

SHORT REPORT

Factors Affecting Production of Haemolysin by Strains of *Vibrio fluvialis*

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ABSTRACT

The in vitro production of haemolysin by *Vibrio fluvialis* was studied using sheep erythrocyte. The effect of the composition of various media and different concentrations of sodium chloride on the production of haemolysin and heat-stability was investigated. Comparatively higher titre of haemolysin production was noted in brain heart infusion (BHI) broth. Adding 0.5% NaCl to BHI broth reduced the production of haemolysin; adding 5.0% NaCl to the medium totally inhibited it. The highest titre of haemolysin was produced at 30 °C and 37 °C, whereas no haemolysin was produced at 50 °C. Haemolytic activity was totally destroyed when heated at 56 °C for 30 minutes. Haemolysin could be assayed easily following this method.

Key words: Haemolysin; Haemolysis, *Vibrio fluvialis*

INTRODUCTION

Haemolysin is one of the pathogenic factors of different bacterial pathogens. Considering haemolytic phenotype as one of the probable virulence markers, initial screening and isolation of virulent heterotrophic bacteria from the environment has been accomplished on blood agar plate (1). Haemolytic activity has been reported from different members of the family *Vibrionaceae*, such as *Vibrio cholerae* O1 El Tor (2), *V. mimicus* (3), *V. vulnificus* (4), *V. cholerae* O139 (5), and *Aeromonas* spp. (6,7). Moreover, some members of the family *Vibrionaceae*, such as *V. cholerae* non-O1 (8), *V. hollisae* (9,10), and *V. mimicus* (11), produce haemolysins related to the thermostable direct haemolysin (TDH) of *V. parahaemolyticus*. Nishibuchi *et al.* (12) made a comparative analysis of the *V. cholerae* non-O1, *V. mimicus* and *V. hollisae* genes that show similarities with the TDH gene of *V. parahaemolyticus*. The authors concluded that the haemolysin genes of the family *Vibrionaceae* are derived from a single common ancestral gene (12). This study was undertaken to look into the factors that affect the production of haemolysin by strains of *V. fluvialis*.

MATERIALS AND METHODS

Bacterial strains

Strains of *V. fluvialis* were mostly isolated from prawn-samples collected from the local fish market and from water-samples of the Buriganga river. The strains were preserved on blood agar slant overlaid with sterile paraffin oil and stored at room temperature (four years) until used.

Media and Culture Conditions

Various broth culture media were used. They include brain heart infusion (BHI) broth, heart infusion broth (HIB), HIB with 1% glycerol (HIBGL), trypticase soy broth with 0.2% yeast extract (TSBYE), and Richardson's medium (recipes per litre were: casamino acid 20.0 g, Na₂HPO₄ 0.15 g, NaCl 2.5 g, Tris-HCl 0.65 g, K₂HPO₄ 3.67 g, yeast extract 6.0 g, trace salt 1.0 ml; pH 8.0). Broths were prepared according to the instruction of the manufacturer (Difco).

The effect of different concentrations of NaCl on the production of haemolysin was studied using the strain that produced the highest titre of haemolysin in a particular medium to which NaCl was added to final concentrations

of 0.5%, 1.0%, 2.0%, 3.5%, 5.5%, 6.5%, 8.5%, and 10.5%. To study the effect of temperature on the production of haemolysin, the best haemolysin-producing strain was grown on the best haemolysin-producing medium at different temperatures: 30 °C, 37 °C, 40 °C, 42 °C, 46 °C, 48 °C, 50 °C, and 56 °C for 18 to 24 hours.

Ten mL of the above mentioned broths in a 50-mL conical flask was inoculated with bacteria 10^7 from young cultures and incubated in a shaking waterbath (80 oscillation per minute) either at 37 °C or at various temperatures as indicated above for 18 to 24 hours. The cultures were then centrifuged for 15 minutes at 22,000 g at a temperature of 4 °C. The supernatants were collected and sterilized by millipore (0.22 µm, Millipore Bedford, USA), filtration. These supernatants were used for detect haemolytic activity against sheep erythrocytes.

Haemolysin assay

Haemolysin assay was performed following the procedure of Tison and Kelly (13). Freshly defibrinated sheep blood was used in this experiment. Sheep erythrocytes were washed thrice in normal saline, and a 1% suspension (v/v) of erythrocytes was made in normal saline. Serial 10-fold dilutions of the culture supernatant were prepared in normal saline. Equal volume of 1% erythrocytes were added into each dilution and incubated at 37 °C for two hours. Following incubation, the suspension was centrifuged, and the absorbance at 540 nm was determined in a Coleman spectrophotometer (model 6/20A Colman Junior®, IIA, Illinois, USA). Haemolysin titres were calculated as the reciprocal of the last dilution which has greater than 50% haemolysis compared to the undiluted supernatant (14).

Heat inactivation

Aliquots of the supernatant were heated at 50 °C and 56 °C for 30 minutes before assay of haemolytic activity.

Table. Production of haemolysin in various culture media

Culture media	Haemolysin titre produced by <i>V. fluvialis</i> strains						
	VF2	VF3	VF4	VF14	VF16	VF17	VF21
BHI broth	256	128	4	16	32	52	16
TSBYE	64	32	-	4	-	8	-
TSB	8	32	-	-	-	-	-
HIB	32	32	-	-	-	32	-
HIB+GL	8	-	-	16	-	-	-
RCH	-	-	-	-	-	-	-

BHI = Brain heart infusion broth
 TSBYE = Trypticase soy broth + yeast extract
 TSB = Trypticase soy broth
 HIB = Heart infusion broth
 HIB+GL = Heart infusion broth + 1% glycerol
 RCH = Recharadson's medium
 - = No production of haemolysin

RESULTS

Effect of different media on haemolysin production

Of the various culture media tested in this study, BHI broth was the best for the production of haemolysin. None of the strains could produce haemolysin in RCH. Strain No. 2 was considered to be the best haemolysin-producing isolate. The titres of haemolysin produced by different strains are shown in Table. The subsequent experiments were conducted with *V. fluvialis* strains VF2 and BHI broth.

