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Isolation and Characterization of *Shewanella putrefaciens* from Farm Reared Freshwater Prawn and Farm Environment

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Farmed *Macrobrachium rosenbergii,* farm water and mud were analysed for hydrogen sulphide producing bacteria including *Shewanella putrefaciens.* The biochemical characteristics and spoilage potential of *S. putrefaciens* strains isolated from *M. rosenbergii* were investigated and compared to marine and brackishwater strains isolated from fish (*Rastrelliger kanagurta*) and black clam(*Villorita cyprinoides)* respectively. The H_2S producing bacteria including *S. putrefaciens* constituted a low proportion (<2%) of the total aerobic flora of pond water and mud and 12% of the total aerobic population on fresh prawn. On icing, there was a lag or slight decrease in the counts of H_2S producing bacteria including *S. putrefaciens* in the muscle of *M. rosenbergii.* The proliferation started on 16th day of iced storage and the count of sulphide producers reached ca. 6.25 log $_{10}$ cfu g $^{-1}$ in prawn muscle at the time of rejection after 3 weeks. Gram-negative, non-fermentative, motile rods constituted 16-25% of the hydrogen sulphide producing bacteria on prawn muscle and intestine and they were identified as *Shewanella putrefaciens.* The results indicate that *S. putrefaciens* is an important spoiler of freshwater prawn. All the *S. putrefaciens* isolates from *M. rosenbergii* were able to produce H_2S and reduce TMAO and exhibited high spoilage potential. The present study suggests that counts of H_2S producing bacteria can be a useful indicator of quality deterioration of farm reared *M. rosenbergii.*

Key words : *Macrobrachium rosenbergii, Shewanella putrefaciens,* spoilage.

Shewanella putrefaciens is a well known spoilage bacteria of fish from marine, temperate and tropical waters. The offensive, fishy, rotten, H_2S off-odours in spoiled fish are generally by Sulphide Producing Bacteria (SPB) mainly *S. putrefaciens* (Lannelongue *et al.*1982; Gram *et al.*1987; Lima dos Sandos 1978; Shamshad *et al.* 1990). Some sulphide producing *Aeromonas* sp. and *Enterobacteriaceae* spp. may occasionally be present (Gram *et al.*1987). The importance of *S. putrefaciens* for spoilage of fish lies in its biochemical action on muscle, namely reduction of TMAO to trimethylamine (TMA), production of hydrogen sulphide from cysteine, formation of methylmercaptane (CH_3SH) and dimethylsulphide $(CH_3)_2S$ from methionine and production of hypoxanthine (H_x) from inosine monophosphate (IMP) or inosine (Herbert & Shewan 1975; Gram & Huss 1996; Boskou & Debevere 1997). *S. putrefaciens* is chiefly involved in the production of putrescine and cadaverine (Suzuki et al. 1988; Rodriguez *et al.* 1994; Lopez caballero *et al.* 2001). Middlebrooks *et al.* (1988) reported major histidine decarboxylase activity in strains of *S. putrefaciens* isolated from Spanish mackerel.

S. putrefaciens has a high spoilage potential, but this organism has a low spoilage activity and high cell concentrations are required to cause spoilage (Dalgaard 1995). Stenstrom and Molin (1990) reported that although occurring in smaller numbers than *Pseudomonas* in spoiled fish, *S. putrefaciens* is considered to have a higher spoilage potential in marine fish because of its higher metabolic activity. This organism together with *Pseudomonas* sp are the specific spoilers of ice-stored marine fish and shrimp from temperate and tropical waters (Gillepsie

& Mac Rae 1975; Lima dos Santos 1981; Reilly *et al.* 1984; Gram 1992; Gram & Huss 1996; Koutsoumanis & Nychas 1999).

S. putrefaciens was isolated from the freshwater sediment of Oneida Lake in USA(Myers & Nealson, 1988). This organism has also been isolated from tropical freshwaters, but does not appear to be important in the spoilage of iced freshwater fish from tropical waters (Lima dos Santos 1978; Gram *et al.* 1990). This may be due to occurrence of very low numbers at the time of spoilage and the inability of the organism to compete with high numbers of antagonistic psuedomonads (Gram 1993; Gram & Melchiorsen 1996). *S. putrefaciens* was found responsible for spoilage of farmed shrimp (Reilly *et al.* 1986). Similar studies on freshwater prawn have not been reported.

