J. Microbiol. Biotechnol. (2001), **11**(3), 491–496

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Improvement of Decarboxylating Agar Medium for Screening Biogenic Amine-Producing Bacteria in Kimchi

MAH, JAE-HYUNG, SOON-YOUNG SHIN, HEUNG-SHICK LEE, HONG-YON CHO, AND HAN-JOON HWANG*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Received: February 6, 2001
Accepted: April 23, 2001

Abstract A modification of decarboxylating agar medium as described by Niven was performed to improve the detection method of biogenic amine-producing bacteria and to eliminate the false-positive. A total of 120 bacterial strains isolated from kimchi were used to evaluate different decarboxylating agar media and for screening biogenic amines. Potential false-positives ranged from approximately 66 to 79% of the strains tested in the already well-known media. In our improved medium, none of the 120 strains showed the potential false-positives. There was a good agreement (81.7%–87.5%) between the results obtained by the improved medium and by HPLC analysis. Consequently, this medium was greatly improved in screening biogenic amine-producing bacteria and discarding false-positives. Of the 120 kimchi isolates, 14.2, 18.3, 37.5, and 0.8% were found by HPLC to be the producers of histamine, tyramine, putrescine (as a form of spermine), and cadaverine, respectively. The proportion of biogenic amine producer during kimchi fermentation increased to a maximum at an immature period and decreased thereafter.

Key words: Biogenic amines, decarboxylating agar medium, false-positive, HPLC, kimchi

Biogenic amines can be produced and degraded as a result of normal metabolic activity in animals, plants, and microorganisms, and are mainly produced by microbial decarboxylation of amino acids in various food products [8]. The frequently observed foodborne intoxications are caused by biogenic amines involving histamine [8]. Common symptoms are nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, a bright red rash, oral burning, and hyper- or hypotensions. Tyramine has been identified as the major mutagen precursor [19]. An additional health risk is recognized as a possible role of biogenic amines, which are the known precursors for carcinogenic nitroso compounds [23].

Biogenic amines can be formed not only in spoiled food but in fermented food as well. In kimchi, a Korean traditional fermented vegetable, the presence of low level of tyramine was reported by Mower and Bhagavan [16]. Biogenic amine-producing lactic acid bacteria have been found in several types of foods including dry sausage and fermented vegetables such as sauerkraut [15, 22]. Therefore, biogenic amines including tyramine can also be produced in kimchi.

The methods used for analyzing individual amines are generally not only extremely difficult, but expensive and time consuming as well. Paper chromatography, thin layer chromatography (TLC), gas liquid chromatography (GLC), and high performance liquid chromatography (HPLC) have all been advocated for the isolation and identification of amines or amine derivatives. On the other hand, differential plating media based on the pH shift by biogenic amine formation have been developed by various authors [5, 6, 10, 14, 17, 18, 24], which are rapid, cheap, and simple methods for detecting biogenic amines. However, some reports have described false-positive microorganisms [1, 11, 20], due to the formation of other alkaline bacterial products [2, 4, 9, 12, 21].

This study was conducted to develop a differential medium that was improved for screening biogenic amine-producing bacteria and discarding the false-positives. It was also performed to determine the ability of kimchi isolates to producing biogenic amines.

Materials and Methods

Test Microorganisms

One hundred and twenty bacterial strains have been isolated from kimchi [13]. Stock cultures were maintained on lactobacilli-MRS (MRS, Difco) agar slants at 4°C.

*Corresponding author
Phone: 82-2-923-7769; Fax: 82-2-3290-3437;
E-mail: hjhwang@korea.ac.kr

Detection of Biogenic Amine Producer and the Potential False-Positive

Biogenic amine formation was tested by inoculating each strain on various decarboxylating agar media as described by Niven *et al.* [18], Yoshinaga and Frank [24], and Joosten and Northolt [10]. Each medium was supplemented with 0.5% (w/v) of different amino acids (L-histidine hydrochloride monohydrate, L-tyrosine, L-ornithine hydrochloride, and L-lysine hydrochloride) (all from Sigma). For evaluating the potential false-positive (PFP), the medium without amino acid was used as the control [1].

