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Screening method for detection of immediate amino acid decarboxylases - producing bacteria implicated in food poisoning

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Abstract. Assessment of amino acid decarboxylase activity can be conducted using tubed broth or plated agar. In this study, the test was carried out in microtitre plates containing lysine, ornithine, arginine, tyrosine, tryptophan, phenylalanine or histidine as biogenic amine precursors. Møller decarboxylase base broth (MDB) with or without 1% of a known amino acid were added to wells of a 96 well-microtitre plate. The wells were inoculated with *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter anitratus* or *Staphylococcus aureus* to the final concentration of 6.0 x 10^7 cfu/ml and incubated at 35°C. The absorbance of the culture broth was read at 570 nm at 0, 1.0, 2.0, 3.0, 4.0, 5.5, 6.5 and 7.5 hour. Comparison of means of A'_{570} between 0 hour and a specified incubation time was determined statistically. Positive decarboxylase activities were detected in the media inoculated with *E. coli* and *K. pneumoniae* in less than 6 hours. The current method is suitable for immediate producers of amino acid decarboxylase enzymes. It costs less as it uses less amino acid and it has the potential to be used for screening aliquots of food materials for amino acid decarboxylase activities.

INTRODUCTION

Biogenic amines (BAs) are formed via decarboxylation of their corresponding amino acids through the action of amino acid decarboxylase enzymes produced by bacteria which are present in a wide range of food products, including fish and meat products (Silla, 1996). The amino acid decarboxylases are found in the genera such as *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Photobacterium*, and some lactic acid bacteria (Rice *et al*., 1976; Brink *et al*., 1990; Huis in't Veld *et al*., 1990). The main BAs encountered in foods include histamine, tryptamine, *p*-phenylethylamine, tyramine, putrescine, cadaverine, spermine, and spermidine (Silla, 1996). The four former amines have important physiological effects in human, either psychoactive which affect the nervous system or vasoactive which act on the vascular system (Lovenberg, 1973). Other nitrosatable amines which form the most known carcinogenic *N*-nitrosamines are secondary amines such as agmatine and the polyamines spermine and spermidine, which occur widely in fish, meat and vegetable products (Smith, 1980, Silla Santos, 1996). Thus, consumption of foods containing high amounts of these amines can have toxicological effects (Shalaby, 1996).

Early detection of amino acid decarboxylases-producing bacteria in food materials is essential in the food industry in order to avoid the risk of amine formation and subsequently, a cause of food-borne disease or food poisoning. Therefore, the use of methods for early and rapid detection of these bacteria is important for preventing BAs accumulation in food products. During the last

two decades, several methods to detect BA-producing bacteria and to quantify the BA produced have been developed (Marcobal *et al.*, 2006).

Amino acid decarboxylase test using Møller decarboxylase broth has been used in standard bacteriological methods to detect activity of bacteria with the ability to produce ornithine and lysine decarboxylase and arginine dihydrolase. In this conventional standard method, pure bacterial isolate is inoculated into glass tubes of basal media (approximately 2.0 ml) with and without amino acids precursor. Inoculated tubes are overlaid with mineral oil and then subjected to incubation at 35 ± 2°C for 18-72 hours. This test is based on the increase of growth medium pH upon BA formation (Maijala, 1993; Bover-Cid & Holzapfel, 1999) with the addition of a pH indicator in the growth medium such as bromocresol purple (Møller, 1954; Niven *et al.*, 1981; Choudhury *et al.*, 1990). Change of colour of the broth to purple indicates a positive amino acid decarboxylase reaction while yellow colour indicates that the test bacterium does not produce the appropriate enzyme. The pH of Møller's medium is 6.0, however, Niven and associates adjusted the pH to 5.3 to increase the differentiation ability of the medium (Niven *et al.*, 1981). Niven's medium have been used for the detection of histamine-producer *Enterobacteriaceae* and other bacteria from fish (Niven *et al.*, 1981; Yoshinaga & Frank, 1982).

