EVALUATION OF SOME NUTRITIONAL REQUIREMENTS FOR THE GROWTH OF *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM CRAB MEAT HOMOGENATE

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Abstract
Vibrio parahaemolyticuswas isolated from crab meat homogenate and inoculated into broth to test the effects of various media components on its growth.The bacterium grew best in a defined medium with malic acid and methionine and in another medium with lactic acid and methionine. Control samples devoid of amino acids gave very poor growth. Carbon sources were substituted in the defined medium in place of sucrose and among all tested carbon compounds, sucrose, glucose, glycerol, fructose, and mannitol caused the production of best bacterial growth. Presence of soluble starch and cellobiose in the media resulted in the production of very low biomasses of 0.18 and 0.16 OD600respectively.When different sucrose concentrations were each tested, it was shown that sucrose concentrations of 40 and 35 mg/ml caused growth repression whereas concentrations of 15 and 20 mg/ml resulted in the best growth. Complete medium with peptone had much better increases in bacterial growth than media without peptone. Vibrio parahaemolyticus grew best at pH7.0 and 8.0 but had the least growth at pH11.0. Acidic pH of 3.0 was also lethal to growth. Growth inhibition occurred at NaCl concentrations of 1 and 10% while best growth of this organism occurred at NaCl concentrations of 3 and 4% at which bacterial biomass reached an OD600 of 0.81 and 0.74 respectively.

Keywords: Vibrio parahaemolyticus, crab meat homogenate, organic acids, amino acids, carbon sources, peptone, pH, NaCl concentration.

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1. INTRODUCTION

Vibrio species are Gram negative, facultative anaerobic, motile rods or curved rod shaped bacteria with a single polar flagellum. The species commonly associated with the contamination of seafoods are *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* (Kim *et al*., 2012). Other species that have been increasingly recognized as food pathogens in years are *V.* mimicusand *V. algonolyticus.* Vibrioscause a spectrum of health conditions including cholera, septicemia and milder forms of gastroenteritis (Desmarchelier, 2003). Up to thirty three species of *Vibrio* has been isolated but only twelve species like; *V. cholera*, *V. parahaemolyticus, V. vulfinicus, V. mimicus, V .alginolyticus* have been associated with foodborne infections of gastrointestinal tract (Oliver and Japer, 1997). These pathogenic species are known to be commonly associated with outbreaks of *V*ibrio infection due to consumption of food and water contaminated with human faeces or sewage, raw fish and seafood or with exposure of skin lesion such as cuts, open wounds and abrasions to aquatic environments and marine animals (Igbinosa and Okoh, 2008).

Vibrio species occur naturally in marine and fresh water environments of both temperate and tropical regions of the world (Tang *et al.*, 2017). They can survive in varying degrees of salinity, *V. cholera* and *V. mimicus* have been found to survive in fresh water while the rest are found in more saline environments such as the seas and the oceans. With regards to this, *Vibrio* species are commonly associated with seafood and food of fresh water origin (Jacksie *et al*, 2002). Cases of *Vibrio* infection have been reported in relation to the consumption of contaminated seafoods or recreation in contaminated water environments. There are several reports of isolation of pathogenic *Vibrio* from seafoods such as crabs, shellfish, fin fishes and bivalves. Seafood constitutes an important food component for a large section

