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The Spoilage Effect of *Shewanella putrefaciens* in Whiting Fishes

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Abstract: The present study was undertaken to determine the relationship between spoilage factors of S. *putrefaciens* at different storage temperature and time in fresh whiting-fish with experimental treatment. The whiting fish samples taken from the Kefken region of Kandıra district in Kocaeli. Two groups were separated as control and test group of whiting-fish. Control group was divided into two parts. First part was stored at 0°C in 9 days and within this period, it was analysed in 1st, 3rd, 5th, 7th and 9th days of storage. Second part was also stored at –18°C for 6 months after freezed at –35°C. Test group was also divided into two groups and stored at the same conditions after contaminated with *S. putrefaciens* (Ref. No: CIP 80.40, Inst. Pasteur) at the level of 10^2 cfu g^{-1} and then all group samples analysed for total viable count, *Enterobactericeae*, *Psycrophilic* bacteria, *Pseudomonas* spp. *S. putrefaciens*, TMA amount and pH level. As *S. putrefaciens* average count was determined at under the detectable level (log_{10}<2.0 cfu g^{-1}) and 2.74 mg/100 g of TMA amount in control group, it determined at log_{10} 3.66 cfu $g^{-1}$and 5.59 mg/100 g of TMA amount in test group at 0°C storage temperature in 3rd day. It was determined that the differences became greater in the last day of storage (9th day). As *S. putrefaciens* average level at log_{10} 8.6 cfu g^{-1} and 33.11 mg/100 g TMA amount in test group, it was found under the detectable level (log_{10}<2.0 cfu g^{-1}) and 12.04 mg/100 g TMA amount in control group. Therefore, *S. putrefaciens* had great effect on to spoilage of fish and TMA formation.

Key words: *Shewanella putrefaciens*, whiting-fish, spoilage

INTRODUCTION

Many biochemical, microbiological and ecological factors are involved in fish spoilage during the storage. The bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella* and *Flavobacterium* dominate the microflora on temperate water fish. *Shewanella putrefaciens* has been identified as the specific spoilage bacteria which determine the shelf life of fish stored aerobically in ice[1-3].

Bacteria on fish caught in temperate waters will enter the exponential growth phase almost immediately after the fish have died. This is also true when the fish are iced, probably because the microflora is already adapted to the chill temperatures. During ice storage, the bacteria will grow with a doubling time of approximately 1 day and will, after 2-3 weeks, reach numbers of 10^8-10^9 cfu g^{-1} flesh or cm^2 skin. During ambient storage, a slightly lower level of 10^7-10^8 cfu g^{-1} is reached in 24 h[4,5].

S. putrefaciens, is a gram-negative, psychotropic, motile and rod shapes bacterium which grows at 0°C to 37°C. Besides, its optimum grown degree is 25°C. It is sensible to NaCl and not grown to 6% NaCl concentration[6,7].

S. putrefaciens is considered as an important factor of spoilage in marine fish because of its biochemical action on muscle, that is, reduction of TMAO to trimethylamine (TMA), production of hydrogen sulphid (H_2S), methylmercaptane (CH_3SH) and dimethylsuphide ($(CH_3)_2S$ from methionine and production of hypoxanthine (Hx) from inosine monophosphate (IMP) or inosine and also other characteristic compounds of the species responsible for spoilage[7,8]. As Stenström and Molin[6] have reported, *S. putrefaciens* has a higher spoilage potential in fish own to its higher metabolic activity. However, it occurs in smaller numbers than *Pseudomonas* spp. in spoiled fish.

In this study, it was undertaken to determine the relationship between spoilage factors of *S. putrefaciens* at different storage temperature and time in fresh whiting-fish with experimental treatment.

MATERIALS AND METHODS

The preparation of the test strain: *S. putrefaciens* CIP 80.40 strain was obtained from the Inst. Pasteur. Tubes containing Nutrient Broth were inoculated with *S. putrefaciens* from the slant cultures with an

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inoculation loop and were incubated for 24 h at 25°C. Next, the *S. putrefaciens* suspension was homogenized at vortex and was diluted with 0.1% sterilized peptone water up to 10^{-8}. Then, these dilutions were inoculated to Plate Count Agar (PCA) and Iron Agar (IA) by the method of drop plate. The plates were incubated for 4 days at 25°C. After the incubation, the colonies grown in the plates were counted in order to identify the amount of *S. putrefaciens* included in 1 mL of culture suspension. This process helped to determine the growth dynamic of *S. putrefaciens*. The dose of inoculation at the level of 10^2 *S. putrefaciens*/mL was detected by diluting the main culture suspension which was obtained via inoculations from the stock culture.