In addition to the conventional standard method, numerous methods have been developed to detect BA produced by bacteria grown on protein substrates. These include thin-layer chromatography (TLC) (Chen *et al.*, 1982, Garcia-Maruno *et al.*, 2005), liquid chromatography [High Performance (HPLC) and Ultra High Pressure (UHPLC)] (Fernández *et al.*, 2007, Latorre-Moratalla *et al.*, 2009; Mazzucco *et al.*, 2010; Sakamoto *et al.*, 2010) gas chromatography (GC) (Fernandes & Ferreira, 2000), capillary electrophoresis (CE) (Kvasnicka & Voldrich, 2006) and Nuclear Magnetic Resonance (NMR) (Schievano *et al.*, 2009). Molecular methods for detection and identification of BA-producing bacteria have also been developed. In most molecular methods, DNA extracted from the bacteria was used as template for Polymerase Chain Reaction (PCR) reaction (Kanki *et al.*, 2002, de las Rivas *et al.*, 2006, Björnsdóttir-Butler *et al.*, 2010).

Detection of amino acid decarboxylase-producing bacteria activities by conventional culture techniques (using glass tubes) is tedious and exhibiting disadvantages such as lack of speed and appearance of false positive/negative results, while methods such as PCR (and other molecular-based methods), HPLC and UHPLC, GC, CE and NMR require expensive instrumentation and reagents which are not available in all testing laboratories. Culture method is still a cheaper method and still being used by researchers. Soler *et al.* (1995) used Niven's broth medium in wells of 96-well microtitre plate and were able to determine BAs formation quantitatively. In their method, BAs production was determined spectrophotometrically by monitoring the changes of the pH indicator in the acid phase of the growth medium at 405 nm for 36 hours. In the present study, we modified Soler's method using Møller decarboxylase medium for determining amino acid decarboxylase activity of bacteria qualitatively, with the aim of developing a simple and cost-effective screening method to detect the immediate amino acid decarboxylase producers within a short period of time.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Acinetobacter anitratus* (IMR A48) and *Staphylococcus aureus* (ATCC 25923) were obtained from Culture Collection Services, Bacteriology Unit, Institute for Medical Research, Malaysia. All bacteria were cultured on Plate Count Agar (Difco) and incubated at 35 ± 2ºC, overnight.

Media Preparation

Møller's Decarboxylase Base broth (MDB, Difco) was prepared according to the

manufacturer's instruction. Decarboxylase medium with amino acid was prepared by adding amino acid to MDB to a final concentration of 1% (w/v). Amino acids used were L-histidine, L-cysteine hydrochloride monohydrate, L-lysine hydrochloride, L-arginine, L-ornithine hydrochloride (MERCK, Germany), L-phenylalanine, L-tyrosine and L-tryptophan (FLUKA, Switzerland) The pH of the media was adjusted to 6.0 and then autoclaved at 121°C for 15 min.

Absoption spectrum of MDB

Møller's Decarboxylase Base broth without amino acid was prepared with pH adjusted to 1. Scanning spectrophotometric examination was done on the broth between 200 to 700 nm and at fixed wavelength of 405, 570 and 620 nm using a UV-visible spectrophotometer (Perkin Elmer, Model Lambda 35, U.S.A.) at room temperature. The examination was repeated after the pH of MDB was adjusted to pH 3, 5, 7, 9 and 11 using either sodium hydroxide or hydrochloric acid.

Amino acid decarboxylase test in 96-well microtitre plates

Several overnight colonies of the test bacteria were picked from their Plate Count Agar and suspended in 9 ml sterile saline solution (0.85% sodium chloride) and the turbidity was adjusted to match McFarland 5.0 standard (estimated to contain approximately 1.5×10^9 cfu/ml). Aliquots of 240 µl MDB and 10 µl saline were added into 6 wells of a 96 wells microtitre plate (Kartell, Italy) designated as MS and acted as microbial negative control; 240 µl MDB with a known amino acid and 10 µl saline were added into another 6 wells designated as MAS and acted as amino acids degradation control; 240 µl MDB with 10 µl of a known bacterial suspension were added into another 6 wells designated as MC and acted as a microbial growth density control; and 240 µl MDB with a known amino acid and 10 µl of a known bacterial suspension were added into another 6 wells designated as MAC and acted as the test wells. Broth in the wells of the microtitre plate was layered with sterile mineral oil and the plate was incubated at 35 ± 2°C. The absorbance of the broth was read using a microplate reader (Anthos Labtec Instruments GmbH, Model 2010, Austria) at 570 nm at 0, 1.0, 2.0, 3.0, 4.0, 5.5, 6.5 and 7.5 hour of incubation. Corrected absorbance after incubation (A'_{570}) was calculated using the following formulae :

$$A'_{570} = \frac{A_{MAC}-[(A_{MAS}-A_{MS})+A_{MC}]}{[(A_{MAS}-A_{MS})+A_{MC}]}$$

Amino acid decarboxylase activity was considered positive when the A'_{570} at one particular time was significantly higher compared to A'_{570} at 0 hour ($p<0.05$).