The objective of the present work has been to enumerate hydrogen sulphide producing bacteria from freshwater prawn and farm environment, to isolate *S. putrefaciens.* and to determine their spoilage potential.

Materials and Methods

Freshwater prawn *M. rosenbergii* weighing 40 – 50 g was purchased from freshwater farms located in central Kerala. Prawns were packed in sterile polythene bags, stored in insulated thermocole boxes containing flake ice and transported within 3hrs after being caught. On arrival at the laboratory, prawns were split as twelve 400g portions , packed in individual plastic bags and stored in flake ice in the ratio of 1:1(w/w) in insulated thermocole boxes and kept in a cold room maintained at 0-4°C. Samples were completely covered with flake ice, without direct contact to avoid microbial cross-contamination and washing of soluble compounds. Icing was done every day after draining out the melted ice and samples were withdrawn after every 1 or 2 days of storage for the first week and thereafter every 3 or 4 d up to 23 d. On each sampling day, 4-5 prawn were analysed.

Bacteriological analysis was performed on whole and headless fresh prawn as well as on head, muscle tissue and intestine. Samples of muscle tissue (25g) of ice stored prawn were aseptically taken and transferred to a stomacher bag (Seward Medical, London, UK), 225 ml of physiological saline (Nacl, 0.85% w/v) was added, and the mixture was homogenised for 60s with a stomacher (Lab blender 400, Seward Medical). Gut was excised, weighed and placed in sterile bags containing enough saline solution to make 1:10 dilutions and homogenised for 60s.

Samples (1ml) of serial dilutions of prawn homogenates were plated on Tryptone Soya Agar (TSA Oxoid, UK), incubated at 20°C for 5d for determination of total aerobic counts and on Iron Agar (IA, BBL) overlaid with the same medium after 5d at 20°C (Gram *et al.* 1987; Gennari & Campanini 1991) for enumeration of hydrogen sulphide producing bacteria. Three replicates of at least three appropriate dilutions were enumerated. Black colonies on Iron Agar were counted as hydrogen sulphide producing bacteria. All plates were examined for *S. putrefaciens* by identifying typical colony types and morphological characteristics (Gram *et al.* 1987). *S. putrefaciens* populations associated with prawn at the time of spoilage were characterized morphologically and biochemically. A total of 212 H_2S producing bacterial isolates and 35 *S. putrefaciens* isolates were randomly selected and isolated from IA agar plates. The strains were tested for gram reaction, catalase and oxidase reactions, motility, oxidative/fermentative metabolism and presence of spores. They were then grouped according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984; Sneath *et al.* 1986), further tested for the most relevant characteristics of each group and identified using the schemes proposed for identification (Dainty *et al.* 1979; Valera & Esteve 2002). Further more, the strains were tested for their ability to reduce TMAO to TMA and to produce H_2S and these strains

were compared with the *S. putrefaciens* isolates from Indian Mackerel and black clam. The ability to reduce trimethyl amine oxide (TMAO) and to produce hydrogen sulphide are regarded as prominent characteristics of fish spoilage bacteria (Grams *et al.*1987). The *S. putrefaciens* cultures were all inoculated in sterile *M. rosenbergii* broth, fish muscle broth and clam broth (Gram *et al.* 1987) and incubated at 7°C for 10 days in order to determine spoilage potential and the development of odours.

The microbial counts were expressed as cfu g^{-1}. The counts were transformed to $\log_{10}$ values.

Results and Discussion

The mean counts ($\log_{10}$ cfu g^{-1}) of total aerobic bacteria and H_2S producing bacteria on farmed freshwater scampi *M. rosenbergii* and farm environment are presented in table 1. The aerobic counts at 20°C on whole prawn and prawn head was ca 6.0 $\log_{10}$ cfu g-1. The highest count was observed in prawn intestine. The H_2S producing bacteria including *S. putrefaciens* constituted a low proportion (<2%) of the total aerobic flora of pond water and mud. The H_2S producing bacteria constituted 12% of the total aerobic population on prawn. *S. putrefaciens* constituted a very low proportion (< 2%) of the total H_2S producers on prawn.

