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Fish flesh agar medium – a suitable experimental medium for the detection of spoilage bacteria

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The spoilage characteristics of bacterial strains were studied by growing them at 28 ± 2 °C in agar and broth media prepared with sterile fish and prawn flesh homogenates. The percentage of spoilers found among the bacterial isolates tested, as shown by odour production and halo zone formation, was independent of the source of flesh used. Indole and fluorescent pigment production were also observed in the broth. *Pseudomonas, Vibrio* and *Acinetobacter* exhibited faster growth in flesh media than in the usual artificial media. Decrease of protein and lipid concentration in the clear zone of agar media suggests the utilization of the available substrate by spoilage bacteria.

INTRODUCTION

A valid assessment of the participation of various groups of bacteria in the spoilage of fish and prawn depends on the ready availability of a suitable experimental substrate allowing the activities of pure and mixed cultures to be studied (Lerke et al., 1963). Several authors have suggested flesh as a substrate for the detection of potential spoilers. These include cooked minced fish muscle and sterile raw fish muscle tissue, for organoleptic assessment of spoilage (Castell and Anderson, 1948; Castell and Greenough, 1957, 1959; Castell et al., 1959; Shewan, 1974); Seitz-filtered sterile muscle press juice for both organoleptic and chemical assessment of spoilage (Lerke et al., 1963); irradiated fish juice agar, for comparing the proteolytic activity of pure strains with that in casein digest

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agar (Kazanas, 1968); autoclaved herring extract, for detecting biochemical changes during anaerobic fish spoilage by bacteria; and herring extract agar, for making viable counts (Strøm and Larsen, 1979).

The use of flesh agar for the detection of potential fish spoilers by the formation of a halo or clear zone, demonstrating breakdown of components of the substrate, has so far not been reported. In earlier investigations (Castell et al., 1959; Shewan, 1974) strains were tested using flesh media prepared from the same source from which the bacteria were isolated (Lerke et al., 1963; Shaw and Shewan, 1968; Herbert and Shewan, 1976). However, the activity of bacterial isolates on flesh media prepared from different sources has not so far been investigated. Hence, in the present study an attempt has been made to elaborate a rapid method of identifying potential spoilers using flesh agar and broth media prepared from different sources.

Materials and Methods

Preparation of test media

Fresh fish (*Etroplus suratensis*) and prawns (*Penaeus indicus, P. monodon* and *Metapenaeus monoceros*) collected from Cochin backwater were washed with sterile tap water and eviscerated, whereupon the flesh was cut into small pieces. Homogenates were prepared separately for each fish and prawn species.

One hundred g of flesh was homogenized with an equal volume of tap water and another 100 g with 1% NaCl solution. The homogenates were made up to 1000 ml and centrifuged at 10000 × *g* for 30 min at room temperature (28 ± 2 °C). One part of supernatant as such (Cox and Lovell, 1973) and another part after boiling and removing the coagulated protein were autoclaved at 121 °C for 15 min and used for the preparation of media (Strøm and Larsen, 1979). To solidify media, 1.5% agar (Difco) was added; of broth media, 10-ml aliquots were dispensed in test tubes.

Test cultures

Bacteria tested in the present study were isolated previously during investigations on prawn spoilage. The cleaned prawns were stored at 28 ± 2 °C, 4 °C and −18 °C and were periodically analysed for changes in the bacterial flora. The cultures obtained were identified to generic level. They belonged to species of the genera *Vibrio, Pseudomonas, Aeromonas, Acinetobacter, Micrococcus* and *Bacillus,* and showed proteolytic (casein and gelatin) and lipolytic activity (Tween 80) and reduction of trimethylamine-*N*-oxide (TMAO) to trimethylamine (TMA). A loopful of 18-h-old broth cultures ($10^3 \cdot ml^{-1}$) was inoculated in flesh broth and in the case of agar media it was placed at the centre of the plate.

Table 1. Efficiency of different flesh homogenate media for the detection of odour production and halo formation by bacterial isolates[1]

Source of flesh media	Percentage of isolates tested being		
	Off-odour producers	Halo zone producers	Odour and halo zone producers
Etroplus suratensis	73.1	57.7	54.3
Penaeus indicus	79.0	62.3	53.3
Penaeus monodon	80.0	63.4	50.0
Metapenaeus monoceros	76.7	60.0	50.0

[1] See Table 2 for distribution of genera amongst these strains.