Amino acid decarboxylase test in glass tubes by conventional standard method

One colony of *E. coli* was picked from its Plate Count Agar and suspended in each 2 ml sterile MDB solution only and MDB with known individual amino acids in capped glass tubes. Broth in the tubes was layered with sterile mineral oil and the tubes were incubated at 35 ± 2°C. Change in colour of the growth media was observed at 0, 1, 2, 3, 4, 5, 7, 9, 11, 18, 24, 48 and 72 hours of incubation.

Statistical analyses

All means and standard deviations (SDs) values in this study were calculated using Statistical Package for the Social Sciences (SPSS) version 15.0 software. The comparison of means of A'_{570} was determined by Scheffe's One-way Analysis of Variance (ANOVA) and $p<0.05$ was accepted as statistically significant.

RESULTS

The absorption spectrum of MDB medium from 200 to 700 nm showed absorbance peaks at 429 nm and 589 nm, with 589 nm being the wavelength maximum (lambda-max) (Figure 1). The absorbance at 405, 570 and 620 nm of MDB at different pH is shown in Figure 2. At 570 nm, a linear plot of absorbance and pH was observed between pH 5 to 9, while there was no linear relationship of absorbance versus pH at 405 and 620 nm. In Figure 3, the absorbance at 570 nm (A_{570}) for MDB inoculated with saline (MS), MDB with lysine

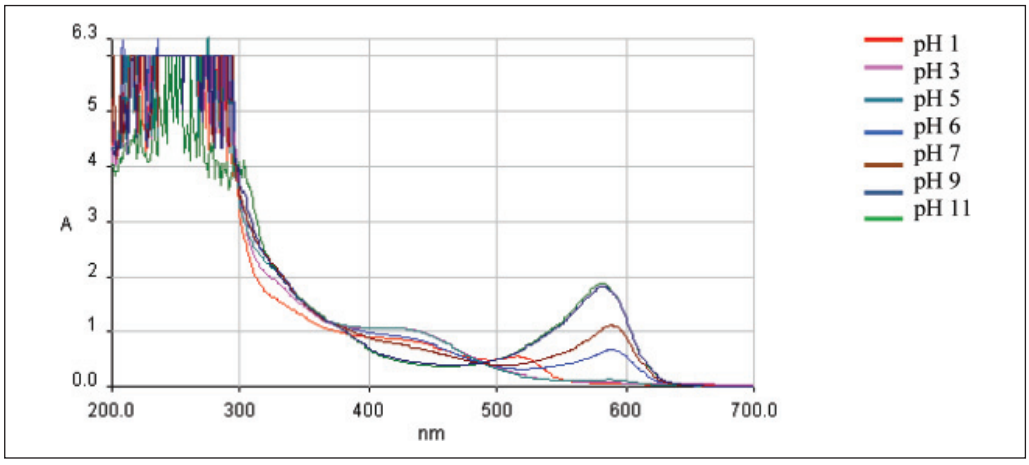

Figure 1. Scanning spectrophotometry of the Moeller decarboxylase medium without addition of amino acids at different pH with the presence of pH indicators, bromocresol purple and cresol red

