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Isolation of Streptococci from Fish and Aquatic Environments

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Abstract

Streptococci were isolated from freshwater and marine aquaculture environments by use of a new developed selective medium, STAN agar. STAN agar is composed of BHI agar with sodium azide and thallous sulphate as selective agents. The deposits in tilapia culture ponds contained 10^2 - 10^3 cfu/g of streptococci. For the raw-fish diets fed to cultured flounder and yellowtail, viable counts of streptococci were 10^2 cfu/g at first and increased to 10^4 cfu/g after they were dipped in sea water for 24h.

Streptococcus strains isolated from freshwater and marine environments were nonhemolytic, did not hydrolyse casein, starch and chitin, and ferment glucose, fructose, sucrose, trehalose and salicin. Marine isolates were different from freshwater ones in the fermentation of some carbohydrates including lactose, galactose, raffinose and arabinose.

Streptococcal infections have become one of the most serious diseases for both freshwater and marine aquacultured fish such as yellowtail, eel, rainbow trout and tilapia in Japan[1-4]. Streptococci are isolated ubiquitously from water, fish and raw fish diets in aquacultural facilities. The genus *Streptococcus* comprises a wide variety of taxonomic groups which were distributed in different environments including soil, water, dairy products and animals[5-7].

Fish pathogenic streptococci are to belong to the species different from those isolated from terrestrial animals, but their taxonomic positions are not yet confirmed distinctly[7]. These streptococci have been isolated from various kinds of sources by use of numerous selective media. Sodium azide, thallous acetate or various kinds of antibiotics were used commonly as selective agents added to isolation media.

In this paper, we attempted to isolate streptococci from various sources in marine and freshwater aquacultural facilities by use of a new selective medium containing sodium azide and thallous sulphate as selective agents.

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Materials and Methods

Bacterial strains

For the determination of colony size on selective media, bacterial strains belonging to *Streptococcus,Micrococcus* and gram-negative rods were used. *Streptococcus faecalis* (IFO 12580) and *St. faecium* (IFO 3181) were used as reference strains.

Media

Z-AII agar composed of polypeptone 5 g, yeast extract 1 g and agar 15 g in one liter of 1/6 strength of artificial sea water (ASW) and Z-CII agar containing same ingredients in 3/4 strength of ASW were used for determination of total viable counts in freshwater and marine environments, respectively. Brain heart infusion (BHI) agar was also used for total viable counts of freshwater samples. EF agar (Nissui) obtained commercially and STAN agars prepared with different concentrations of selective agents were compared for the growth and colony size of streptococci. The compositions of STAN agars are shown in Table 1.

Table 1. The compositions of STAN agar media

Ingredients	Variations of STAN agar		
	STAN/L*3	STAN/S*4	STAN/H*5
BHI broth (Nissui)	35 g	35 g	35 g
Glucose	8	8	8
Sodium azide	0.03	0.05	0.1
Thallous sulphate	0.3	0.5	1.0
BTB*1	0.032	0.032	0.032
TTC*2	0.1	0.1	0.1

*1 BTB = bromothymol blue
*2 TTC = 2, 3, 5–triphenyltetrazolium chloride
*3 STAN/L = low concentration STAN agar
*4 STAN/S = standard STAN agar
*5 STAN/H = high concentration STAN agar

Viable counts

Raw-fish diets (sardine or mackerel) were homogenized in a mortar and diluted with a ten times volume of dilution solution. An aliquot of the suspension was diluted in series and then 0.1 ml of the appropriate dilution solution was smeared on agar plates. Deposit samples from aquaculture ponds were collected together with the overlayered water and stood until solid materials were sunk on the bottom. After an extra volume of water was discarded, equal volumes of water and solid materials were vigorously mixed and diluted in series. Water samples were directly mixed with dilution solution and smeared on agar plates.

Characteristics of bacterial isolates

Bacterial isolates grown in BHI broth or on BHI agar were submitted to bacteriological characterization by means of the standard methods described in bacteriological manuals[8]. Bacterial characteristics examined in this study were as follows: Gram stain, cell form, motility, oxidase, catalase, carbohydrate fermentation, growth temperature, growth pH, utilization of citric acid, VP and MR tests, hydrolysis of hippuric acid, esculin, casein, starch and chitin, and arginine dehydrolase.