The obtaining the sample and the preparation of the research groups: The whiting fish samples were taken for 6 times (approximately 12-15 kg) from the Kefken region of Kandıra district in Kocaeli between September and November 2001. The fish were delivered at the laboratory approximately 4 h after they were caught. Two groups were separated as control and test group of whiting fish that couldn't be determined the *S. putrefaciens*. Control group was divided into two parts. First part was stored at 0°C in 9 days and within this period, it was analysed in 1st, 3rd, 5th, 7th and 9th days of storage. Second part was stored at –18°C for 6 months after freezed at –35°C. Test group was also divided into two groups and stored at the same conditions after contaminated with *S. putrefaciens* at the level of 10^2 cfu g^{-1} by the method of injection and then analyzed. Whiting fishes that inherent of *S. putrefaciens* were analyzed separately.

The identification of *S. putrefaciens*: The fish samples were divided into the groups of 400 g approximately. Twenty five gram of dorsal muscles from each group were separated and then added to 225 mL of diluent (0.85% NaCl with 0.1% peptone water) and homogenized using a Lab. Blender 400. A 1.0 mL aliquot of the homogenate was serially 10 fold diluted and 0.1 mL aliquots of appropriate dilutions were spread onto duplicate plates of Iron agar. Plates of Iron agar were incubated at 25°C for 4 days. After incubation, 3-5 suspicious sulfide-reducing black *S. putrefaciens* colony were selected and were inoculated at Nutrient broth which was then incubated at 25°C for 24 h. Biochemical analysis used at the identification of *S. putrefaciens* are shown in Table 1[6,7].

The study also examined the presence and the growth of the total viable count, *Enterobacteriaceae* spp. *Psycrophilic* bacteria, *Pseudomonas* spp. by the method of drop plate[9]. The medium used for the identification of the microflora of the fish samples and incubation conditions are shown in Table 2.

TMA identification: It was made as to the method reported by Association of Official Analytical Chemists[10].

pH: The pH value was recorded by a pH meter (pH 900, NEL Electronic, Ingold LOT 406-MG-DXK-57/25) and the glass electrode was applied directly to the flesh.

RESULTS

S. putrefaciens average count was determined at under the detectable level ($\log_{10}$<2.0 cfu g^{-1}) in control group at 0°C storage temperature (Table 3). pH value was determined at 6.21 in first day of storage. It was determined at 6.67 in 5th day and 7.12 in last day of storage (Table 4). TMA amount in control group, which was at 0.8 mg/100 g at day 1, was determined as 6.96 mg/100 g at day 5 and 19.04 mg/100 g in last day of storage (Table 5). It was also seen that in test group the average number of *S. putrefaciens* which was determined at under the detectable level ($\log_{10}$<2.0 cfu g^{-1}) in first day of storage, reached the level of $\log_{10}$ 5.84 cfu g^{-1} at day 5 and the level of $\log_{10}$ 8.6 cfu g^{-1} at day 9 (Table 3). pH value was determined at 6.23 in first day of storage. It was determined at 6.77 at day 5 and 7.42 at day 9 (Table 4). TMA amount in test group, which was at 0.81 mg/100 g at

Table 1: Analysis used at the identification of *S. putrefaciens*[6,7]

Bacteria	Gram reaction	Motility	Oxidase	Catalase	Gelatin	Glucose	Arabinose	Maltose	Citrate
S. putrefaciens	-	+	+	+	-	-	-	-	-

Table 2: The medium used for the microbiological analysis and incubation conditions

	Conditions of incubation			
Micro-organism	Time (day)	Temperature (°C)	Atmosphere	Medium
Total viable count	2-3	30	Aerobe	Plate Count Agar, Mast Diagnostics (DM 195)
Enterobactericeae	1-2	37	Anaerobe	Violet Red Bile Glucose Agar, Oxoid (CM 485)
Psycrophil	4-5	4	Aerobe	Plate Count Agar, Mast Diagnostics (DM 195)
Pseudomonas	1-2	30	Aerobe	Pseudomonase Agar Base, Oxoid (CM 559); Pseudomonas C.N Supplement, Oxoid (SR 102)
S. putrefaciens	4-5	25	Aerobe	Iron Agar, Lyngby; Oxoid (CM 876)

Table 3: Mean±SE of *S. putrefaciens* $\log_{10}$ cfu/g during whiting fishes storage at 0°C