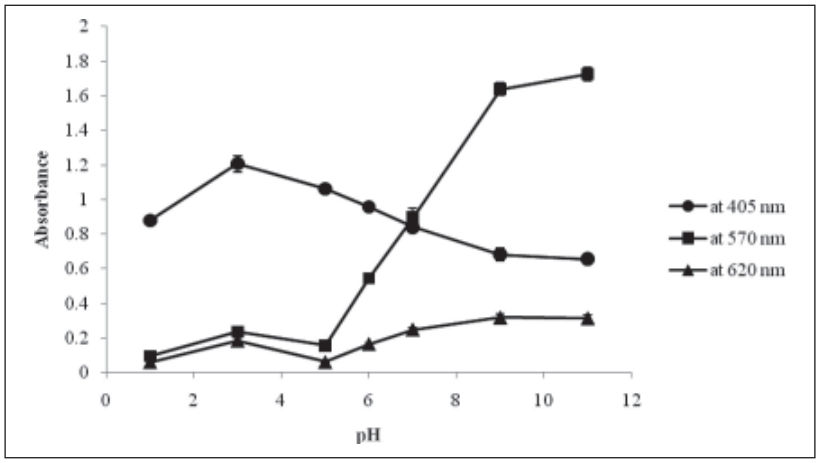

Figure 2. Absorbance of Moeller decarboxylase base broth at different pH read at 405, 570 and 620 nm (mean ± SD; n=3)

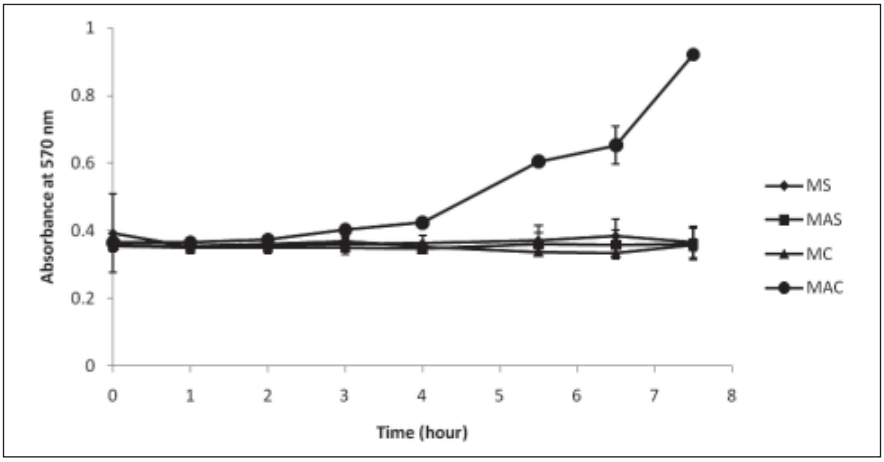

Figure 3. Absorbance at 570 nm for set of media+saline (MS), media+amino acid+saline (MAS), media+culture (MC) and media+amino acid+culture (MAC) for Moeller decarboxylase medium supplemented with lysine and inoculated with *E. coli*

and saline (MAS), MDB with culture (MC) and MDB with lysine and inoculated with *E. coli* (MAC) showed that there was no absorbance elevation in MS, MAS and MC; while the A'_{570} in MAC increased during 7.5 h incubation with highest increment followed by A'_{620} and A'_{405} (Figure 4).

There was a significant increase ($p<0.05$) in absorbance of the MDB medium containing histidine, lysine or ornithine when inoculated with *E. coli* from 5.5 hours of incubation onwards (Figure 5). In the case of *K. pneumoniae*, there was a significant increase in absorbance ($p<0.05$) in the medium containing histidine or lysine from 4.0 hours of incubation onwards (Figure 6). There was no significant increase in absorbance in media containing any of the eight amino acids when the media were inoculated with *A. anitratus* or *S. aureus* (Figures 7 & Figure 8). In the conventional method using MDB inoculated with *E. coli* (Figure 9), positive results were observed at 5 to 7 hours when histidine was present, but