Results

Effect of selective media on colony formation

Average colony diameters on agar plates for streptococci, gram-negative rods and coccus isolates obtained from the sediments in tilapia aquaculture ponds are shown in Fig. 1. *Streptococcus* isolates formed red-colored colonies on EF agar, which were smaller than 0.5 mm in diameter. Any gram-negative rods and coccus isolates could not form colonies, and

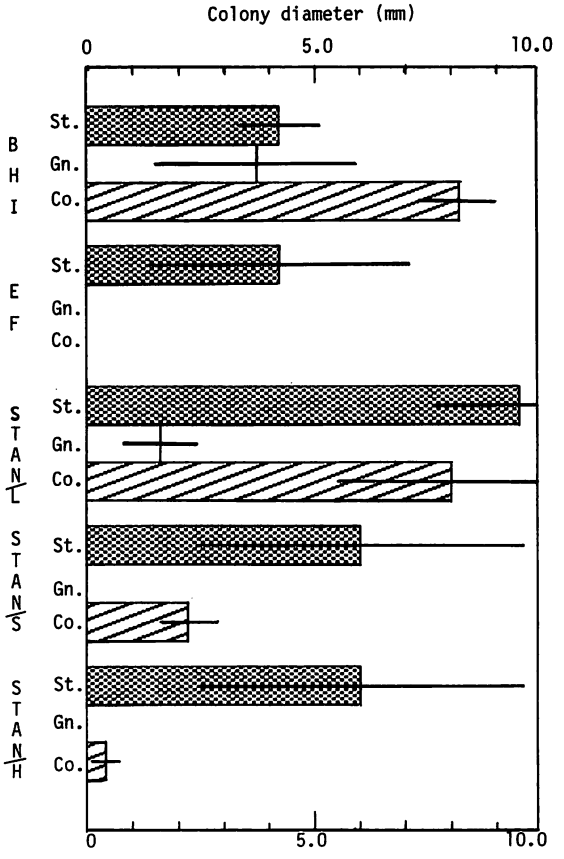

Fig. 1. Colony diameter of streptococci, gram-negative rods and micrococci isolated from the sediments in tilapia culture ponds on selective agar media.
St.: streptococci, Gn.: gram-negative rods, Co.: micrococci.

Table 2. Viable counts of total bacteria (Z–A Ⅱ) and streptococci (EF and STAN) isolated from the deposits in tilapia aquaculture ponds.

Pond	Date (1987)	Viable counts (cfu/g) Z–A Ⅱ	EF	STAN
No. 23	12/11*	3.9×10^8	1.8×10^3	2.3×10^3
	12/23*	1.7×10^8	5.4×10^2	8.7×10^2
No. 14	12/11	1.4×10^8	3.9×10^2	4.8×10^2
	12/23	1.2×10^8	1.9×10^2	2.3×10^2
No. 28	12/11	8.7×10^8	9.5×10^2	1.3×10^3
	12/23	3.6×10^8	2.0×10^2	2.2×10^2
Average		3.4×10^8	6.8×10^2	9.0×10^2

* water temperature at 23.5°C.

Table 3. Viable counts of total bacteria (Z–C Ⅱ) and streptococci (EF and STAN) isolated from the raw–fish diets for cultured flounder.

Samples	Date (1987)	Viable counts (cfu/g) Z–C Ⅱ	EF	STAN
Raw–fish diets	10/18*	4.2×10^5	1.9×10^3	4.5×10^2
at 0 h	11/4*	1.6×10^4	1.0×10^3	2.0×10^2
Raw–fish diets	10/18	5.7×10^5	5.5×10^2	1.5×10^3
at 6 h	11/4	4.4×10^5	4.7×10^3	2.7×10^3
Raw–fish diets	10/18	4.2×10^8	6.6×10^4	7.0×10^4
at 24 h	11/4	5.0×10^8	4.2×10^4	3.6×10^4
Pond water	10/18	1.7×10^4	< 10	< 10
	11/4	3.6×10^2	< 10	< 10
Bottom sands	10/18	3.6×10^5	< 10	< 10
	11/4	3.2×10^6	8.0×10^2	4.5×10^3

* water temperature at 23°C (10/18) and at 22°C (11/4).