Analytical methods

Inoculated media were incubated at room temperature (28 ± 2°C) for up to 48 h. Production of off-odour was observed and evaluated at 18, 24 and 48 h with the help of a panel of five judges. Halo zone formation around the colony on agar media was checked after 12, 18, 24 and 36 h. Indole production in flesh broth was directly tested by adding three to four drops of Kovacs' reagent after 48 h of incubation. Diffusible pigment production and fluorescence in flesh broth and agar media were observed with the aid of an ultraviolet lamp.

Portions of flesh agar (10-mm diam) from halo (cleared zones) and opaque zones in agar plates were cut out, using a sterile cork borer, and the protein and lipid contents estimated according to Snow (1950) and Folch et al. (1957), respectively.

Results

The efficiency of different flesh homogenate media, prepared with and without NaCl, to detect off-odour production and halo formation was similar, regardless of their source. Data obtained for the production of putrid and off-odours, and halo formation around the colony are presented in Table 1. Off-odour production was shown by 73–80%, and halo formation was observed with 58–63% of the 138 bacterial isolates tested. Although a difference of nearly 16% exists between odour producers and halo formers, efficient utilization of the flesh media is seen with at least 60% of the cultures. Further 50–54% of the cultures were able to produce both odour and a halo zone, suggesting their ability to degrade the components of the flesh as well as to release odour due to their action on the substrates. Probably, these isolates are active spoilers which demonstrate their activity directly on the flesh in its pure form.

The taxonomic affiliation of the isolates tested is presented in Table 2. Gram-negative forms were more frequent spoilers than gram-positive forms. The bacteria having spoilage activity were able to produce halos around the colony within

Table 2. Taxonomic affiliation of the isolates tested for odour and halo zone production in flesh homogenate media

Genus	Total isolates tested	% of odour producers	% of halo producers	% of odour and halo producers	Characteristic of odour
Pseudomonas	46	87.0	58.7	41.3	fruity, ammoniacal, pungent, sulphidy
Vibrio	34	94.1	58.8	52.9	fruity, pungent, sulphidy
Acinetobacter	29	79.3	86.2	37.9	ammoniacal, pungent
Enterobacteriaceae	5	80	40	20	pungent, stale
Alcaligenes	2	100	–	–	pungent, stale
Aeromonas	1	100	100	100	pungent
Micrococcus	9	33.3	66.7	22.2	pungent, stale
Bacillus	9	33.3	44.4	–	pungent, stale
Corynebacterium	3	33.3	33.3	–	pungent, stale
Gram-negative	117	87.2	64.1	42.7	
Gram-positive	21	33.3	52.4	9.5	

12–18 h. They also showed faster growth. The cleared zone produced by a representative culture of a *Pseudomonas* species around the colony is shown in Fig. 1. The cleared area was detected more easily as compared with that on casein digest agar.

Putrid, ammoniacal and fruity odours were detected in high intensity from 18 h onwards up to 36 h. Later, the odour intensity decreased. All the isolates which produced indole from tryptophan in the synthetic medium were capable of producing indole from prawn flesh broth. During the course of this experiment seven isolates of *Pseudomonas* spp. which elaborated diffusible fluorescent pigments on King's agar B (King et al., 1954) also showed production of such greenish yellow pigments in flesh broths.

Results of estimations of protein and lipid concentrations in flesh agar are presented in Table 3. A reduction in protein and lipid concentrations was observed in the flesh media prepared with supernatants as well as in those prepared after boiling and removing the coagulated protein. Although the reduction was not substantial, it was significant, indicating a definite degradation or utilization of the components by the flesh-spoiling bacteria.

Discussion

The suitability of sterile muscle press juice as an experimental medium and the efficiency of flesh broth for detecting odour production has been demonstrated (Lerke et al., 1963). There are differences in the methods used in the preparation of sterile flesh media, viz. Seitz filtration (Lerke et al., 1963), irradiation