of the world population. In fact they are major sources of animal protein. Thus, their contamination with pathogenic *Vibrio* makes them a risk factor in dissemination of *Vibrio* diseases. This necessitates surveillance for *Vibrio* species in both the water environment and in the sea and fresh water animals. There have been many reports of the association of *Vibrioparahaemolyticus* with crabs and also reported cases of infections caused by the consumption of crab meat contaminated with this bacterium (Yano *et al*., 2006; Wang 2011).
V. parahaemolyticus is common cause of diarrheal disease worldwide. The infection is typically acquired through consumption of contaminated seafoods. These could be raw, inadequately cooked or cross-contaminated by improper handling. The bacterium is recognized as the leading cause of gastroenteritis associated with seafood consumption in the United States (Farmer *et al*, 2003).The illness caused by *V. parahaemolyticus* food poisoning is a gastroenteritis characterised by watery diarrhoea and abdominal cramps in most cases, with nausea, vomiting, fever and headache. The incubation period is usually between 12 and 24 hour (Heymann 2004).
Organic acids are known antimicrobial agents and are utilised as food preservatives for preventing food deterioration and extending shelf life of perishable foods (Mahmoud 2014). They are cheap, available, and areclassified as generally regarded as safe substances (GRAS). Organic acids including citric and lactic acid are accepted preservatives in various meat and poultry industries(Salem and Amin 2012). There is dearth of information on the use of little amounts of these organic acids in combination with amino acids for the growth of *V. parahaemolyticus*. This work examines some nutritional factors and their effects on the growth of *Vibrio parahaemolyticus*.

2. MATERIALS AND METHODS

Vibrio parahaemolyticus used for this work was isolated from fresh crab meat sample. Crab meat homogenatewas prepared for inoculation by blending the meat for 3 min in a Waring blender in a ratio of 1:15 Triptic soy salt broth (pH7.3). The inoculum was prepared by growing the organism for 24 h at 35^{o}C on Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar (Merck, Darmstadt, Germany) followed by serial transfer of pure cultures to tryptic soy broth with 2.5% NaCl. The isolates were incubated with shaking in a Gallenkamp orbital incubator for 24 h at 35°C and later identified based on their morphological, physiological and biochemical characteristics as outlined in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Colonies were confirmed to be *Vibrio parahaemolyticus* using API 20NE test strips (Biomerieux, Marcy L'Etoile, France). A1.6 x10^{2} colony forming units/ml of the bacteriumwas inoculated into a defined medium in conical flasks which was designated as medium M and had the following composition (g/L): sucrose, 20; Yeast extract, 5; Sodium thiosulphate, 10; Acetic acid; 5, Methionine, 2.5. The medium was dispensed into 100 ml amounts in conical flasks and each flask inoculated with 3% NaCl solution. The medium pHwas then adjusted to 7.3 using sterile 0.2 M sodium hydroxide solution before sterilization.

Effects of the combinations of organic acids and amino acids on the growth of *Vibrio parahaemolyticus*

Acetic acid in Medium M,when not under test was replaced with either lactic, malic or citric acid. Methionine in the Medium M was replaced with either proline, tryptophan or alanine.Control media containing each organic acid without amino acids were similarly prepared. The medium pH was adjusted to 7.3and eachflask was inoculated with 1.6 x10^{2}CFU/ml of *Vibrio parahaemolyticus*. The broth was incubated with shaking for 24 h at 35°C.

Effects of carbon sources on the growth of *Vibrio parahaemolyticus*

The sucrose in medium M when not under test

was replaced with either glucose, soluble starch, cellobiose, fructose, glycerol or mannitol each at 20g/L concentration. The medium pH was adjusted to 7.3 and inoculated with 1.6 x10^2CFU/ml of *Vibrio parahaemolyticus*. The broth was incubated with shaking for 24 h at 35°C.

Influence of various concentrations of sucrose on the growth of *Vibrio parahaemolyticus*

In the preliminary experiment, sucrose in Medium M when used as a carbon source caused the best growth of the bacterium after incubation. Therefore, 0-40mg/ml of sucrose were each added into aliquot portions of fresh Medium Mdevoid of its original sucrose content. Then the medium pH was adjusted to 7.3 and inoculated with 1.6 x10^2CFU/ml of *Vibrio parahaemolyticus*, incubated with shaking for 24 h at 35°C.