Table 4. Viable counts of total bacteria (Z–C Ⅱ) and streptococci (EF and STAN) isolated from the raw–fish diets for cultured yellowtail.

Samples	Date (1987)	Viable counts (cfu/g) Z–C Ⅱ	EF	STAN
Raw–fish diets	11/18	4.5×10^5	3.5×10^2	6.9×10^2
at 0 h	12/1	1.3×10^6	7.3×10^3	6.9×10^3
Raw–fish diets	11/18	2.0×10^6	9.0×10^2	1.2×10^3
at 6 h	12/1	1.4×10^6	1.1×10^2	2.5×10^2
Raw–fish diets	11/18	3.0×10^8	2.6×10^5	2.9×10^5
at 24 h	12/1	1.4×10^8	2.6×10^5	2.9×10^5
Moist pellets	11/18	7.2×10^5	1.3×10^2	1.1×10^3
	12/1	1.2×10^6	1.9×10^4	2.2×10^4
Sea water	11/18	2.0×10^4	< 10	< 10
	12/1	1.3×10^4	< 10	< 10

* water temperature at 23°C (11/18) and at 22°C (12/1).

some strains of streptococci could not also on EF agar. On the other hand, *Streptococcus* isolates produced larger colonies on STAN/S as compared with those on EF agar, While some coccus isolates could produce tiny colonies on STAN/S agar. However, it is easy to distinguish streptococci from micrococci on the basis of their colony forms and colors. We principally used STAN/S agar in order to isolate streptococci from aquaculture environments.

Isolation of streptococci from aquaculture environments

Table 2, 3 and 4 present viable counts in water, sediments and raw-fish diets from tilapia, flounder and yellowtail aquaculture environments, respectively. In general, viable counts on STAN(STAN/S) agar were somewhat more than those on EF agar and it was easy to dis-

Table 5. Main characteristics of freshwater and marine isolates of *Streptococcus*.

Characters	Freshwater isolates(20)	Marine isolates(24)	Reference strains A	B	C	D	E*
Cell form	Co	Co	Co	Co	Co	Co	Co
Gram stain	+	+	+	+	+	+	+
Motility	−	−	−	−	−	−	−
Oxidase	−	−	−	−	−	−	−
Catalase	−	−	−	−	−	−	−
OF test	F	F	F	F	F	F	F
Growth at 10°C	d	d	+	+	+	+	+
45°C	−	−	+	+	+	+	−
Growth in 3% NaCl	+	+	+	+	+	+	+
6.5%	d	d	+	+	+	+	+
10%	−	−	−	−	−	−	−
Growth at pH 4.6	d	−	+	+	+	+	+
pH 9.6	+	+	+	−	+	+	+
Growth after 60°C	+	d	+	+	+	+	+
Citrate util.	−	−	−	−	−	−	−
VP test	d	d	+	−	−	−	−
MR test	d	d	+	+	+	+	+
Hydrolysis of							
hippurate	−	d	−	−	−	−	+
esculin	+	d	+	+	+	+	+
arginine	d	+	−	+	+	+	−
casein	−	−	−	−	−	−	−
starch	−	−	−	−	−	−	−
chitin	−	−	−	−	−	−	−
Hemolysis(β)	−	−	−	−	−	−	−

*A : *St. faecalis* (IFO 12580), B : *St. faecium* (IFO 3181)
C, D, E : *Streptococcus* isolates from tilapia.
Abbreviation : Co ; coccus, F ; fermentation, d ; variable, + ; positive, − ; negative.

tinguish streptococcus colonies owing to their growth rate and colony morphology on STAN agar. Viable counts of streptococci were 10^2 - 10^5 cfu/ml or g while total viable counts were 10^4 -10^8 cfu/ml or g. Especially, viable counts of streptococci in raw-fish diets increased after dipped in sea water for 24 h.

