Production of Hydrogen Sulphide and Other Volatile Sulphides by Spoilage Bacteria from Fish

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The performance of five agar media for enumerating hydrogen sulphide producing, spoilage bacteria in marine fishes was evaluated and the results are discussed Comparison of filter paper strip method using two reagents lead acetate and dithio- bisnitro benzoic acid (DTNB), with the iron agar slants showed that the lead acetate paper strips were more sensitive than other methods for detecting minute quantities of hydrogen sulphide. The nature and concentration of the substrate were found to influence the production of hydrogen sulphide. Majority of the isolated cultures attacked cysteine and produced hydrogen sulphide alone. Bacterial isolates which produced volatile sulphides other than hydrogen sulphide were very few and the volatile sulphides were produced when methionine was used as the substrate. The hydrogen sulphide producers from fish belonged to genera *Aeromonas, Vibrio, Alteromonas, Enterobacteriaceae, Pseudomonas, Acinetobacter, Moraxella* etc.

The production of hydrogen sulphide and other volatile sulphides is considered to be characteristic of spoilage bacteria. The importance of bacteria in the production of volatile sulphides in fish was established by the works of Herbert *et. al.*, (1971). Miller *et. al.*, (1973), Herbert & Shewan (1976) and Kadota & Ishida (1972). In determining hydrogen sulphide production by bacteria, the type and availability of sulphur source, sensitivity of the test for its detection, growth of the organism in the basal medium and the presence of the necessary enzyme system in the organism being tested must be taken into account (Mac. Faddin, 1980). The influence of method on the production of hydrogen sulphide by some bacterial species has been evaluated (Kuster & Williams, 1964; Lee & Simmard, 1984).

Methods for the detection of sulphide by bacteria in foods have traditionally relied on the use of iron or lead salt in the medium. These metal salts can detect only hydrogen sulphide whereas other volatile sulphides like methyl mercaptan or dimethyl sulphide remain undetected. The Ellmans reagent 5, 5' dithio-bis-2 nitrobenzoic acid (DTNB) which reacts with aliphatic thiols to yield yellow compounds (Ellman, 1959) have been used by Mc Meekin *et. al.*, (1978) to assess volatile sulphides produced by bacteria from poultry. Gillespie (1976) used DTNB method to detect volatile sulphides other than H_2S by bacteria from fish and Sharp *et. al.*, (1977) for bacterial isolates of dairy origin.

The present study evaluates the methods for qualitative and quantitative detection of hydrogen sulphide or other volatile sulphides. It also deals with the production of hydrogen sulphide and other volatile sulphides from organic and inorganic sources by tropical marine bacteria.

Materials and Methods

Sample consisted of the skin and muscle portion of the fishes procured from fish markets in and around Cochin and included most of the commonly available fish species in this area.

Peptone iron agar (PIA) (Levin, 1968), lead acetate agar (LA) (Difeo), Kliger iron agar (KIA) (Oxoid), modified Long and Hammer's medium (L & H) (Vanspreekens, 1974) and tryp-

tone soytone agar (TSA) (Thampuran & Iyer, 1989) were used for the enumeration of hydrogen sulphide producers. The total plate count was taken on sea water agar (SWA).

For determining the total count of bacteria and total hydrogen sulphide producing bacteria, 1 ml aliquots of the serial dilutions in saline were pour plated in duplicate. Plates were incubated at room temperature (28 ± 1°C) for 48 h. The hydrogen sulphide producers were enumerated by noting the number of colonies with black centre.

Colonies were randomly picked from sea water agar into tubes of sea water peptone broth. They were purified and subsequently tested for hydrogen sulphide and other volatile sulphides, by streaking on tryptone soytone agar slents and by hanging filter papers of DTNB (Mc Meekin *et. al.*, 1978) and lead acetate (Harrigan & Mac Cance, 1976) in the head space of the liquid broth containing peptone 10g, L-cysteine hydrochloride 0.5g, sodium chloride 7.5g, magnesium sulphate 0.5g per litre distilled water. pH was adjusted to 7.0 and sterilized at 0.7kg/cm^2 for 20 min. The isolates giving a positive reaction for H_2S or volatile sulphides were identified upto generic level as per the classification scheme given in Bergeys manual (Buchanan & Gibbons, 1974).

For studying the effect of specific substrates on sulphides, production, media mentioned in Table 1 were used. The lead acetate paper was used to detect hydrogen sulphide.

Results and Discussion

In this study, for judging the performance of each medium, the factors considered were the actual number of black colonies developed in each medium, ease of detection and enumeration and the time required for the development of black colonies.