On icing, there was a lag or slight decrease in the counts of H_2S producing bacteria including *S. putrefaciens* in the muscle of *M. rosenbergii* followed by a definitive increase after 12 days of storage (fig 1). These results suggest that the original sulphide producing bacterial flora on prawn was dominated by mesophilic bacteria being able to grow at refrigeration temperatures only after a long lag phase. Similar observations were reported by Gram(1989), Leitao & Silveira (1993) and Leitào & Rios (2000) on psychrotrophic counts on freshwater fish and prawn. The slow growth rate is considered as one of the main factors explaining the longer shelf life of tropical fish when first stored at refrigeration temperatures. Koutsoumanis *et al.* (1999) attributed this initial lag phase of H_2S producing bacteria in fresh mediterranean fish stored in ice to the inhibition of growth by *Pseudomonas* spp. which produce siderophores. *S. putrefaciens* and *Pseudomonas* spp. are the two strongly competitive psychrotrophic microorganisms in fish (Koutsoumanis *et al.* 1999).

The low numbers of sulphide producers were consistent with the lack of H_2S like off-odours and off flavours in prawn on 16th day in ice. However, meat separated from the shell in 25-30% of the samples and hanging head was noticed in prawn. Their count of sulphide producers reached ca. 6.25 $\log_{10}$ cfu g^{-1} in prawn muscle when the prawn were rejected after 3 weeks. Gram *et al.* (1987) have identified *S. putrefaciens* as the strongest spoiler of seafood from temperate waters and they observed spoilage of fish and production of significant amounts of

Table 1. Counts of H_2S producing bacteria ($\log_{10}$ cfu g^{-1}) and *Shewanella putrefaciens* on farmed *Macrobrachium rosenbergii* and farm environment.

Microbial parameter	Bacterial count (mean $\log_{10}$ cfu g^{-1}) Farm water*	Farm mud	Prawn whole	Prawn headless	Prawn muscle	Prawn intestine	Prawn head
Total aerobic count	3.66	5.57	6.143	5.513	4.7	6.951	6.372
H_2S producing bacterial count	1.845	1.778	5.231	4.612	3.662	5.740	5.568
Percentage of H_2S producers in total aerobic flora	1.5	0.01	12	12.6	9	6	15.7
Shewanella putrefaciens count	0.301	1.301	3.079	3.041	1.924	3.602	3.017

* mean $\log_{10}$ cfu ml^{-1}

Table 2. Composition of the H_2S producing bacterial flora of freshwater prawn after 0 (fresh sample), 8 and 19 days of ice storage.

H_2S producing bacteria	Percentage of H_2S producing bacterial flora					
	Shell with muscle			Intestine		
	0d	8d	19d	0d	8d	19d
Enterobacter cloacae	20	7.7	0	13.3	16.7	0
Citrobacter freundii	11.4	7.7	0	23.3	8.3	0
Aeromonas hydrophila	7.1	7.7	11.1	6.7	16.7	20.5
A. veronni biovar. *sobria*	20	30.8	66.7	20	33.3	46.2
A. veronni biovar. *veronni*	14.3	0	0	6.7	0	2.6
A. trota	2.9	0	0	8.2	0	0
A. jandai	14.2	15.0	0	6.7	4.2	0
Shewanella putrefaciens	1.4	7.7	16.7	1.7	8.3	25.6
Micrococcus sp.	2.9	7.7	0	3.4	0	0
Enterococcus sp.	2.9	0	5.5	5	8.3	5.1
Bacillus sp.	2.9	7.7	0	5	4.2	0

sulphur compounds when the *S. putrefaciens* numbers exceeded 6 $\log_{10}$ cfu g^{-1}. Counts of sulphide producers in the range of log 6- 6.7 are reported on ice stored fish from temperate and tropical waters at sensory rejection (Capell *et al.* 1997).