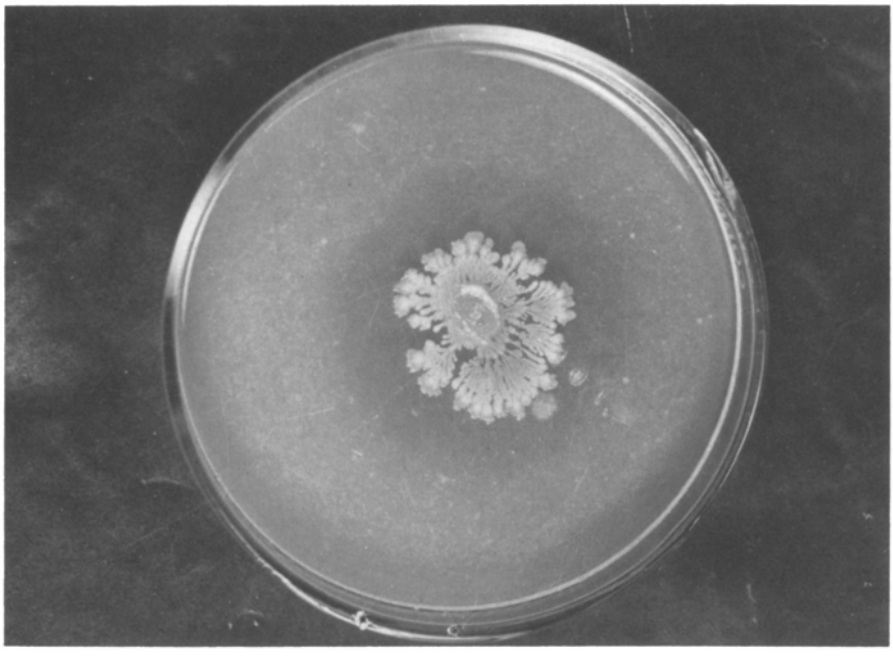

Fig. 1. Clear zone produced by *Pseudomonas* sp. on flesh agar medium.

(Kazanas, 1968; Van Spreekens, 1977) and autoclaving (Alur et al., 1971; Cox and Lovell, 1973; Strøm and Larsen, 1979). In the present study, sterilization by autoclaving gave satisfactory results and enhanced rapid determination of potential spoilers amongst the bacterial flora.

Though flesh media with and without precipitated protein showed a difference in biochemical composition (Table 3) and turbidity, both proved equally suitable

Table 3. Protein and lipid concentrations in halo and opaque areas in flesh agar media after growth of spoiler bacteria (average of ten samples)

Flesh agar medium	Substrate	Concentration in the opaque zone ($mg \cdot g^{-1}$)	Concentration in the halo zone ($mg \cdot g^{-1}$)	Difference ($mg \cdot g^{-1}$)	% of the content reduced
Medium prepared after boiling and removal of coagulated protein	protein	15.0	13.5	1.5	10.0
	lipid	4.7	1.9	2.8	59.6
Medium prepared directly	protein	42.3	36.3	6.0	14.2
	lipid	6.5	2.3	4.2	64.6

to detect spoiling bacteria. Hence, for the purpose of detecting the spoilage potential of bacteria the flesh media may be prepared in either way.

Bacteria isolated from *P. indicus* showed the same activity in media prepared with flesh of either prawn or fish. This suggests that the activity of the spoiler is not specific for the fish species from which it was isolated; more likely, specificity is towards the chemical nature of the substrate. Hence, any fish or prawn muscle may be used as raw material for the purpose of detecting potential spoilers.

Production of off-odour as well as indole, as by-products of the bacterial action on flesh components as observed in this investigation agrees well with the results of Lerke et al. (1963) and supports the suitability of this medium for detecting organoleptic and chemical changes of sea-food caused by fish spoilage bacteria. Enhancement of fluorescent pigment production in the flesh broth by *Pseudomonas* spp. suggests the feasibility of the medium for detecting fluorescent pigment-producing spoilers directly.

Testing proteolytic and lipolytic activity of bacteria in artificial media and in this way relating presence of bacteria to spoilage (Castell and Mapplebeck, 1952) is common practice. Lerke et al. (1965) reported a complete lack of correlation between spoilage activity in fish juice and the results of biochemical tests, and no pattern of biochemical activities could be detected which could reliably characterize either the spoiler or the non-spoiler group. However this may be, proteolysis is important in fish spoilage, primarily as a means of replenishing the amino acid content of the non-protein nitrogen pool on which the spoilage bacteria are growing (Liston, 1973). In the present study, clear zones formed by the bacterial strains and changes in protein and lipid concentrations in the media suggest the production of hydrolytic enzymes thus confirming the validity of flesh agar media for rapid determination of spoilers.

In conclusion, potential spoilers existing as a part of the associated microflora in fresh and processed fish can be easily and directly detected with flesh agar and broth media, which are readily available at low cost and easy to prepare. Flesh agar may further be used for total viable counts, as Strøm and Larsen (1979) have reported, to study populations of potential spoilers on fish and prawn samples directly.

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