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Evaluation of modified Zobell marine agar for differential isolation of histamine-forming bacteria from fresh fish

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Histamine formed in fish due to the activities of histamine-forming bacteria is a health hazard. In this study, the modified Zobell marine agar (mZMA) containing histamine and a pH indicator dye helped in better discrimination and isolation of histamine-forming bacteria from fresh fish.

Histamine fish poisoning is one of the major health risks associated with the consumption of scombroid fishes like tuna, seer fish and mackerel. Exogenous histamine formed in fish due to bacterial activities is responsible for scombroid poisoning (Feng et al., 2016). Some groups of bacteria are capable of producing histidine decarboxylase enzyme which converts free histidine present in the fish tissue to histamine by decarboxylation reaction (Hungerford, 2010). Early detection of histamine forming bacteria is necessary for assuring the quality of seafood.

Many different selective media have been proposed for the isolation of histamine-forming bacteria from fish. Niven's medium, which is a modification of Moeller's decarboxylase medium, is routinely used for the isolation of histamine-forming bacteria (Niven et al., 1981). Subsequently, this medium was modified by replacing the indicator dye bromocresol purple (pH range 5.2–6.8) with cresol red (pH range 7.2–8.8) which resulted in modified Niven's medium (MNM) (Yoshinaga and Frank, 1982). MNM is widely used for the detection and quantification of histamine-forming bacteria. However, certain limitations are associated with MNM. The low pH (5.3) of the medium restricts the growth of some acid-sensitive histamine-forming bacteria (Bjornsdottir-Butler et al., 2011; Niven et al., 1981). The occurrence of false positive reaction with this media has been reported to be as high as 63% (López-Sabater et al., 1996) which could be due to the production of non-histamine alkaline compounds (Tembhurne et al., 2013). Compared to Moeller's medium, Niven's medium is devoid of glucose. The absence of carbohydrate can force the microorganisms to use simpler proteins like tryptones leading to the formation of ammoniacal compounds which increase the pH of the medium. Thus, the addition of glucose as a carbon source can reduce false positives (Kim et al., 2001). However, the reduction in pH due to the production of acid from glucose can mask the pH increase by histamine leading to false negative results (Maijala and Eerola, 1993). In some cases, high histamine producers were found to be negative on Niven's medium and its modified versions (da Silva et al., 2002; Roig-Sagués et al., 1997). Thus, the existing media and cultivation methods need constant improvement for accurate detection and quantification of histamine-forming bacteria in fish. In this context, the present study was carried out to investigate the efficiency of Modified Zobell marine agar (mZMA) for the isolation of histamine-forming bacteria from fish.

Fresh fish were collected from local markets and brought to the laboratory. About 30 g of fish muscle containing 10 g each from dorsal, ventral and caudal regions of an individual fish was mixed briefly in a blender. From this, 10 g was taken and homogenized with 90 ml of sterile physiological saline (0.85% NaCl) for two minutes in a stomacher (Seward Inc., UK), serially diluted and spread plated on Modified Niven's medium (MNM) (Mavromatis and Quantick, 2002). The plates were incubated 30 °C for 18–24 h. Bacterial colonies surrounded by a pink halo were considered as presumptive histamine producers, while those which did not produce the typical colony phenotypes were considered as histamine negative bacteria. The selected isolates were purified on trypticase soy agar (TSA) plates containing 0.1% L-histidine. The histidine decarboxylase activity of the isolates was determined as previously described (Tembhurne et al., 2013). The isolates were stored in glycerol broth at −80 °C until further use.

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Table 1
Compositions of modified Niven's medium and Modified Zobell marine agar.

Modified Niven's medium (MNM)	Quantity g/L
Components	
Tryptone	5.0
Yeast extract	5.0
L- histidine hydrochloride	20.0
Sodium chloride	10.0
Cresol red	0.2
Agar	15.0
pH	6.5 ± 0.2
Modified Zobell Marine agar (mZMA)	
Peptone	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
L- histidine hydrochloride	-[a]
Bromothymol blue	-[b]
Agar	15.0
pH	6.5 ± 0.2

[a] L- histidine hydrochloride was used at 2.5, 5 and 10 g/l concentrations separately.
[b] Bromothymol blue was used at 0.2, 0.4 and 0.8 g/l levels concentrations separately.

Histamine production by selected bacteria was estimated using an ELISA kit (Immunolab, GmbH, Germany). Bacteria were inoculated in tuna fish infusion broth prepared from tuna meat to simulate the seafood characteristics and incubated for 24 h at 30 °C. The genomic DNA from the isolates was extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) and used in PCR amplifications. The *hdc* gene was amplified using primers and conditions as described previously (Takahashi et al., 2003). *Morganella morganii* MTCC-662 (hdc^+) was used as the positive control. For identification of bacteria, the partial 16SrRNA gene sequences were amplified using universal primers and sequenced (Bioserve Biotechnologies, Secunderabad, India).

Zobell marine agar (Hi-Media, Mumbai, India) was selected for modification because of its ability to support a range of marine bacteria. The final pH of the medium was adjusted to 6.5. Compositions of mZMA and MNM are given in Table 1. MNM was prepared as previously described (Yoshinaga and Frank, 1982). ZMA (Zobell, 1941) was modified by adding bromothymol blue indicator and L-histidine hydrochloride (Table 1). L-histidine hydrochloride (Sigma-Aldrich, India) was used at three different concentrations of 0.25%, 0.5% and 1%, while bromothymol blue was used at 0.02%, 0.04% and 0.08%. Both media were sterilized by autoclaving at 121 °C for 15 min at 15 lbs. pressure. Three histamine-producers namely *Morganella morganii*, *Klebsiella variicola*, *Staphylococcus capitis* and two histamine negative bacteria *Bacillus subtilis* and *Proteus vulgaris* were spot inoculated on both MNM and mZMA. The plates were incubated at 30 °C for 24 to 48 h and the colony colors were recorded.