Time-course of the growth *Vibrio parahaemolyticus* with or without peptone

Aliquots of Medium M with or without 5g/L of peptone were each added into flasks and inoculated with 1.6 x10^2CFU/ml of *Vibrio parahaemolyticus* and medium pH adjusted to 7.3 and incubated with shaking at the following time durations (4, 8, 12, 16, 20 and 24 h).

Influence of pH on the growth of Vibrio parahaemolyticus

The pH effect on the growth of *Vibrio parahaemolyticus* was determined by adjusting the pH of Medium M from pH 3.0-11.0 with either dilute H_2SO_4or 0.2M NaOH solution in conical flasks. The flasks were each inoculated with1.6x10^2CFU/ml of *Vibrio parahaemolyticus* and incubated with shaking for 24 h at 35°C.

Effects of NaCl concentrations on the growth of Vibrio parahaemolyticus

Sodium chloride concentrations (1-10%) were each added intoflasks containing 100ml of Medium M devoid of its original NaCl content. A control experiment devoid of NaCl was similarly prepared. The tubeswere inoculated with 1.6 x10^2CFU/ml of *Vibrio parahaemolyticus* and medium pH was adjusted to 7.3 and incubated with shaking for 24 h at 35°C.

Growth measurements: Cultures diluted in phosphate buffered saline were measured at optical density of 600nm using Spectrumlab 23A spectrophotometer.

3. RESULTS AND DISCUSSION

Vibrio parahaemolyticus was grown in media which had various combinations of organic acids and amino acids. Data in Table 1 shows that the bacterium grew best in the defined medium with malic acid and methionine and in another medium with lactic acid and methionine.

Table 1: Influence of the combinations of organic acids and amino acids on the growth of *Vibrio parahaemolyticus* in the defined medium

OD after 24 h at 35°C

Organic acid	Amino acid				
	None	Proline	Tryptophan	Alanine	Methionine
Acetic acid	0.35	0.58	0.68	0.71	0.81
Lactic acid	0.39	0.69	0.81	0.68	0.84
Malic acid	0.33	0.62	0.71	0.81	0.87
Citric acid	0.29	0.45	0.60	0.76	0.79

Control sample which lacked amino acids in them showed very poor growth. Requirement of organic acids and amino acids for the growth of *Vibrio* species had previously been reported (Ramirez et al., 2017). Beuchat (1976) reported that growth of *Vibrio parahaemolyticus* were similar in media adjusted to identical pH values, regardless of whether citric, ascorbic or malic acid was used to attain these values. This work has shown that optimum growth of this bacterium was achieved when organic acids were combined with amino acids in growth media.

A medium which contains basal salts, carbon sources and amino acids fulfils the primary goal of a defined medium for the growth of *Vibrioparahaemolyticus* (Cherwonogrodzky and Clark 1982). Various carbon sources were substituted in Medium M in place of sucrose for the growth of *Vibrio parahaemolyticus* (Table 2).

Table 2: Effects of carbon sources on the growth of *Vibrio parahaemolyticus* in medium M

Carbon sources	OD_{600}nm
None	0.11
Glucose	0.77
Sucrose	0.81
Soluble starch	0.18
Cellobiose	0.16
Fructose	0.70
Glycerol	0.63
Mannitol	0.70

Among all the tested compounds, sucrose caused the production of the best growth of 0.81 OD_{600} after 24 h incubation at 35^{o}C. Glucose, glycerol, fructose, and mannitol also caused the production of good bacterial biomasses. Soluble starch and cellobiose caused the production of very low biomasses of 0.18 and 0.16 OD_{600}respectively. It appears that the bacterium failed to increase growth because it was unable to metabolize these compounds.

The fact that best bacterial growth occurred in the defined medium which contained 20g/l sucrose prompted the testing of other concentrations of sucrose for their ability to promote good growth of this bacterium. The effects of using various concentrations of sucrose for the growth of *Vibrio parahaemolyticus* is presented in Table 3. It is evident from the results presented in Table 3 that sucrose concentrations of 40 and 35 mg/ml caused growth repression whereas concentrations of 15 and 20 mg/ml resulted in the best growth of this organism.