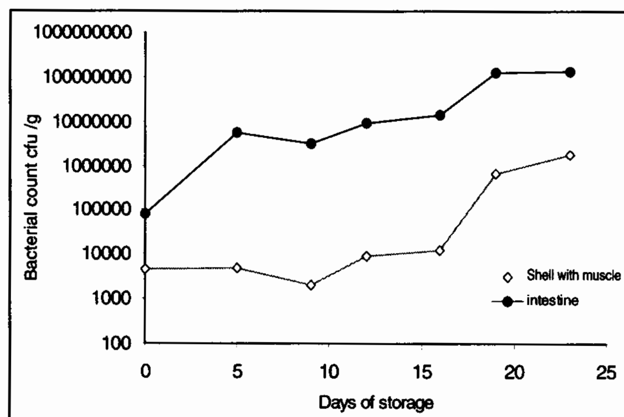

Fig. 1. Hydrogen sulphide producing bacterial count on ice stored *Macrobrachium rosenbergii*

Counts of H_2S producing bacteria, though constituting a small proportion of the total aerobic flora, provided a useful indicator of quality deterioration of prawn and can be used to determine the time to rejection as reported earlier by Lougovois *et al.* (2003) for iced gilthead sea bream. The H_2S producing bacteria, although constituted 12% of the total micro flora initially, decreased significantly (<2%) during icing. They constituted 23-25% of the total flora at the time of rejection (fig 2). *S. putrefaciens* represented 16-25% of the H_2S producing bacteria on spoiled ice stored prawn whereas they constituted very low proportion on fresh prawn. The results indicate that *S. putrefaciens* is an important spoiler of freshwater prawn and organisms other than *S. putrefaciens* have been involved in the spoilage of freshwater prawn. Stenstrom & Molin (1990) reported a high spoilage potential for *S. putrefaciens* in marine fish because of its higher metabolic activity although occurring in smaller numbers than *Pseudomonas* in spoiled fish. In Atlantic Moroccan sardines (El Marrakchi *et al.* 1992), *S. putrefaciens* was recognised as an important spoiler (22.7% of the total micro flora at spoilage).

The present study suggests that freshwater prawn could be kept in iced condition for 12-16 days without significant deterioration in bacteriological quality. Angel *et al.* (1981) reported no deterioration in *M. rosenbergii* for 14 days in iced condition where as Ninan *et al.* (2003) reported acceptability up to 12 days. The main H_2S producing bacterial groups detected among 120 isolates from fresh prawn were 1. Aeromonadaceae 2. Enterobacteriaceae (*Citrobacter* and *Enterobacter*)3. Gram-negative aerobic motile rods (*Shewanella*) 4. Gram-positive cocci (*Micrococcus*, *Enterococcus*) 5. Gram-positive spore-forming bacteria (*Bacillus*). The predominant H_2S producing

Table 3. Spoilage potential and biochemical characteristics of *Shewanella putrefaciens* strains isolated from Indian mackerel (marine), black clam (brackishwater) and freshwater prawn (freshwater).

Characteristics	Marine isolates n=25	Brackishwater isolates n=24	Freshwater isolates n=35
Pink pigmentation on Nutrient Agar	100	100	100
Ornithine decarboxylase	100	100	100
Gelatinase	100	100	100
H_2S production	100	100	88
Reduction of TMAO	98	100	100
Casein hydrolysis	100	100	98
Growth in 6% NaCl	4	0	0
Acid from			
1. Glucose	0	0	0
2. Sucrose	0	0	0
3. Lactose	0	0	0
4. Mannitol	0	0	0
Starch hydrolysis	100	100	100
Off odour production in			
1. Sterile fish muscle medium	100	100	100
2. Sterile clam medium	100	100	100
3. Sterile freshwater prawn medium	100	100	100

bacterial flora in fresh prawn were Gram-negative belonging to groups I and II (Table 2). Members of the Aeromonadaceae *A. Hydrophila, A. veronii* biovar. *sobria, A. veronii* biovar *veronii* and *A. jandai* were represented in the microflora. These species produced offensive odours (sulphide, putrid, rotten egg) and exhibited proteolytic activity. Bacteria belonging to Enterobacteriaceae family were identified as *Cirobacter freundii* and *Enterobacter cloacae.* The numbers of *Enterobacter* and *Citrobacter* decreased in prawn muscle during icing and after 19 days of iced storage, these species could not be detected. It may be due to inability of these bacterial species to survive and / or grow at low temperatures. The activity of *Enterobacter* strains was reported to be scarce at low temperature (Gennari *et al.* 1999).