The PFP means that a strain shows a positive result even in the control medium without corresponding amino acid. To avoid PFP and false-positives (FP) and to improve the differentiation of biogenic amine-producing bacteria from a nonproducer, various modifications of Niven's medium were carried out in this study. The composition of the basal medium was 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, and 3% agar.

An improved decarboxylating agar medium was used for qualitative detection of the biogenic-amine-producing capacity of the strains isolated from kimchi. This contained 0.125% tryptone, 0.125% yeast extract, 0.75% $(NH_4)_2SO_4$, 0.5% NaCl, 0.1% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$, 0.004% $FeSO_4 \cdot 7H_2O$, 0.05% Tween 80, 0.02% cresol red, 3% agar, and 2% of corresponding amino acid, adjusted to pH 5.3, and sterilized at 121°C for 10 min.

Colonies were picked and seeded directly onto each medium and incubated at 30°C for 24/48 h, and the plates were observed every 3 h during the first 24 h period. The presence of red colonies surrounded by a purple or deep red halo on a yellowish background indicated a positive reaction.

Detection of Individual Biogenic-Amine-Producing Bacteria

All strains were investigated for their ability to produce histamine. Strains were seeded in tryptic soy agar (TSA, Difco) plates enriched with 0.1% L-histidine hydrochloride and incubated at 30°C for 24 h. A loopful from the culture was then inoculated in 9 ml of tryptic soy broth (TSB, Difco) with 2% histidine (pH 5.8) supplemented with 0.0005% pyridoxal-HCl (Sigma). One-milliliter aliquots of these cultures were transferred to new tubes containing 9 ml of the same broth, which were incubated. Five milliliters of these broth cultures were taken with a sterile syringe, filtered through a 0.2 μm membrane (Millipore), and kept at −25°C until it was assayed by HPLC. Other biogenic amines – tyramine, putrescine, and cadaverine – were also determined in TSB with 1% L-tyrosine, 2% L-ornithine hydrochloride, and 2% L-lysine hydrochloride, respectively, and supplemented with 0.0005% pyridoxal-HCl at pH 5.8.

Determination of Biogenic Amine by HPLC

Determinations of histamine, tyramine, putrescine, and cadaverine in the filtered broth cultures were carried out by HPLC, according to the procedure developed by Eerola *et al.* [7] and modified by Ben-Gigirey *et al.* [3, 4].

Preparation of amine standard solutions. Amine standard solutions were prepared as follows. First, stock solutions of histamine, tyramine, putrescine, and cadaverine (all from Sigma) were prepared separately in Milli-Q water at a concentration of 10,000 ppm. Next, working solutions of amines at concentrations of 100 or 1,000 ppm were prepared by diluting 100 μl or 1,000 μl of each stock solution in Milli-Q water to bring to a final volume of 10 ml.

Preparation of extracts. Biogenic amine extracts were prepared by adding 9 ml of 0.4 M perchloric acid (Merck) to 1 ml of the filtered broth culture, and the mixture was homogenized with a vortex mixer. After centrifugation of the homogenate at 3,000 rpm for 10 min, the supernatant was filtered through the Whatman paper No. 1.

Derivatization of sample extracts and mixed standards. One milliliter of the biogenic amine extract was mixed with 200 μl of 2 N sodium hydroxide and 300 μl of saturated sodium bicarbonate. Two milliliters of dansyl chloride (Sigma) solution (10 mg/ml) prepared in acetone were added to the mixture, which was then incubated at 40°C for 45 min. Residual dansyl chloride was removed by adding 100 μl of 25% ammonium hydroxide. After 30 min, the extracts were adjusted to 5 ml with acetonitrile. Finally, the mixture was centrifuged at 2,500 rpm for 5 min and the recovered supernatant was filtered through 0.5 μm pore-size filters (Millipore).