Among the isolates tested in this study, *Morganella morganii* produced highest level of histamine, while *Staphylococcus capitis* produced the least (data not shown). After 24 h of incubation, there was a visible distinction between the histamine formers and the non-histamine formers on mZMA, while on MNM the difference was not obvious (Fig. 1). After 48 h, the characteristic halo was visible around the histamine-forming bacterial colonies on MNM and histamine formers could be distinguished based on the colony color (Fig. 1).

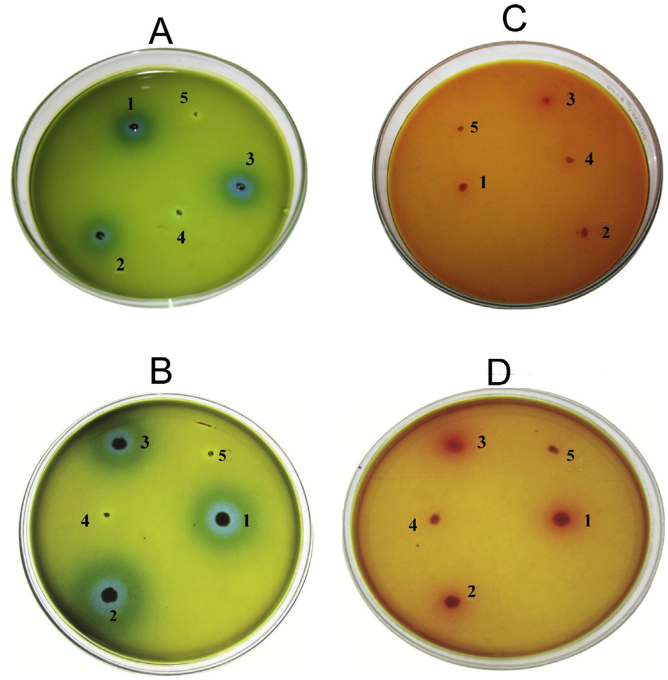

Fig. 1. Growth and colony morphologies of spot inoculated histamine-producing *Morganella morganii* (1), *Klebsiella variicola* (2), *Staphylococcus capitis* (3) and the histamine negative bacteria *Bacillus subtilis* (4), *Proteus vulgaris* (5) on (A) mZMA after 24 h (B) mZMA after 48 h (C) MNM after 24 h and (D) MNM after 48 h.

Fig. 2 shows colony morphologies on MNM and mZMA containing

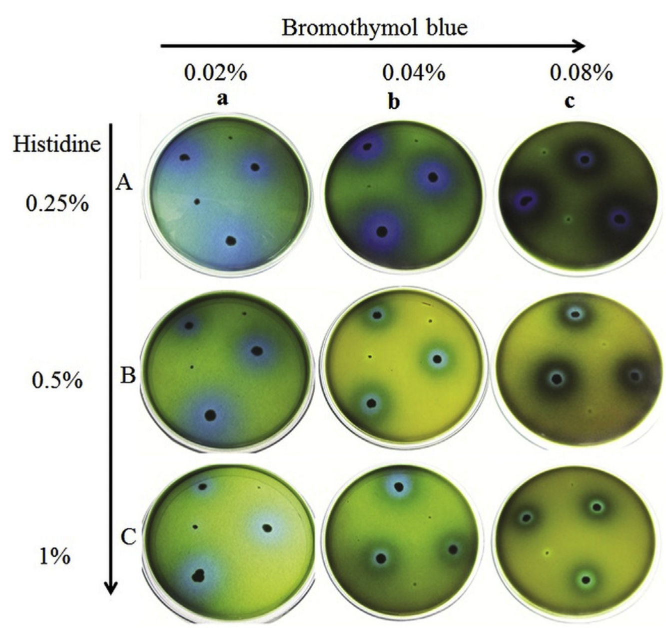

Fig. 2. Colony characteristics of histamine-forming bacteria on mZMA containing varying concentrations of L-histidine hydrochloride and bromothymol blue (BTB). Rows A, B, C represent plates with 0.25, 0.5 and 1% histidine, respectively while plates in columns a, b, c contain 0.02, 0.04 and 0.08% BTB. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

different levels of histamine and bromothymol blue. The indicator is discriminatory at a pH range of 6.0 to 7.6. At pH 6, bromothymol blue is yellow in color which gradually changes to blue as the pH increases. It was observed that as the dye concentration increased, the blue halos formed around the histamine-forming colonies became narrower and darker, and were less discriminatory. At reduced dye concentrations, the halos formed were comparatively broader and brighter allowing clear discrimination of histamine producing bacteria from histamine negative bacteria (Fig. 2). Thus, bromothymol blue at 0.04% and histidine at 0.25% in mZMA resulted in better contrast between the histiamine-forming bacterial colonies and the background color of the medium (Fig. 2). Histidine levels of > 0.25% did not significantly change the colony characteristics of histamine formers (Fig. 2). A comparative study using mZMA and MNM showed higher recovery of histamine-forming bacteria on mZMA from fresh fish collected from a local market (data not shown). Identification of histamine-forming colonies was found to be much easier on mZMA compared to MNM. Further, identification of histamine-forming bacteria can be accomplished within 24 h on mZMA. Thus, we conclude that mZMA agar could be routinely used for the isolation of histamine-forming bacteria from fresh fish.

Conflicts of interest

None.

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