Table 3: Influence of sucrose concentrations on the growth of *Vibrio parahaemolyticus* in the defined medium

Sucrose (mg/ml)	OD_{600}nm
0	0.12
10	0.79
15	0.88
20	0.81
25	0.44
30	0.43
35	0.38
40	0.31

The composition of medium M was adjusted with or without 5% peptone to test whether this compound had any effect on the growth of the bacterium and to also reveal the best incubation duration for this organism (Table 4).

Table 4: Time-course of the growth of *Vibrio parahaemolyticus* in Medium M with or without peptone measured at OD_{600nm}

Incubation time (h)	With peptone	Without peptone
0	0.11	0.11
4	0.43	0.31
8	0.55	0.38
12	0.68	0.45
16	0.70	0.51
20	0.85	0.60
24	0.91	0.63

It was noted that medium which contained peptone had much better increases in bacterial growth than media without peptone. Best growth occurred between 20 and 24 h incubation. This is evident of the fact that *Vibrio parahaemolyticus* requires peptone as a nitrogen source for cell biomass formation as had been previously reported (Twedt and Novelli, 1971).

The test organism grew best at pH7.0 and 8.0 and it had the least growth at pH11.0 (Fig. 1).

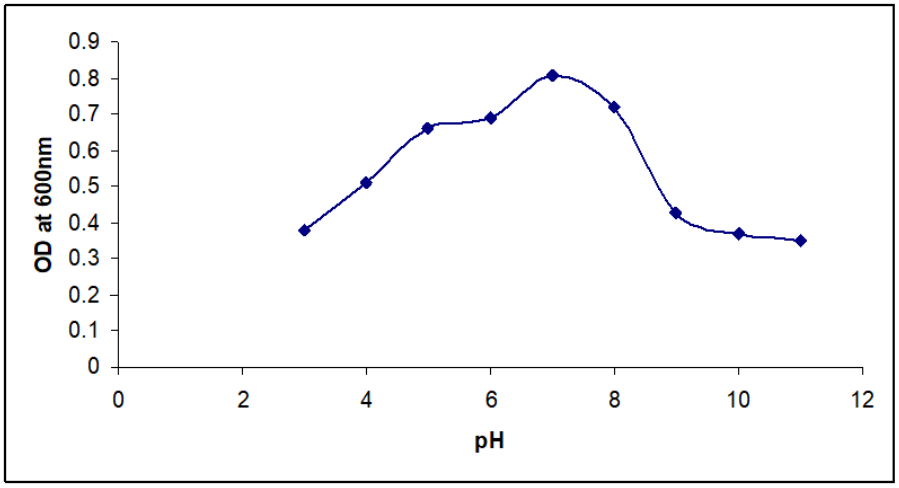

Fig. 1: Influence of pH on the growth of *Vibrio parahaemolyticus*

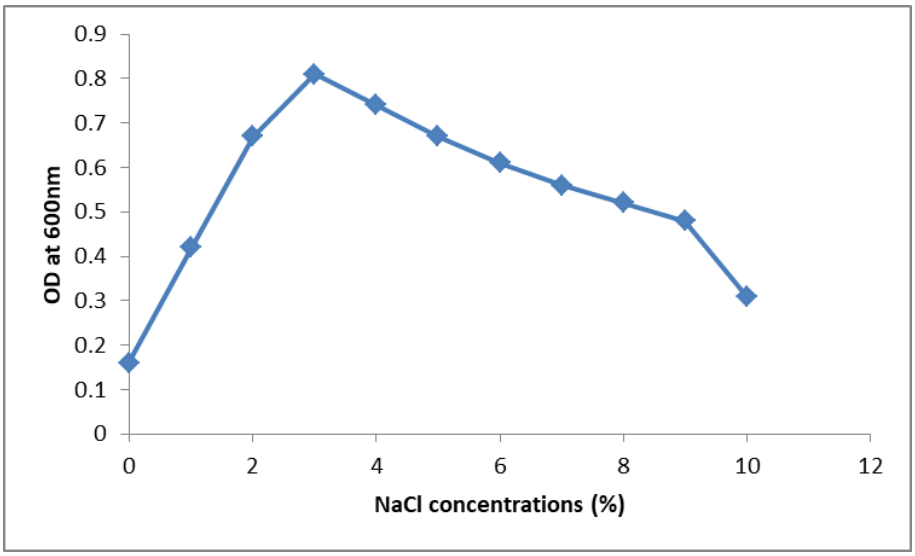

Fig. 2: Influence of NaCl concentrations on the growth of *Vibrio parahaemolyticus*

Elevated pHs beyond 9.0 showed to be lethal to the bacterium while acidic pH of 3.0 also resulted in very poor growth. The best pH for the growth of *Vibrio parahaemolyticus* was between 7.6 and 8.6 but growth was also reported in media at pH5.0 to 11.0 (Beuchat, 1975). There were reports of a sharp drop in viable cells of *Vibrio parahaemolyticus* at pH 5.0 with no survivors after 15 min of incubation but viable population of the bacterium in culture adjusted to pH 6.0 to 10.0 remained about the same for 2 h (Vanderzant and Nickelson, 1972).

The concentrations of NaCl in growth media were adjusted from 1-10% (Fig. 2). It was observed that the most pronounced growth inhibition occurred at concentrations of 1 and 10%. Best growth of this organism occurred at NaCl concentrations of 3 and 4% at which bacterial biomass reached an OD_{600} of 0.81 and 0.74 respectively. Whitaker et