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Effect of Temperature, pH and Media on the Recovery of Aerobic Heterotrophic Bacteria from the Rabbitfish *Siganus rivulatus*

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ABSTRACT. As part of ongoing studies on the microbiology of the rabbitfish *Siganus rivulatus*, farmed in sea cage sites at Jeddah, methods have been devised for the enumeration and recovery of dominant aerobic heterotrophic bacteria. Thus, from a comparison of 11 media at pH 6.5-9.5, incubation temperatures of 5-40°C and statistical analysis of the data by means of the student's t-test, the highest number of aerobic heterotrophic bacteria was consistently recovered on medium A3, which comprised 2.4% (W/V) sodium chloride, 0.7% (W/V) magnesium sulphate, 0.075% (W/V) potassium chloride, 0.1% (W/V) bacteriological peptone (Oxoid) and 1.5% (W/V) agar (Oxoid No. 1) at pH 7.2-7.8 with incubation at 20-25°C for 14 days. Using this regime, the aerobic heterotrophic bacterial populations on the gills of *S. rivulatus* and from the surroundings water (fish cages and open sea) were estimated at 6×10^5/g, 3.3×10^4/ml and 6.6×10^2/ml, respectively. The presence of higher quantities of a diverse range of organic nutrients (including bacteriological peptone, yeast extract, beef extract and casein) led to the recovery of reduced numbers of bacteria from both gill and water samples.

Introduction

Methods of bacterial enumeration have a direct influence on the resultant quantitative data. However, the most commonly used and widely accepted methods for enumerating aerobic heterotrophs from water and soils involve plate counts (Buck and Cleverdon, 1960; Jannasch and Jones, 1959). The main problem of pour plate methods is that many marine bacteria are heat sensitive. Consequently, damage to and even death of the cells may occur after exposure to the temperatures of molten

cooled agar (Austin, 1988). Thus, spread plating is the main alternative for the recovery of native aquatic bacteria (Jones, 1970). However, a number of factors influence viable counts of bacteria, namely, medium composition, pH and incubation conditions. It is conceded that there is a complex interaction involving the precise incubation temperature and the nature of media used. For example, Mudarris and Austin (1988) noted higher counts at 15-25°C than at 30°C. According to Frobisher *et al.* (1974) bacteria have certain optimal pH limits for growth. For example, the bacterial counts from turbot gills were significantly affected by the pH value of the isolation medium (Mudarris and Austin, 1988).

The objective of the present study was to discover the most suitable medium and cultural conditions for the recovery of aerobic heterotrophic bacteria from *S. rivulatus* gills.

Materials and Methods

Experimental Animals

Live rabbit fish of approximately 55 g average weight, were obtained from the mariculture facility at Jeddah. The fish were maintained in aerated sea water at approximately 19°C.

Bacteriological Examination

Healthy animals were killed by physical destruction of the brain, and portion of gills, each weighing 1 g were carefully excised and placed in sterile petri dishes. For comparison, volumes of the surrounding water (fish cages and open sea) were also collected. Following gentle washing with 10 m volumes of sterile sea water (121°C/15 min), gill filaments were pulverized (Giffiths tubes) for 3 min in 9 ml volumes of sterile sea water. For all the sample types, dilution were prepared to 10^{-4}. Thence 0.1 ml volumes were spread out of the surface of triplicate plates of many types of media. In the ensuing experiments, 11 media and various incubation conditions were assessed for the recovery of maximal numbers of bacteria. The media tested were as follows: marine 2216E agar (Difco); brain heart infusion agar (BHIA, Oxoid); nutrient agar (Oxoid); medium K (Mudarris and Austin, 1988); iron peptone agar (FEPA, Ferrer *et al.*, 1963) prepared with and without aged (for 30 days) sea water, and 5 specially formulated products including; medium A1, this comprised NaCl, 2.4% (W/V); $MgSO_4 . 7H_2O$, 0.7% (W/V); KCl, 0.075% (W/V); bacteriological peptone, 0.5% (W/V); agar (Oxoid No. 1) 1.5% (W/V); distilled water, 100 ml; medium A2, containing bacteriological peptone, 0.25% (W/V); medium A3, comprising bacteriological peptone, 0.1% (W/V); medium B1, this comprised bacteriological peptone, 0.5% (W/V); agar No. 1, 1.5 (W/V); aged sea water, 75 ml; distilled water, 25 ml, and medium B2, containing bacteriological peptone, 0.25% (W/V). In addition, the effect of acidity-alkalinity was determined by adjusting the pH to 6.5, 7, 7.2, 7.4, 7.6, 7.8, 8, 8.5, 9 and 9.5. Incubation was at 5, 10, 15, 20, 25, 30, 35 and 40°C up to 14 days, whereupon the number of colonies was counted.