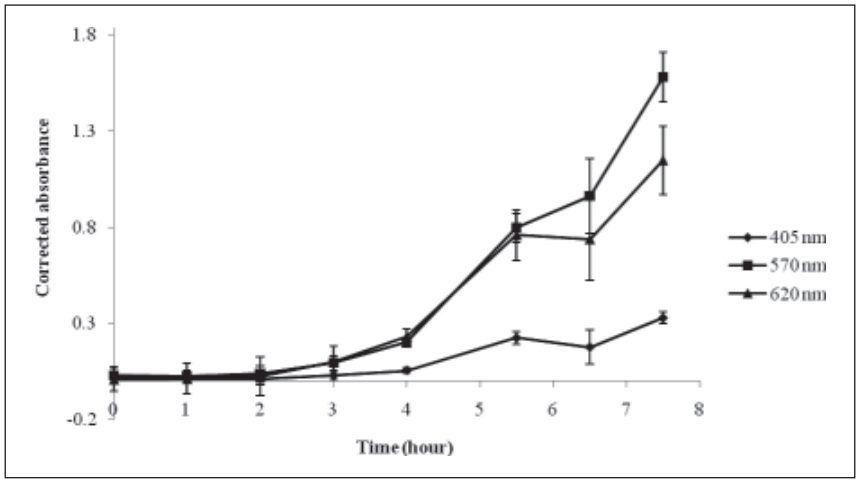

Figure 4. Corrected absorbance (mean ± SD; n=6) of Moeller decarboxylase base medium added with lysine and inoculated with *E. coli* at 405, 570 and 620 nm

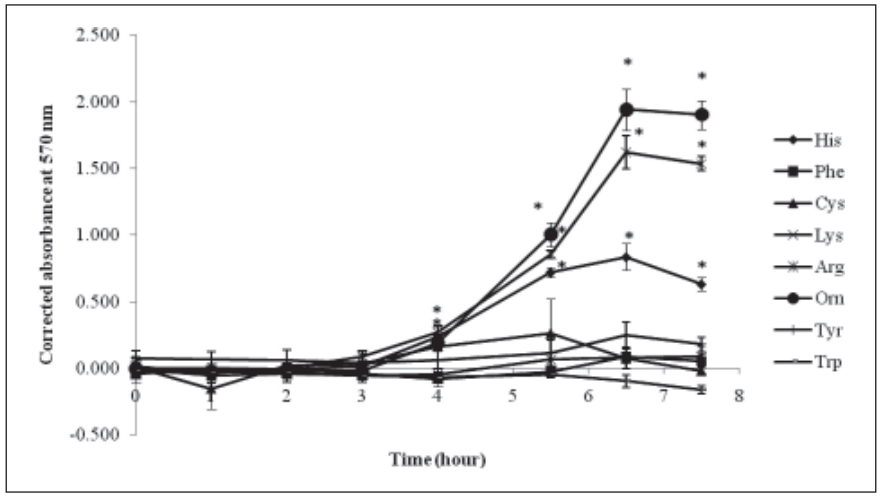

Figure 5. Corrected absorbance at 570 nm of the Moeller decarboxylase medium (mean ± SD; n=6) in the presence of various amino acids (His: histidine, Phe: phenylalanine, Cys: cysteine, Lys: lysine, Arg: arginine, Orn: ornithine, Tyr: tyrosine and Trp: tryptophan) and inoculated with *E. coli* for 7.5 hours at 35 ± 2°C. * indicates statistically significancy at $p<0.05$ compared to 0 hour

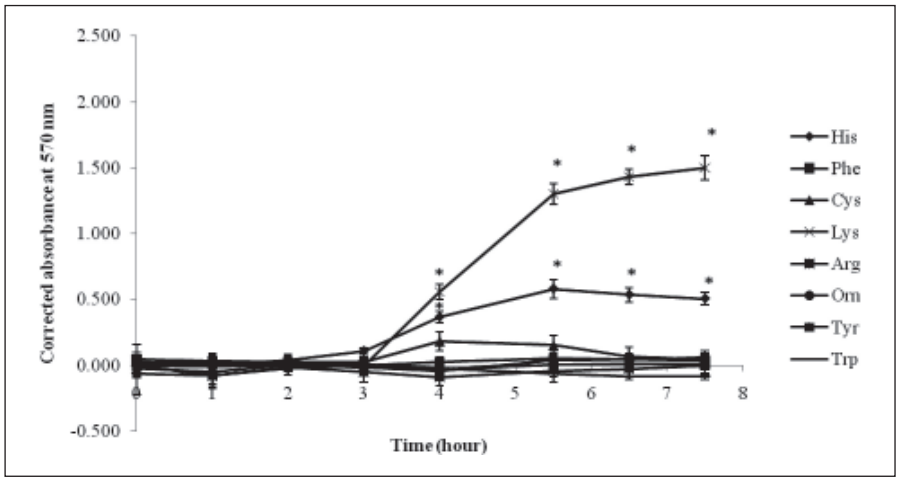

Figure 6. Corrected absorbance at 570 nm of the Moeller decarboxylase medium (mean ± SD; n=6) in the presence of various amino acids (His: histidine, Phe: phenylalanine, Cys: cysteine, Lys: lysine, Arg: arginine, Orn: ornithine, Tyr: tyrosine and Trp: tryptophan) and inoculated with *K. pneumoniae* for 7.5 hours at 35 ± 2ºC. * indicates statistically significancy at $p<0.05$ compared to 0 hour