The black colonies on iron agar isolated from prawn stored 19 days in ice were identified as either *S. putrefaciens* or *Aeromonas* spp. *A. hydrophila* and *A. veronii* biovar *sobria* were the dominant flora on prawn muscle and intestine and these strains were found to be active on proteins and TMAO suggesting a relevant role in the spoilage. 16-25% of the hydrogen sulphide producing bacteria on prawn muscle and intestine were characterized as *Shewanella putrefaciens.* This is the first report of the isolation of *S. putrefaciens* from freshwater environments in India. Myers and Nealson (1988) isolated *S. putrefaciens* from freshwater sediment of Oneida Lake, New York, USA. The literature concerning fish from North European waters shows *S. putrefaciens* as the main spoiler of aerobic ice stored fish (Gennari *et al.* 1999).

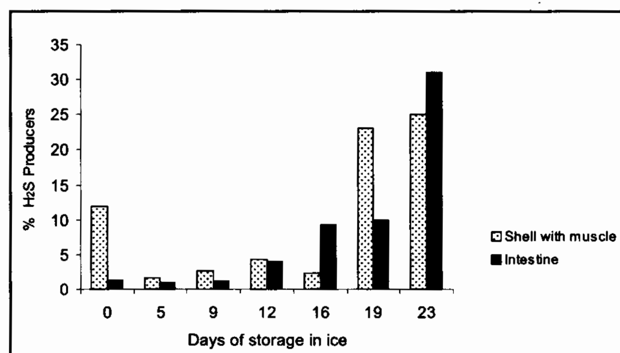

Fig. 2. Percentage of H_2S Producing bacteria in the total aerobic bacterial population (20°C) on ice stored farmed *Macrobrachium rosenbergii*

Conversely, their spoiling role was reported to be less marked in tropical iced fish (Poulter *et al.* 1981., Gram *et al.* 1989). Gennari *et al.* (1999) reported incidence range of *S. putrefaciens* on different fish samples at the time of spoilage to be 2-28% and they considered these organisms as the main specific spoilage organism. A similar incidence range (6%) was observed in ice stored *M. rosenbergii* during spoilage indicating *S. putrefaciens* as one of the SSO of farmed freshwater prawn. Reilly *et al.* (1984) reported *S. putrefaciens* as one of the dominant spoilage organism in ice stored pond reared *P.monodon.* In another study, Reilly & Dangla (1986) reported. *A. hydrophila* as the principal spoilage organism of farm reared *P. monodon* held at 0°C.

S. putrefaciens strains isolated from *M. rosenbergii* were characterized and their biochemical characteristics and spoilage potential were compared to marine strains isolated from fish (*Rastrelliger kanagurta*) and black clam (*Villorita cyprinoides)* (Table 3). All the marine and freshwater strains tested produced salmon pink pigment on agar media intensifying with age. All the strains exhibited cytochrome oxidase, catalase and gelatinase activities, optimal growth at ca.4°C, ability to reduce TMAO and inability to ferment glucose. Except 4 marine strains, all the strains were unable to grow in 6% NaCl. Only 88% of the freshwater isolates produced H_2S and 98% hydrolysed casein. All the *S. putrefaciens* isolates from *M. rosenbergii* were potential spoilage organisms due to their ability to produce H_2S and reduce TMAO. Furthermore, off-odours were produced when grown in sterile fish muscle, clam and prawn media.

The present study indicates that counts of H_2S producing bacteria can be useful in quality determination of farm reared *M. rosenbergii*. This study reveals that *S. putrefaciens* is an important spoiler of farmed freshwater prawn, but the role of organisms other than *S. putrefaciens* cannot be excluded.

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