Chromatographic separations. A Waters 2690 separation module equipped with a Waters 996 photodiode array detector and Millenium 2010 software was employed. A Nova-Pak C18, 4 μm, 150 by 3.9 mm column (Waters) was employed. Ammonium acetate (0.1 M) (Merck) (solvent A) and acetonitrile (Merck) (solvent B) were used as mobile phases. The program involved a linear elution gradient starting at 50% of solvent B and reaching up to 90% of the solvent over 19 min. Flow rate was 1 ml/min. The sample volume injected was 20 μl and the column effluent was monitored at 254 nm.

RESULTS

Detection of Potential False-Positives (PFP) and Development of Decarboxylating Agar Medium for Detection of Biogenic-Amine-Producing Bacteria

As shown in Fig. 1, the PFP strains were detected in 65.8, 79.2, and 72.5% of the strains tested in the decarboxylating agar media described by Niven *et al.* [18], Yoshinaga and Frank [24], and Joosten and Northolt [10], respectively.

As shown in Fig. 2, PFP was reduced from 65.8% to 39.2% by adding 0.1% glucose. Cresol red that was substituted for bromocresol purple showed a decrease

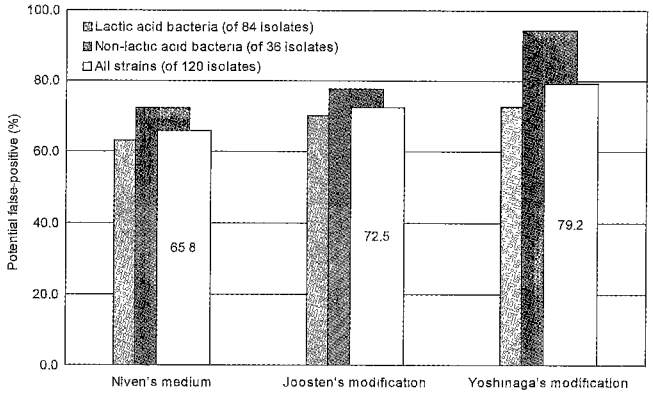

Fig. 1. Evaluation of three decarboxylating agar media to detect biogenic-amine-producing bacteria.

in PFP from 65.8% to 35.8%. Besides this, PFP slightly diminished to 31.7% by adding both glucose and cresol red at the same time. The complete substitution of ammonium sulfate for organic compounds as the nitrogen source seemed to be problematic because almost all the tested bacteria were not well-grown, while the combination of 0.75% ammonium sulfate, 0.125% tryptone, and 0.125% yeast extract (6:1:1, w/w/w) was highly effective not only for growth of the bacteria but also for eliminating PFP (Table 1). Additionally, some metal sulfates (Mg, Mn, and Fe) and Tween 80 were added to enhance the growth of lactic acid bacteria. To increase the intensity of positive reaction by biogenic amines, the amino acid concentration of 2.0% was also established. When lower concentrations were used, a weak color change was observed. According to the result, it was shown that none of the 120 strains showed PFP in this improved medium. This indicated that the medium was improved remarkably in screening biogenic-amine-producing bacteria and discarding PFP when compared with the already well-known decarboxylating agar media.

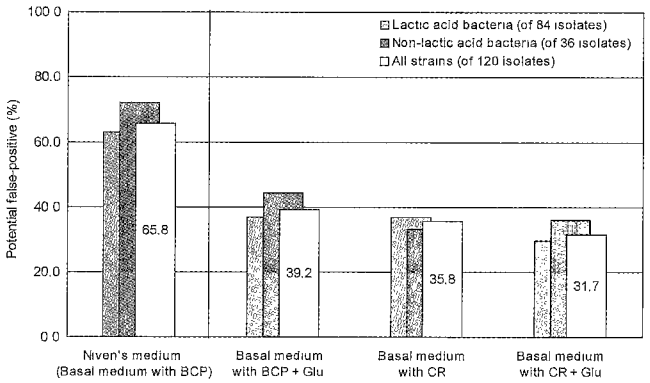

Fig. 2. Modification of Basal medium to decrease potential false-positives by the addition or substitution of components.
BCP: 0.006% bromocresol purple; CR: 0.02% cresol red; Glu: 0.1% glucose.

Table 1. Modification by substitution of different N-sources of basal medium with 0.02% cresol red and 0.1% glucose.