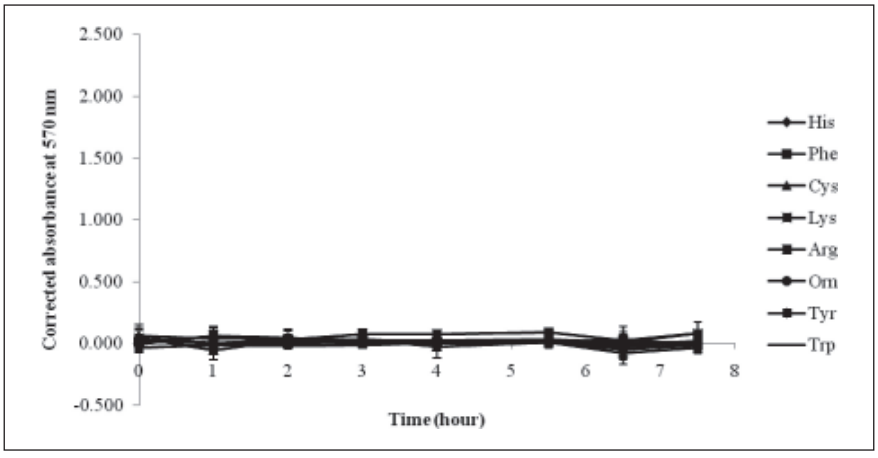

Figure 7. Corrected absorbance at 570 nm of the Moeller decarboxylase medium (mean ± SD; n=6) in the presence of various amino acids (His: histidine, Phe: phenylalanine, Cys: cysteine, Lys: lysine, Arg: arginine, Orn: ornithine, Tyr: tyrosine and Trp: tryptophan) and inoculated with *A. anitratus* for 7.5 hours at 35 ± 2ºC. * indicates statistically significancy at $p<0.05$ compared to 0 hour

subsequently subsided and negative results were detected after 9 hours of incubation time. With the presence of arginine, the positive results were only detected after 48 hours of incubation time.

DISCUSSION

Møller's base broth (MDB) contains dextrose as a source of carbohydrate, peptone and beef extracts to supply the nutrients necessary to support bacterial growth, pyridoxal as the enzymatic reaction co-factor, and bromocresol purple and cresol red as the pH indicators (Møller, 1954). The pH 6.0 set at initial incubation time is optimum for the bacterial growth (Arnold & Brown, 1978; Møller, 1954). In Møller's method, results are based solely on visualization of media colour change. When a bacterium ferments dextrose under anaerobic condition,