al., (2010) reported that *Vibrio parahaemolyticus* is a moderately halophilic bacterium with an absolute requirement for salt and this organism grew best in neutral media containing 3% NaCl and when the bacterium was grown in broth containing NaCl concentration of 0.5%, the organism failed to grow. Kalburge et al., (2014) confirmed that this bacterium requires a minimum of 0.5% NaCl for growth and could grow in media containing 10.5% NaCl. *Vibrio parahaemolyticus* grew in media containing as

little as 0.5% NaCl but 3% NaCl concentrationwasthe optimal level for itsgrowth (Beuchat 1975). It was noted that a criterion used to separate *Vibrio parahaemolyticus* from *V. alginolyticus* and some other Vibrios is the inability of *Vibrio parahaemolyticus* to grow well in broth containing 10% NaCl and this inability was reported by many researchers (Johnson et al., 1971; Kaneko and Colwell 1973) but Lee 1972 showed that the maximum NaCl concentration tolerated by *Vibrio parahaemolyticus*is 8%. Other reports suggest that this bacterium can grow and tolerate NaCl in media exceeding 10% concentration (Kampelmacher et al., 1972) and these discrepancies were related to other media components, temperature of incubation, pH and strain variability.

4. CONCLUSION

This work demonstrated that the best growth of *Vibrio parahaemolyticus* occurred in media which contained a combination of organic acids and amino acids than in media devoid of amino acids. Sucrose was superior to other carbon sources and the best sucrose concentrations for optimal growth were 15 and 20 mg/ml. Media with peptone had much better increases in bacterial growth than media without peptone. Best growth occurred at pH7.0 and 8.0 while the least growth occurred at pH11.0. Sodium chloride concentrations of 3 and 4% were optimal for growth at which bacterial biomass reached an OD_{600} of 0.81 and 0.74% respectively. Lower or higher NaCl concentrations did not give better bacterial growth. This work will educate food scientists on the nutritional conditions that are lethal to this bacterium and will therefore help mitigate high growth and risks associated with the presence and multiplication of this organism in foods.

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