N-sources[1)]	Incidence of potential false-positives from test microorganisms cultivated on each media (%)		
	ALL	LAB	NON
8:0:0	37.1	29.8	36.1
3:3:2	17.5	14.3	25.0
2:2:4[a]	0.0	0.0	0.0
1:1:6[b]	0.0	0.0	0.0
0:0:8[c]	0.0	0.0	0.0

[1)]Tryptone:yeast extract:ammonium sulfate (w/w/w). ALL: all strains of 120 isolates; LAB: lactic acid bacteria of 84 isolates; NON: non-lactic acid bacteria of 36 isolates. [a]Combination of 0.25% Tryptone, 0.25% yeast extract, and 0.5% ammonium sulfate as N-source showed potential false-positives when test microorganisms were incubated for 21 h. [b]Combination of 0.125% Tryptone, 0.125% yeast extract, and 0.75% ammonium sulfate was established as the N-source for both growth of bacteria and discarding false-positives. [c]1% ammonium sulfate as N-source was problematic because almost all the tested bacteria were not well-grown.

Confirmation of the Improved Decarboxylating Agar Medium

It was found that there was nearly complete agreement between the results obtained by the improved differential medium and HPLC (Fig. 3).

In an improved differential medium for histamine, 8 strains (6.7%) of false-positives (FP) and 7 strains (5.8%) of false-negatives (FN) were detected. In the case of tyramine, 18 strains (15.0%) and 4 strains (3.3%) occurred as FP and FN, respectively. The peak with the retention time of spermine was detected by HPLC in the culture in TSB that was supplemented with ornithine, although putrescine was not detected. In the improved medium, 7 strains (5.8%) of FP were detected. Among 17 strains recognized as a positive strain for cadaverine production by improved differential medium, only one strain was confirmed by HPLC.

Determination of Biogenic-Amine-Producing Bacteria in Kimchi by HPLC

The proportion of biogenic amine producer, except the cadaverine producer, in kimchi during the fermentation process increased to its maximum at the immature period and decreased after that (Fig. 3).

Histamine producers were detected in 3.3, 30.0, and 23.3% of the strains in kimchi at after-preparation, immature period, and optimum period, respectively, while it was not detected at the over-ripening period. 6.7 and 66.7% of the strains isolated at the after-preparation and immature periods were identified as tyramine producers. Of the strains isolated at after-preparation, immature period, optimum period, and over-ripening period, respectively, 6.7, 56.7, 43.3, and 43.3% produced spermine. Also, only one strain that was isolated at after-preparation was recognized as a cadaverine producer.