The total bacterial count and the count of hydrogen sulphide producers are given in Table 2. The counts of hydrogen sulphide producers were almost comparable in all the media except Kliger iron agar where lowest count was noticed. However the colonies developed in Kliger iron agar were intense black so that even the microcolonies could be counted. They developed within 18 h. The colonies that developed in lead acetate agar had a greyish colour and took 48 h for counting. Modified Long & Hammers medium and tryptone soytone agar were equally good as far as the count,

Table 1. *Composition of liquid media for testing the production of hydrogen sulphide*

	Medium numbers								
Components (g/100 ml)	1	2	3	4	5	6	7	8	9
Peptone	1	1	1	1	1	1	1	1	1
Cysteine hydrochloride	0.05	0.05	0.05	0.05	0.05	-	0.05	0.05	0.05
Sodium chloride	-	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Sodium sulphate (Na_2SO_4)	0.05	-	0.05	-	-	-	-	-	-
Calcium chloride ($CaCl_2$)	-	-	0.05	-	-	-	-	-	-
Magnesium sulphate ($MgSO_4$ $7H_2O$)	-	-	-	0.05	0.05	0.05	0.05	0.05	0.05
Sodium sulphite (Na_2SO_3)	-	-	-	-	0.05	0.05	-	-	-
Sodium meta bisulphite ($NaHSO_3$)	-	-	-	-	-	-	0.05	-	-
Sodium thiosulphate ($Na_2S_2O_3$)	-	-	-	-	-	-	-	0.05	-
Manganese sulphate ($MnSO_4$)	-	-	-	-	-	-	-	-	0.05
Ferrous sulphate ($FeSO_4$)	-	0.05	-	-	-	-	-	-	-

Table 2. *Total bacterial count and the count of hydrogen sulphide producers in different media*

Sample	Part	SWA	PIA	KIA	LA	L & H	TSA
Mackerel *(Rastrelli ger kanagurta)*	Skin and	2.53×10^5	2.03×10^3	1.97×10^3	1.24×10^4	1.42×10^4	2.28×10^4
Decapterus	"	8.82×10^7	7.40×10^3	2.10×10^4	7.90×10^5	8.6×10^5	9.10×10^5
Parava *(Lactarius lactarius)*	"	2.10×10^5	4.50×10^2	4.86×10^3	2.60×10^2	-	-
Johnius sp.	"	1.76×10^6	3.20×10^4	2.50×10^4	7.70×10^4	1.00×10^5	4.94×10^4
Johnius sp.	Intestine	4.11×10^5	2.10×10^4	5.82×10^4	2.30×10^4	1.18×10^4	1.20×10^4
Jew fish *(Otolithes argenteus)*	Skin and muscle	8.45×10^5	6.20×10^4	5.85×10^4	2.20×10^4	3.00×10^4	7.08×10^4
Otolithes sp.	"	9.30×10^6	3.60×10^3	2.15×10^4	3.09×10^4	9.31×10^4	1.10×10^5
Barracuda	"	9.00×10^5	1.10×10^4	2.65×10^4	7.20×10^4	9.04×10^4	8.40×10^4
Metapenaeus dobsoni	Muscle	5.46×10^7	8.30×10^4	2.65×10^5	1.11×10^6	3.49×10^6	3.20×10^6
Kilimeen *(Nemipterus japonicus)*	"	5.25×10^5	7.40×10^2	2.73×10^3	7.40×10^3	7.79×10^3	1.24×10^4
Penaeus indicus	"	8.53×10^6	7.90×10^3	$1.83\ 10^4$	9.20×10^4	1.01×10^5	2.27×10^5
Kadiran *(Sillago sihama)*	Skin and muscle	1.15×10^6	1.08×10^4	3.19×10^4	4.20×10^4	4.80×10^4	5.92×10^4

Table 3. *Sensitivity of lead acetate and DTNB filter paper strips and iron agar slants on the detection of hydrogen sulphide by various bacteria*

Bacterial species	No. tested	No. of positive isolates		
		Lead acetate strip	DTNB strip	Iron agar slants
Aeromonas spp.	29	26	22	17
Vibrio spp.	15	5	6	9
Alteramonas putrefaciens	6	5	5	5
Pseudomonas spp.	5	2	2	1
Entero bacteriaceae	4	2	2	3
Acinetobacter spp.	4	1	1	2
Moraxella spp.	2	1	1	Nil
Flavobacterium spp.	1	Nil	Nil	Nil
Alcaligenes spp.	2	2	1	Nil
Total	69	44	40	37

colony appearance and incubation period were concerned. But probably due to the presence of high levels of nutrients such as gelatin, the colonies formed a black mass on the surface of the L & H plates and it could not be counted accurately.

In colony characteristics and time of development TSA resembled Kliger iron agar. The salmon pink colour of the potent fish spoiler *Alteromonas putrefaciens* was enhanced by the presence of soytone and low levels of ferric ammonium citrate used in the medium. On the basis of the above findings this medium could be considered suitable for enumerating hydrogen sulphide producers of fishery origion.

The sensitivity of the filter strip method in liquid broth using two reagents is compared with agar slant method and the results have been presented in Table 3. When the individual culture were tested for sulphide production by the three methods, 60% of the isolates were found to be positive according to lead acetate filter paper strips, 55% by DTNB strips and 51% by iron agar slants. However, the detection rate was found to vary among different genera. For *Aeromonas* and *Alcaligeness* spp. lead acetate strips provided maximum positive results. *Pseudomonas* spp. had no difference in results between the two reagents; however the iron agar showed lower detection rate of hydrogen sulphide.

