1} recovered on TSA. Values followed by different superscript letters are statistically different ($P \leq 0.05$).

ential substrate (TSA). All media included 4-methylumbelliferyl-α-D-glucoside as a substrate.

Since α-glucosidase activity is not unique to *E. sakazakii*, the performance and utility of 4-methylumbelliferyl-α-D-glucoside as a selection marker cannot be separated from the selective aspects of the medium. Also, reducing background noise produced by other bacteria is another important technique for increasing sensitivity of medium. Therefore, selection was performed using two criteria: improving selectivity and reducing background noise. The degree of selectivity was calculated as the ratio between the colonial counts on the reference unselective medium (TSA) and the counts observed on VRBG agar and tryptone bile agar (Table 2). When the *E. sakazakii* culture mixed cocktail was used for enumeration, tryptone bile agar had a higher recovery ratio (92.2%) than did VRBG agar (69.7%).

Background noise was defined as the ratio of fluorescent colonies (in the absence of *E. sakazakii*) versus total colonies. Background noise was monitored for fluorescent colonies that would indicate false-positive results. VRBG agar (52.4%) and TSA (43.5%) yielded a high percentage of false-positive colonies, but tryptone bile agar had a low background noise calculated at 1.0%.

To optimize the basal medium for isolation of only *E. sakazakii*, different nitrogen sources were tested to reduce background noise. A background culture mixed cocktail (excluding *E. sakazakii*) was plated onto medium containing different nitrogen sources (20 g/liter) (Table 3). Proteose Peptone III (72.66%) produced the highest background noise, followed by Bacto Peptone (56.35%) and Proteose Peptone I (11.60%). Tryptone (0.68%) yielded the lowest background noise; after that, the tryptone concentration was also optimized. The lowest level of tryptone produced the highest background noise. As tryptone concentration increased, background noise decreased. Tryptone concentrations of 40 g/liter (0.37%) and 20 g/liter (0.62%) produced low levels of background noise that were not statistically different from each other ($P \leq 0.05$). But tryptone at a concentration of 40 g/liter increased the medium turbidity that negatively affected the discrimination of fluorescent colonies under UV light.

TABLE 3. Selection of nitrogen source and optimization of tryptone concentration at incubation of 30°C for 24 h tested with background culture mixed cocktail

Nitrogen source	Background noise (%)[a]	Tryptone concentration (g/liter)	Background noise (%)
Bacto Peptone	56.35 ± 2.48[c]	40	0.37 ± 0.65[a]
Tryptone	0.68 ± 0.59[a]	20	0.62 ± 0.54[a]
Proteose Peptone I	11.60 ± 3.38[b]	10	10.03 ± 4.01[b]
Proteose Peptone II	1.38 ± 0.74[a]	5	48.85 ± 4.28[c]
Proteose Peptone III	72.66 ± 10.19[d]	2.5	77.33 ± 12.20[d]

[a] Occurrence of nontarget fluorescent colones expressed as percentage of total CFU ml^{-1} recovered on TSA; means ± standard deviations of three replicated plates. Values followed by different superscript letters are statistically different ($P \leq 0.05$).

TABLE 4. Effect of incubation time and temperature on the recovery of fluorescent *E. sakazakii* colonies and total microorganisms tested with total culture mixed cocktail

Time (h)	Fluorescent colonies[a]		Total colonies		Fluorescent colonies/total colonies (%)[b]	
	30°C	37°C	30°C	37°C	30°C	37°C
18	0.67 ± 0.58	5.33 ± 1.53	77.33 ± 6.66	87.67 ± 4.51	0.87[a]	6.08[b]
24	2.33 ± 1.15	7.33 ± 3.06	78.67 ± 6.43	88.67 ± 3.51	2.96[a]	8.27[b]
48	5.33 ± 1.53	7.33 ± 3.06	84.33 ± 7.51	89.33 ± 3.06	6.32[b]	8.21[b]

[a] Means ± standard deviations of three replicated experiments.
[b] Values followed by different superscript letters are statistically different ($P \leq 0.05$).

The newly developed OK medium is formulated as follows: tryptone, 20.0 g; bile salts no. 3, 1.5 g; agar, 15.0 g; sodium thiosulfate, 1.0 g; ferric citrate, 1.0 g; and 4-methylumbelliferyl-α-D-glucoside, 50.0 mg per liter. Sodium thiosulfate and ferric citrate were added as secondary selective markers for differentiation of H_2S-producing *Enterobacteriaceae* (*Citrobacter*, *Salmonella*, *Edwardsiella*, *and Proteus*) (12).

The effect of incubation times (18, 24, and 48 h) and temperatures (30 and 37°C) on the microorganisms was evaluated (Table 4). At 30°C, the number of fluorescent colonies increased dramatically up to 48 h but the total number of colo-

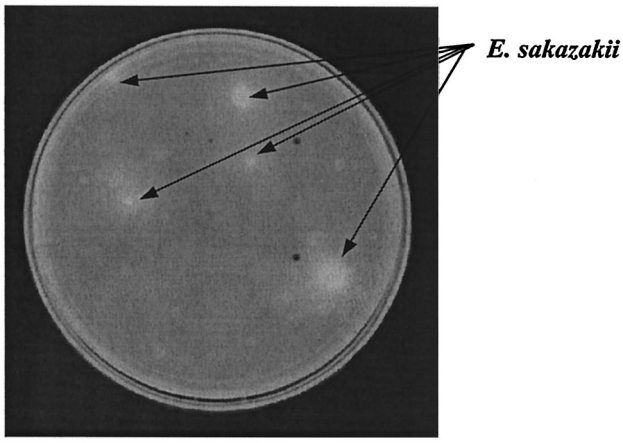

FIG. 1. Differentiation of *E. sakazakii* using the newly developed OK medium. The distinct fluorescent colonies are identified as *E. sakazakii*, and black spotted colonies are H_2S-producing microorganisms.

TABLE 5. Verification of fluorescent and nonfluorescent colonies on OK medium

Temperature (°C)	Fluorescent colonies		Nonfluorescent colonies	
	Examined	Verified (%)[a]	Examined	Verified (%)
30	24	24 (100.0)	22	0 (0.0)
37	24	24 (100.0)	22	0 (0.0)

[a] Verified as *E. sakazakii* by API 20E test and oxidase test.

nies did not. But at 37°C, a restricted increase in fluorescent colonies occurred, while total colonies increased with incubation time. Incubation for 24 h yielded the highest fluorescent-to-total colony ratio and further incubation (48 h) made it difficult to discriminate due to fluorescence diffusion. Villari et al. (21) reported almost the same phenomena in an experiment with other fluorogenic substrates. More fluorescent colonies were observed after 37°C incubation than after 30°C. Thus, 24-h incubation at 37°C was determined as the optimal growth conditions for differentiation of *E. sakazakii*.

When the total culture mixed cocktail (including *E. sakazakii*) was plated onto OK medium and incubated at 37°C for 24 h, distinct fluorescent colonies appeared when exposed to long-wavelength UV light (Fig. 1).

In verification tests, the fluorescent colonies were selected and subcultured on TSA in order to assess the likelihood of false positives and false negatives. The fluorescent colonies were confirmed by using API 20E biochemical systems and the oxidase test (Table 5). A total of 48 fluorescent colonies were examined, and all colonies were verified as *E. sakazakii* (100.0%), while none of the 44 nonfluorescent colonies were identified as *E. sakazakii*.

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