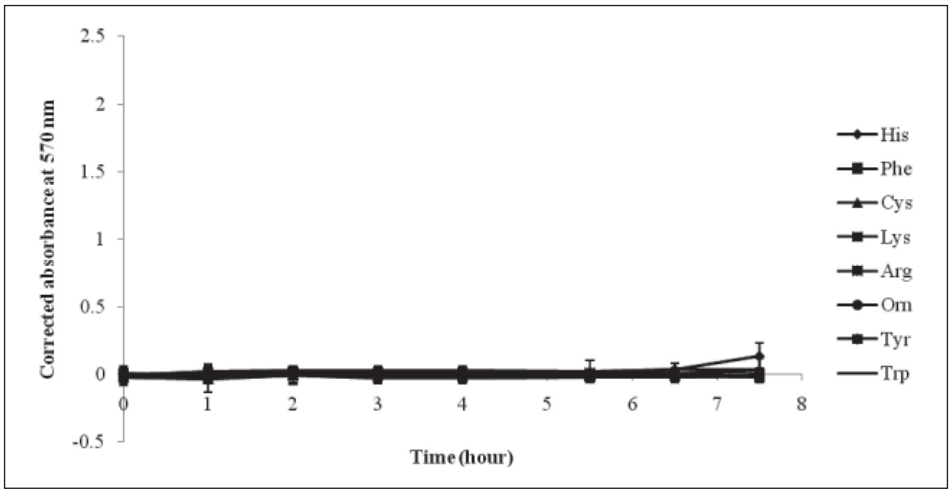

Figure 8. Corrected absorbance at 570 nm of the Moeller decarboxylase medium (mean ± SD; n=6) in the presence of various amino acids (His: histidine, Phe: phenylalanine, Cys: cysteine, Lys: lysine, Arg: arginine, Orn: ornithine, Tyr: tyrosine and Trp: tryptophan) and inoculated with *S. aureus* for 7.5 hours at 35 ± 2°C. * indicates statistically significancy at $p<0.05$ compared to 0 hour

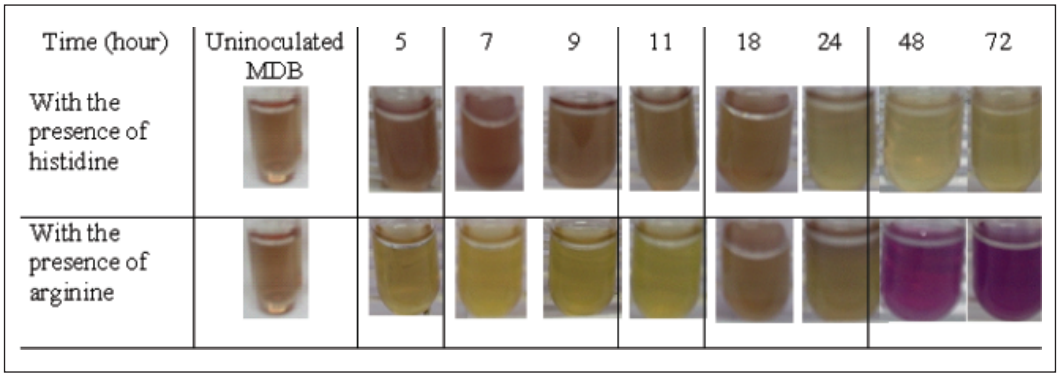

Figure 9. Results obtained through conventional method using MDB when inoculated with *E. coli* in the presence of histidine and arginine

pH of the medium decreases to pH 5.0 - 5.5 and changes colour from yellowish-red to yellow. The pH 5.0 - 5.5 is also optimum for amino acid decarboxylases activities (Kawabata & Suzuki, 1959b). An amino acid if present in the medium will be decarboxylated by a bacterium producing the enzyme, amino acid decarboxylase, to its alkaline biogenic amine, causing an elevation of pH which results in a colour change to purple or violet (Møller, 1955; Thornley, 1960). In the media where amino acid decarboxylases activities are absent, purple colour is not developed.

The presence of bromocresol purple and cresol red in the Møller's base broth contributed to absorbance peaks exhibition at 429 nm and 589 nm (wavelength maximum, lambda-max), as observed in the absorption spectrum (Figure 1). In order to choose the single best wavelength to use, fixed point spectrophotometry of the medium at pH 1, 3, 5, 6, 7, 9 and 11 were determined at 405, 570 and 620 nm. These are the wavelengths for the filters available in the microplate reader used in the study. Within the critical pH range of 5 to 9 (pH range of the decarboxylase medium during the test), a linear relationship

between absorbance and pH was observed at 570 nm while the relationship at the other two wavelengths was non-linear. Plot of A'_{570} against time was found to be best portraying the absorbance increment of the medium inoculated with *E. coli* in the presence of lysine, compared to A'_{405} and A'_{620} (Figure 3). Based on these findings, 570 nm was used as the wavelength when measuring the absorbance of MDB in the current method. In the previous study done by Soler *et al.* (1995), a miniature analytical method in the 96-well microtitre plate using Niven's medium, 405 nm wavelength was used when measuring the absorbance. This was due to the pH indicator, bromocresol purple, which was present in the medium in high concentration (0.006%), thus, exhibiting high absorbance at lambda-max and was unreliable due to violation of the Beer-Lambert's Law (Swinehart, 1962).