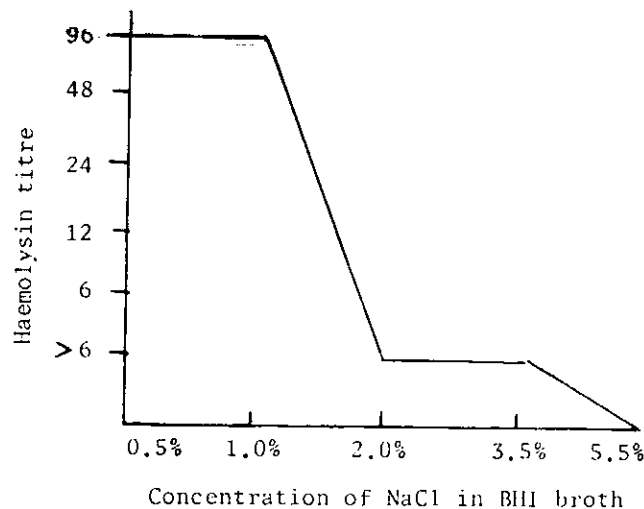


Fig. 1. Effect of different concentrations of NaCl on the production of haemolysin

Effect of NaCl concentration on haemolysin production

Adding 0.5% NaCl to BHI broth decreased the production of haemolysin. Further increase of the NaCl concentration gradually decreased the production of haemolysin. The production of haemolysin totally ceased in BHI broth containing 5.0% NaCl (Fig. 1).

Effect of incubation temperature on haemolysin production

The production of haemolysin was optimal at 30 °C and 37 °C, and totally ceased at 50 °C (Fig. 2).

Heat inactivation of haemolysin

Haemolytic activity decreased (haemolysin titre 24) when the culture supernatant was heated at 50 °C for 30 minutes compared to the unheated culture supernatant (haemolysin titre 192). Haemolytic activity in the culture supernatant was totally abolished when heated at 56 °C for 30 minutes.

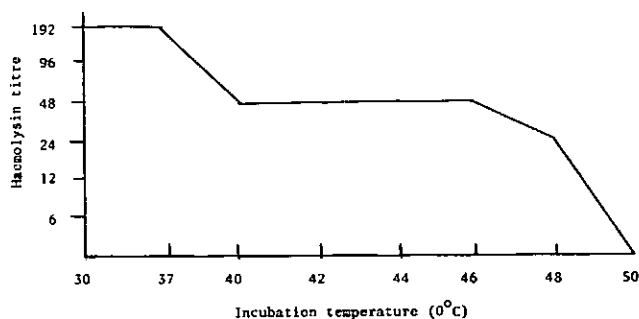


Fig. 2. Effect of incubation temperature on production of haemolysin

DISCUSSION

Haemolysins, like other metabolites, are excreted in the medium during growth. In case of *V. vulnificus*, the optimum production of haemolysin takes place during the logarithmic growth phase (13). *A. caviae*, however, produces optimum quantities of haemolysin in the stationary growth phase (6). Haemolytic activities of *Aeromonas* and *V. cholerae* O1 biotype El Tor were induced by repeated passage in RIL (15,16). Moreover, chelating agents, such as iron, when added to the medium, enhance the production of haemolysin (6). Media composition also affects the production of haemolysin by different members of the family *Vibrionaceae*. HIB induces the optimum production of haemolysin in *V. vulnificus* (13). The present study has shown that *V. fluvialis* displays the optimal production of haemolysin in BHI broth. This has also been shown for *V. mimicus* (3).

Sodium chloride is one of the important components of bacteriological culture media. Addition of NaCl to the medium enhances the production of virulence factors, such as enterotoxin (17) and haemolysin (13), but excess amounts of NaCl adversely affect the bacterial growth. Addition of 0.5% NaCl to HIB broth induced the increased production of haemolysin in *V. vulnificus*, but the gradual increase of the NaCl concentration inhibited the production of haemolysin (14). In *V. mimicus*, the production of haemolysin was optimal in BHI broth containing extra NaCl (0.5% to 1.5%) (3). In the present study, the optimal quantities of haemolysin were produced in BHI broth with its usual NaCl concentration. The production of haemolysin was affected by increasing the concentrations of NaCl in BHI broth.

Bacterial metabolites are excreted at optimal rates when they grow at an optimal temperature. 36 °C has been considered optimal for all members of the family *Vibrionaceae* (13). To find the optimal temperature for the production of haemolysin, the bacterial cultures were incubated at various temperatures, and the production of haemolysin was measured (3,13). In this study of *V. fluvialis*, temperatures between 30 °C and 37 °C were associated with the highest haemolysin titres.

Haemolysin is a protein. When heated, it like other proteins changes its structural conformation through coagulation and loses its haemolytic potential. This occurs at a temperature around 60 °C. Like *V. vulnificus* (13) and *V. mimicus* (3), haemolysin of *V. fluvialis* was inactivated when heated at 56 °C for 30 minutes. A further study is needed to find out any immunological cross-reactivity of *V. fluvialis* haemolysin with that of *V. cholerae*, *V. mimicus*, and *V. vulnificus*.

In conclusion, the heat-labile haemolysin of *V. fluvialis* is produced optimally in BHI broth with its usual concentration of NaCl. The production of haemolysin was affected after adding NaCl that contained in the medium.

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