Statistical Examination of the Data

Plate count data were analyzed by means of student's t-test, incorporated into a program package on an IBM microcomputer.

Results

Of the eleven types of media examined, the highest counts were consistently obtained on medium A3 (Table 1). Thus, in one comparative exercise, counts of 5.2×10^5 wet weight of gill tissue were obtained with this medium. Conversely, the lowest numbers were recovered on medium B1. Moreover, these findings were reenforced by the results of the t-test (95% confidence level).

TABLE 1. Numbers of aerobic heterotrophic bacteria recovered on the various media (all at pH 7.4) following incubation at 20°C for 14 days.

Medium	No. of bacteria ($\chi 10^5$/g fresh weight of gill)
Marine 2216E agar (Difco)	1.97
Brain heart infusion agar (Oxoid)	1.9
Nutrient agar (Oxoid)	1.43
Medium K	1.46
Iron peptone agar (FEPA)	2.63
Iron peptone agar prepared in aged sea water	1.7
Medium A1	1.53
A2	2.3
A3	5.2
B1	1.33
B2	1.43

From Table 2, it is apparent that the nature of the microbiological methods used exerted a pronounced effect on the recovery of bacteria from *S. rivulatus* gills. In the comparison of incubation temperatures, most bacteria were recovered at 20°C. However, statistical examination of the data (significant at the 95% level in the t-test) also pointed to the benefit of using 25°C. Both these temperatures were vastly superior, in terms of recovery of the maximal number of bacteria to the other incubation temperatures.

TABLE 2. Effect of temperature of incubation on the numbers of bacteria recovered from *Siganus rivulatus* gills. Medium A3 at pH 7.4 was used, with incubation for 14 days.

Temperature (°C)	No. of aerobic bacteria ($\times 10^5$/g)
5	0
10	0.26
15	1.26
20	3

TABLE 2. (continued).

Temperature (°C)	No. of aerobic bacteria ($\times 10^5$/g)
25	2
30	1.56
35	1.1
40	0.66

Concerning acidity-alkalinity, the highest plate counts were obtained with media at pH 7.2 (Table 3), although the t-values highlighted pH 7.4, 7.6 and 7.8 (at the 95% confidence level). Consequently, it was apparent that the greatest number of aerobic bacteria were recovered from *S. rivulatus* gills by the use of medium A3 at pH 7.2-7.8 with incubation at 20-25°C for 14 days. Using the technique, the number of aerobic heterotrophic bacteria on gill filaments and from the surrounding water (fish cages and open sea) were estimated at 6×10^5/g, 3.3×10^4/ml and 6.6×10^2/ml, respectively.

TABLE 3. Effect of pH of the medium on the numbers of bacteria recovered from *S. rivulatus* gills. Medium A3 used, with incubation at 20°C for 14 days.

pH	No. of aerobic bacteria ($\times 10^5$/g)
6.5	2.86
7	3.76
7.2	5.26
7.4	4.26
7.6	4.23
7.8	4
8	3.6
8.5	2.53
9	1.96
9.5	1.5