The effect to different medium components on the production of nitrogen sulphide is given in Table 4. 76% of the cultures gave positive results when magnesium sulphate was added to the basal medium containing peptone and sodium chloride. Exclusion of cysteine from the medium greatly reduced the production of hydrogen sulphide and only 10% of the cultures could produce H_2S in the absence of cysteine.

Table 4. *Influence of liquid medium composition on the detection* of hydrogen sulphide*

Medium No.**	Total no of isolates tested	No. of positive isolates	%
1	92	27	29
2	92	32	35
3	92	59	64
4	92	70	76
5	92	23	25
6	92	9	10
7	92	43	47
8	92	37	40
9	92	10	20

* Lead acetate paper strip method was used throughout this study.

** Composition of the medium given in Table 1

The concentration of methionine affected the production of volatile sulphides. But in the case of cysteine, the effect was less conspicuous, though the incubation time was affected. In many instances, when 500 mg% cysteine was added to the basal medium, positive reaction could be observed in 5 to 7 h. It was also noticed that addition of 1% peptone to the cysteine medium enhanced the detection rate. Eventhough the precuesor of hydrogen sulphide is cysteine, according to Jocelyn (1972) true teactant is cystine which is required only in catalytic amounts. The enhancement of the detection rate by the addition of peptone may be due to the presence of cystine.

The data also shows that fewer number of cultures could utilize methionine as the source of sulphur. These cultures showed positive result with DTNB and negative result with lead acetate method. This shows that these cultures produced volatile sulphides other than H_2S. For cysteine, most of the cultures produced positive reaction with DTNB and lead acetate indicating the production of H_2S with or without dimethyl sulphide and methyl mercaptan, since DTNB can detect both H_2S and other volatile sulphides.

Methionine is the precursor of methyl mercaptan and dimethyl sulphide (Segal & Starkey, 1969; Kadota & Ishida, 1972). On the other hand H_2S is reported to be produced from cysteine only (Kadota & Ishida, 1972; Herbert & Shewan, 1976). The results given in Table 5 are in agreement with the above reports.

A positive test with lead acetate and negative result with DTNB is controversial, since both reagents detect H_2S. But four isolates were found to give this type of reaction. In the medium in which methionine was sole source of sulphur, all these isolates failed to give a positive test with DTNB indicating that no methyl mercaptan or dimethyl sulphide is being formed. Since no reaction was observed with DTNB in methionine medium, it is to be inferred that they produced only H_2S from cysteine. These

Table 5. *Effect of substrate concentration and incubation period on the production of hydrogen sulphide and other volatile sulphides*

Incubation period h	Method of testing	% of positive isolates						
		Methionine mg/100 ml		Cysteine mg/100 ml		Peptone (1%)	+ cysteine	(mg/100ml)
		100	500	100	500	50	100	500
24	Pb Ac	Nil	Nil	42	50	ND	ND	ND
	DTNB	6	16	38	44	ND	ND	ND
48	Pb Ac	Nil	Nil	44	53	45	48	60
	DTNB	8	22	40	47	ND	ND	ND
72	Pb Ac	Nil	Nil	47	54	59	60	62
	DTNB	9	23	42	49	ND	ND	ND
96	Pb Ac	2	2	52	56	60	64	64
	DTNB	9	23	46	51	ND	ND	ND
Total no. of isolates tested		118	108	118	108	102	118	108

ND - not determined
Pb Ac - lead acetate

isolates belonged to the genera *Aeromonas, Vibrio* or *Alcaligenes*. It is possible that the production of hydrogen sulphide is very little and it does not produce sufficient colour change in DTNB impregnated filter paper for visual detection. Sharp *et. al.*, (1977) reported that when washed cell suspensions were incubated with methionine and DTNB, the yellow colour developed by weakly positive cultures were not visible to naked eye; same may be applicable here also. This shows that as far as sensitivity for H_2S is concerned, lead acetate is superior to DTNB in detecting minute traces of H_2S.

The liquid medium using lead acetate is superior to other methods in detecting H_2S of bacterial origin. It is also more advantageous that actually incorporating it into the agar medium since it alleviates any possible toxic effect produced by lead (Mac Faddin, 1980). In many instances, when hydrogen sulphide production was evident in liquid medium, the agar slant failed to give a positive result. Bulmash & Falton (1964) had noticed blackening of lead acetate paper for some *Citrobacter* cultures, though no blackening developed on the butt of TSI slant. They suggested that the iron sulphide indicator in the medium was masked.

This study also points to the different type of spoilage flora of fish on the basis of the production of volatile sulphides. *Aeromonas* species were the major flora, followed by *Vibrio, Alteromonas Putrefaciens* and the *Pseudomonas* species. The *Pseudomonas* species though considered as active spoilers formed minor fraction of the sulphide producers. This is because eventhough Gr I *Pseudomonas* are strong producers of H_2S, Gr III and IV are weak producers or even non-producers of H_2S. Gr II *Pseudomonas* produced sulphides other than H_2S according to the report of Mc Meekin *et. al.*, (1978).

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