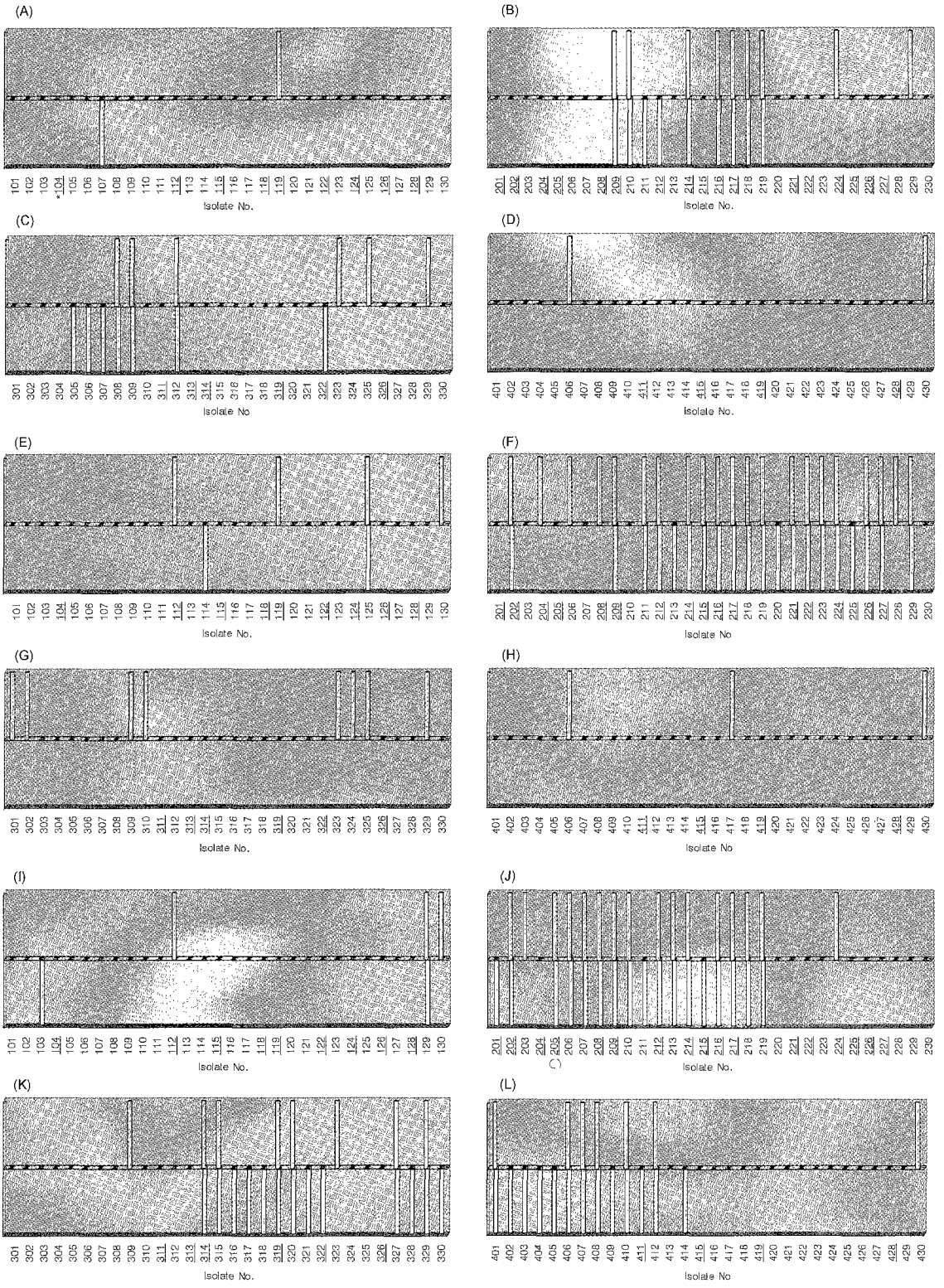

Fig. 3. Correlation between the qualitative detection of biogenic amines by the improved decarboxylating agar medium (top) and that by HPLC (bottom) for isolates from kimchi at each ripening period.

Histamine producer at after-preparation (A), the immature period (B), the optimum period (C), and the over-ripening period (D); Tyramine producer at after-preparation (E), the immature period (F), the optimum period (G), and the over-ripening period (H); Putrescine (spermine converted from putrescine was detected by HPLC) producer at after-preparation (I), the immature period (J), the optimum period (K), and the over-ripening period (L). *36 isolates of non-lactic acid bacteria are underlined.

DISCUSSION

False-positive (FP) results in the Niven's medium have been reported by several authors [1, 12, 20, 21], and some authors [2, 4, 9, 12, 21] suggested that the results might be caused by production of other alkaline bacterial products in the medium. Rodríguez-Jerez *et al.* [20] described a lower incidence of FP results in the Niven's medium, indicating that many FP results may appear afterwards. In our study, we observed that a high percentage (65.8%–79.2%) of strains showed a false-positive reaction in the decarboxylating agar media as described by various authors [10, 18, 24], without amino acid (Fig. 1). What this means is that the media are not sufficiently suitable to detect the biogenic amine producer.