In the current method, wells designated MS, MAS and MC were included to determine any possible absorbance increments due to microbial contamination, amino acid degradation other than by the enzyme produced by the test organism and/or bacterial growth density during the experiment, respectively. MAC well was the reaction wells containing medium with the individual amino acid and bacteria. It was observed that the A'_{570} of MS, MAS and MC wells showed no significant increase from 0 hour within the 7.5 hours incubation (Figure 3). To accommodate any possibilities of substantial effects coming from the three former wells to the latter reaction well, we have derived a formula which ruled out all the three confounding factors. Thus, we determined that positive A'_{570} with significant difference statistically from 0 hour of incubation time ($p<0.05$) indicates presence of amino acid decarboxylase activity.

In this study, *E. coli* and *K. pneumoniae* which are *Enterobacteriaceae* family members were used as positive controls for their respective amino acid decarboxylases. Results showed that *E. coli* exhibited positive amino acid decarboxylase activity for histidine, lysine and ornithine within the 7.5 hours incubation time (Figure 5). This result is in accordance to the previous reports that *E. coli* has histidine, lysine and ornithine decarboxylases (Kawano & Asakawa, 1964; Lawson & Quinn, 1967). Our results also showed that *K. pneumoniae* exhibited positive results for histidine and lysine decarboxylases and negative results for the arginine decarboxylase (Figure 6) and were parallel with the previous studies (Maccani, 1979; Baranowski, 1985; Bergey & Holt, 2000). Negative decarboxylase activity was observed for *S. aureus* and *A. anitratus* (Figure 7 and Figure 8), which had not been known to produce amino acid decarboxylases.

The positive results for *E. coli* histidine decarboxylase activity at 5 to 7 hours of incubation time was successfully detected with the present method (Figure 9), parallel with the previous results (Kawano & Asakawa, 1964). However, using the conventional method, the amino acid decarboxylase activity was not detected when the purple colour changes back to yellow and observation done after 18 hours missed capturing the medium purple colour at early stage of incubation. *Escherichia coli* was found to be negative for arginine decarboxylase using the current method (Figure 5), but was positive after 48 hours of incubation using the conventional tube method (Figure 9). Based on these results, the current method with incubation period of 7.5 hours was successfully applied for detecting only the immediate amino acid decarboxylases-producing microorganisms. Work is underway to determine the applicability of the current method if the incubation period is extended in order to capture late amino acid decarboxylase activity.

In the standard bacteriological method, Møller decarboxylase broth is added with lysine, ornithine or arginine. In the current method, we used the medium with other amino acids such as tyrosine, tryptophan, phenylalanine and histidine due to their important roles as biogenic amines precursors for carcinogenic nitrosamines (ten Brink *et al.*, 1990 B. ten Brink, C. Damink, H.M. Joosten and J.H. Huis in 't Veld, Occurrence and formation of biologically active amines in foods, International Journal

of Food Microbiology (1990), pp. 73–84. Abstract | PDF (579 K) | View Record in Scopus | Cited By in Scopus (216) ten Brink, *et al*., 1990) and indicator of food spoilage (Halasz *et al*., 1994). Cysteine which is not a substrate for biogenic amines production had been used as the amino acid negative control. Results showed that the current method was able to detect histidine decarboxylase activity and this implies that it is applicable for the detection of histamine-producing bacteria. With the inclusion of eight amino acids in the method, all bacterial decarboxylases associated with biogenic amines production in food poisoning incidents can be detected using the current method.

Some precautions were made along the study. Precipitate from limited insolubility of tyrosine and bubbles should be avoided when pipetting the medium into the reaction wells since it would result in high absorbance reading. If bubbles are formed, they have to be removed before putting the mineral oil on top of the medium. Chen *et al*. (1982) and Baranowski (1985) had pointed out that the increase in the pH of the medium could also be confused with production of other alkaline compounds such as through the ammonia production or decarboxylation of amino acids which led to false positive reactions. The presence of biogenic amines degrading enzymes also should be taken into account in the case of false negative results.

In conclusion, a rapid detection method for amino acid decarboxylases was developed using Møller decarboxylase broth. This method detects amino acid decarboxylase activity within 7.5 hours incubation for eight amino acids associated with bacterial biogenic amines that have effects on health, such as histamine.

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