Discussion

The results of this study have demonstrated the marked effect of methodology on the number of bacteria isolated from the gills of *S. rivulatus*. The data appear to correspond closely with previous studies, which demonstrated a relationship between temperature and nutrients in the recovery of aerobic marine bacteria (Jones, 1960; Mudarris and Austin, 1988). In this study, the highest number of colonies was consistently recovered at an incubation temperature of 20°C. However, statistical examination of the data also pointed to the benefit of using 25°C. Certainly, this indicates that mesophiles are more abundant than thermophiles on *S. rivulatus* gills. Bacteria have definite optimal pH limits for growth (Frobisher *et al.*, 1974). In the present

study, the highest plate counts were obtained with slightly alkaline media, *i.e.* at pH 7.2. However, statistical examination of the data also pointed to the benefit of using the slightly higher pH of 7.4, 7.6 and 7.8. This observation corresponds well with the report of Carpenter (1977), who noted that generally the optimum pH of bacteria is close to neutrality. In fact, numerous authors have used a similar pH for isolation purposes (Anderson and Conroy, 1969; Kaper *et al.*, 1978; Mudarris and Austin, 1988). The results of this study have clearly demonstrated that the commercially available version, or the specialized formula of Ferrer *et al.* (1963) and Mudarris and Austin (1988) incorporated into the dilution plate technique, are inadequate for recovery of maximal number of aerobic heterotrophic bacteria from the gills. Instead, optimal conditions centered on using medium A3 (at pH 7.2-7.8 with incubation at 20-25°C), which contained low quantities of peptone.

It is relevant to note that Floodgate (1964) also recognized that low levels of nutrients, notably peptone, generated higher counts of marine bacteria than other recipes incorporating higher amounts of organic material. It should be emphasized that the results of this study suggested that, in some instances, high concentrations of organic components may be inhibitory to some marine bacteria. Thus, with the methods described in this study, the maximal number of aerobic heterotrophic bacteria recovered from the gills of *S. rivulatus* was estimated as 6×10^5/g. This population size approximated the number of bacteria recovered from the gills of fresh water salmonids (Trust, 1975) and from the gills of the marine fish turbot (Mudarris and Austin, 1988).

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تأثير درجات الحرارة والأس الهيدروجيني والأوساط الغذائية على عزل البكتيريا الهوائية عضوية التغذية من أسماك السيجان النهيري

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المستخلص . تم في هذه الدراسة تحديد الطرق المناسبة لعزل البكتيريا الهوائية ذات التغذية العضوية والمتواجدة على أسماك السيجان النهيري وذلك كجزء من الأبحاث الجارية عن الأحياء الدقيقة لهذه الأسماك والتي تم تربيتها في أقفاص على ساحل مدينة جدة .

وعليه فقد تم استخدام إحدى عشر وسطًا غذائيًا مختلفًا ودرجات مختلفة من الأس الهيدروجيني ابتداءً من ٦,٥ وحتى ٩,٥ ودرجات حرارة حضانة من ٥م° وحتى ٤٠م° .

وبتحليل النتائج إحصائيًا باستخدام Student's t-test وجد أن أعلى الأرقام قد سجلت على الوسط الغذائي A_3 والمعد معمليًا والذي يتضمن التالي :

٢,٤٪ (وزن/جم) كلوريد الصوديوم و ٠,٧٪ (وزن/جم) كبريتات المغنسيوم و ٠,٠٧٥٪ (وزن/جم) كلوريد البوتاسيوم و ٠,١٪ (وزن/جم) بيبتون بكتيري (Oxiod) و ١,٥٪ (وزن/جم) آجار (Oxoid No. 1) وذلك عند درجة أس هيدروجيني من ٧,٢ إلى ٧,٨ ودرجات حرارة حضانة ٢٠م° و ٢٥م° ولمدة أربعة عشر يومًا .

وباتباع المعايير السابقة قدرت الأعداد البكتيرية على خياشيم الأسماك والمياه المحيطة بها بالإضافة إلى مياه البحر المفتوح كالتالي : ٦×١٠^{٥}/جرام و $٣,٣ \times ١٠^{٤}$/ملم و $٦,٦ \times ١٠^{٢}$/ملم وذلك على التوالي .

كما أشارت نتائج هذه الدراسة إلى أن استخدام التراكيز العالية من المواد العضوية في الأوساط الغذائية قد يؤدي إلى نتائج عكسية وإلى انخفاض واضح في الأعداد البكتيرية سواء المعزولة من الأسماك أو المياه المحيطة بها .