Ben-Gigirey *et al.* [4] suggested that the composition of tryptic soy broth (TSB) itself may permit the bacterial decarboxylation of other amino acids, because TSB contains a high concentration of peptones. Although supplementary amino acids are not added, they can be released from the organic nitrogen sources of Niven's medium that contains organic nitrogen sources at concentration of 10 g/l. In our study, the more ammonium sulfate replaced organic nitrogen source in the medium, the less PFP reactions were formed (Table 1). Biogenic amines which did not have any relationship to the supplemented amino acid were also detected qualitatively in the TSB culture by HPLC. Therefore, FP results in the Niven's medium may be due not only to the production of other alkaline bacterial products, but also to the bacterial decarboxylation of an amino acid released from the medium.

In the medium where ammonium sulfate was replaced entirely with both tryptone and yeast extract as a nitrogen source, bacteria were not properly grown although PFPs were not observed. To eliminate this problem the medium was supplemented with only a minimum amount of organic nitrogens in comparison with the basal medium, so that the final nitrogen source with combination of 0.75% ammonium sulfate, 0.125% tryptone, and 0.125% yeast extract (6:1:1, w/w/w) was established. It was highly effective not only on the growth of bacteria but also on eliminating false-positive. Adding glucose also showed a decrease in PFP, since the color contrast surrounding the colonies in the medium was improved. Cresol red, which showed the color change at higher pH levels than bromocresol purple, was a superior indicator to detect an increase in the pH of the medium by biogenic amines formed, since FP decreased tremendously, even though this indicator showed slightly higher FN results than bromocresol purple. Therefore, cresol red is favorable for detecting biogenic amine formation and eliminating FP results.

Several differential plating media have been developed for Enterobacteriaceae [6, 17, 18], lactic acid bacteria [5, 10, 14], and acid-sensitive clostridia [24], but they have only a limited value for the screening of general microorganisms in foods. However, our improved medium was found to be sufficiently suitable in detecting various biogenic amine-producing microorganisms.

Concentrations of biogenic amines were very low as determined by HPLC. Moreover, there was a poor agreement for cadaverine between the results obtained by the improved differential medium and HPLC, which might be caused by low concentrations of these amines being far below a detection limit. Spermine was detected by HPLC in TSB culture supplemented with ornithine (Fig. 3), which showed a good correlation with the results observed in the improved medium for a putrescine producer. These results suggest that putrescine can easily be converted into spermine by its producer. Spermine, a biogenic polyamine produced by condensation of two molecules of putrescine and one molecule of propylamine, is proposed to be a potential precursor for the carcinogenic N-nitrosopyrrolidine formation [23].

Among all the tested kimchi isolates, spermine producers (37.5%) were the highest, although the presence of histamine (14.2%) and tyramine producers (18.3%) were considerably high. However, the production ability of these biogenic amine producers can be suppressed in kimchi during the fermentation process, which may be caused by microbiological and physicochemical factors in kimchi. Therefore, it should be investigated whether or not the biogenic-amine-producing capacity of the kimchi isolates could be maintained in kimchi during the fermentation process, and whether spermine produced by the isolates was converted to N-nitrosopyrrolidine in the food system. Studies conducted on starter cultures without any biogenic-amine-producing activity are urgently needed.

Acknowledgments

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-00-B-22000-0149).

REFERENCES

1. Ababouch, L., M. E. Afilal, S. Rhafiri, and F. F. Busta. 1991. Identification of histamine-producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperature (25°C). *Food Microbiol.* **8:** 127–136.
2. Baranowski, J. D., P. A. Brust, and H. A. Frank. 1985. Growth of *Klebsiella pneumoniae* UH-2 and properties of its histidine decarboxylase system in resting cells. *J. Food Biochem.* **9:** 349–360.
3. Ben-Gigirey, B., J. M. V. B. DE Sousa, T. G. Villa, and J. Barros-Velazquez. 1998. Changes in biogenic amines and