Characteristics of streptococcus isolates

Table 5 indicates the main characteristics of freshwater and marine isolates of streptococci could not grow at 45°C and at the concentration of 10% NaCl. Reference strains (*St. faecium* and *St. faecalis*) were able to grow at 45°C. All strains tested did not hydrolyse casein, starch and chitin, and were non-hemolytic. As shown in Fig. 2, most of freshwater isolates fermented galactose, lactose and raffinose. On the other hand, most of marine isolates did not ferment lactose and there were marine isolates positive and negative for the fermentation of arabinose, galactose and raffinose depending on raw-fish diet samples used to isolate streptococci.

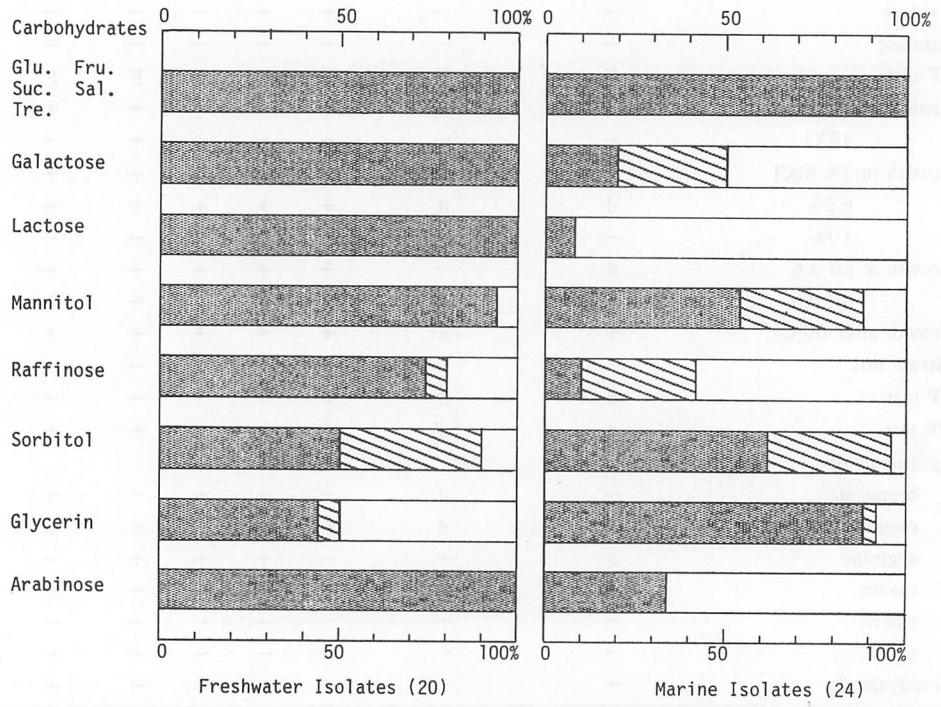

Fig. 2. Fermentation of various carbohydrates by freshwater (20 strains) and marine isolates (24 strains) of streptococci.
▨: positive, ▨: weak positive, □: negative.

Discussion

Streptococcus infection is one of the most serious problems for the aquaculture of yellowtail, eel, flounder and tilapia in Japan. EF agar has been usually used for the isolation of streptococci from diseased fish and aquaculture environments. However, EF agar is considerably suppressive for streptococci to grow on it. STAN agar developed in this study was found to be suitable for streptococci to form colonies on it in comparison with EF agar because streptococci can grow faster and produce distinguishable colonies on it. In this study, we examined the distribution of streptococci in aquaculture environments by use of STAN agar.

Streptococci were isolated commonly from freshwater and sea water sources and most of isolates were non-hemolytic ones. Especially, viable counts of streptococci were higher in raw-fish diets fed to cultured yellowtail and flounder. In raw-fish diets, streptococci were isolated at 10^2 - 10^3 cfu/g when samples were taken out of the freezer and thawed. However, after raw-fish diets were dipped in sea water of fish culture ponds for 24 h, the number of streptococci associated with them increased up to 10^4 - 10^5 cfu/g. These results indicate that streptococci were principally transferred to aquaculture environments through raw-fish diets and increased during the period when they were left under the water. Therefore, the storage conditions of raw-fish diets and rapid removal of them lefted in aquaculture environments are important for the supression and protection against streptococcus infection to cultured fish.

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