microbiological analysis in albacore (*Thunnus alalunga*) muscle during frozen storage. *J. Food Prot.* **61:** 608–615.
4. Ben-Gigirey, B., J. M. V. B. DE Sousa, T. G. Villa, and J. Barros-Velazquez. 1999. Histamine and cadaverine production by bacteria isolated from fresh and frozen albacore (*Thunnus alalunga*). *J. Food Prot.* **62:** 933–939.
5. Bover-Cid, S. and W. H. Holzapfel. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.* **53:** 33–41.
6. Chen, C.-M., C. Wei, J. Koburger, and M. Marshall. 1989. Comparison of four agar media for detection of histamine-producing bacteria in tuna. *J. Food Prot.* **52:** 808–813.
7. Eerola, S., R. Hinkkanen, E. Lindfors, and T. Hirvi. 1993. Liquid chromatographic determination of biogenic amines in dry sausages. *J. Ass. Off. Anal. Chem. Int.* **76:** 575–577.
8. Halász, A., Á. Baráth, L. Simon-Sarkadi, and W. Holzapfel. 1994. Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* **5:** 42–49.
9. Hernández-Herrero, M. M., A. X. Roig-Sagués, J. J. Rodríguez-Jerez, and M. T. Mora-Ventura. 1999. Halotolerant and halophilic histamine-forming bacteria isolated during the ripening of salted anchovies (*Engraulis encrasicholus*). *J. Food Prot.* **62:** 509–514.
10. Joosten, H. and M. Northolt. 1989. Detection, growth and amine-producing capacity of lactobacilli in cheese. *Appl. Environ. Microbiol.* **55:** 2356–2359.
11. López-Sabater, E. I., J. J. Rodríguez-Jerez, M. Hernández-Herrero, A. X. Roig-Sagués, and M. T. Mora-Ventura. 1996. Sensory quality and histamine formation during controlled decomposition of tuna (*Thunnus thynnus*). *J. Food Prot.* **59:** 167–174.
12. López-Sabater, E. I., J. J. Rodríguez-Jerez, M. Hernández-Herrero, and M. T. Mora-Ventura. 1996. Incidence of histamine-forming bacteria and histamine content in scombroid fish species from retail markets in the Barcelona area. *Int. J. Food Microbiol.* **28:** 411–418.
13. Mah, J. H. and H. J. Hwang. 1998. Microflora during cabbage-kimchi fermentation. *J. Sci. Technol.* **16:** 173–187.
14. Maijala, R. L. 1993. Formation of histamine and tyramine by some lactic acid bacteria in MRS-broth and modified decarboxylation agar. *Lett. Appl. Microbiol.* **17:** 40–43.
15. Maijala, R. L., S. H. Eerola, M. A. Aho, and J. A. Hirn. 1993. The effect of GDL-induced pH decrease on the formation of biogenic amines in meat. *J. Food Prot.* **56:** 125–129.
16. Mower, H. F. and N. V. Bhagavan. 1989. Tyramine content of Asian and Pacific foods determined by high performance liquid chromatography. *Food Chem.* **31:** 251–257
17. Møller, V. 1954. Distribution of amino acid decarboxylase in Enterobacteriaceae. *Acta Pathologica et Microbiologica Scandinavica* **35:** 259–277.
18. Niven, C. F. Jr., M. B. Jr. Jeffrey, and D. A. Jr. Corlett. 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. *Appl. Environ. Microbiol.* **41:** 321–322.
19. Ochiai, M., K. Wakabayashi, M. Nagao, and T. Sugimura. 1984. Tyramine is a major mutagen precursor in soy sauce, being convertible to a mutagen by nitrite. *Gann.* **75:** 1–3.
20. Rodríguez-Jerez, J. J., E. I. López-Sabater, A. X. Roig-Sagués, and M. T. Mora-Ventura. 1994. Histamine, cadaverine and putrescine forming bacteria from ripened Spanish semipreserved anchovies. *J. Food Sci.* **59:** 998–1001.
21. Rodríguez-Jerez, J. J., M. T. Mora-Ventura, E. I. López-Sabater, and M. M. Hernández-Herrero. 1994. Histidine, lysine and ornithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. *J. Food Prot.* **57:** 784–787, 791.
22. Shalaby, A. R. 1996. Significance of biogenic amines to food safety and human health. *Food Research Int.* **29:** 675–690.
23. Smith, T. A. 1980/81. Amines in food. *Food Chem.* **6:** 169–200.
24. Yoshinaga, D. H. and H. A. Frank. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). *Appl. Environ. Microbiol.* **44:** 447–452.