Days	Control[A]	Test[B]	Normal[C]	General
1	0±0	0±0	0±0	0[a]±0
3	0±0	3.6600±0.0600	2.3000±0.1732	1.9867±0.5367[b]
5	0±0	5.8433±0.0348	3.3700±0.1629	3.0711±0.8481[c]
7	0±0	6.5067±0.1035	4.4433±0.2248	36500±0,9625[d]
9	0±0	86067±0.1733	5.9600±0.3301	4.8556±1.2771[e]

a, b, c, d, e: The difference between the days shown by different labels is significant

A, B, C: The difference between the groups shown by different labels is significant

day 1, was determined as 10,32 mg/100 g in 5th day and as 33, 11 mg/100 g in last day of storage (Table 5). The *S. putrefaciens* average count was determined at under the detectable level ($\log_{10}$<2.0 cfu g^{-1}) in whiting fish that inherent of *S. putrefaciens*. However the average level of *S. putrefaciens* was 3.37 cfu g^{-1}, in 5th day of storage and reached $\log_{10}$ 5.96 cfu g^{-1} at day 9 (Table 3). pH value was determined at 6.21 in first day of storage. It was determined at 6.72 at day 5 and 7.15 at day 9 (Table 4). TMA amount in test group, which was at 0.79 mg/100 g at day 1, was determined as 7.05 mg/100 g in 5th day and as 21.23 mg/100 g in last day of storage (Table 5). Microbiological growth was limited during 6 months in frozen storage (-18°C) of fishes and also pH levels and TMA amounts were not shown as important changes. The growth of further microorganisms was shown in Table 6.

DISCUSSION

El-Marrakchi *et al.*[2] have carried out sensory, chemical and microbiological analysis on sardines during 18 day of storage in ice. They reported that the limit counts of 10^6-10^7 cfu g^{-1} were reached at day 9 for fish stored in ice. This particular flora was reported to constitute an important part of the spoilage microorganisms with *S. putrefaciens* being its major representative. The levels of TMA during sardine storage in ice were determined as 0.16, 4.84 and 10.78 mg/100 g of flesh at days 0, 9 and 18. Sardines were graded according to their content in TMA. First grade: TMA lower than 1 mg/100 g: very good freshness. Second grade: TMA of

Table 4: Mean±SE of pH values of whiting fishes stored at 0°C

Days	Control[A]	Test[B]	Normal[C]	General
1	6.2167±0.04410	6.2333±0.02728	6.2167±0.03756	6.2222±0.01869[a]
3	6.5100±0.02082	6.5367±0.02333	6.5000±0.02082	6.5156±0.01215[b]
5	6.6700±0.02082	6.7767±0.03180	6.7200±0.03055	6.7222±0.02087[c]
7	6.7667±0.03756	7.1767±0.01856	6.7933±0.01453	6.9122±0.06745[d]
9	7.1267±0.02906	7.4200±0.01155	7.1567±0.02333	7.2344±0.04793[e]

Table 5: Mean±SE of TMA values during whiting fishes storage at 0°C

Days	Control[A]	Test[B]	Normal[C]	General
1	0.8000±0.04163	0.8133±0.04485	0.7933±0.02333	0.8022±0.01913[a]
3	2.7433±0.1584	5.5900±0.1457	2.7300±0.09539	3.6878±0.4804[b]
5	6.9600±0.09609	10.3267±0.4004	7.0533±0.04807	8.1133±0.5663[c]
7	13.5000±0.3972	21.2633±0.5732	14.4033±0.2783	16.3889±1.2446[d]
9	19.0400±0.2951	33.1167±1.7350	21.2367±0.7782	24.4644±2.2556[e]

a, b, c, d, e: The difference between the days shown by different labels is significant

A, B: The difference between the groups shown by different labels is significant

Table 6: The microflora ($\log_{10}$ cfu/g) in whiting fish stored at 0°C

Group	Day	TVB*	Enterobact.*	Psycrophilic	Pseudomonas	*S. putrefaciens*	pH	TMA
Control	1	3.18	3.24	3.36	2.36	<2.0	6.21	0.80
	3	4.21	3.42	4.50	3.20	<2.0	6.51	2.74
	5	5.00	3.66	5.24	4.22	<2.0	6.67	6.96
	7	5.48	3.70	6.35	5.46	<2.0	6.76	13.50
	9	6.54	3.46	6.79	6.09	<2.0	7.12	19.04
Test group	1	3.37	3.32	3.48	2.64	<2.0	6.23	0.81
	3	4.27	3.60	4.20	3.42	3.66	6.53	5.59
	5	4.97	3.64	5.45	4.74	5.84	6.77	10.32
	7	5.74	3.46	6.18	5.07	6.50	7.23	21.26
	9	6.07	3.80	6.79	5.71	8.60	7.42	33.11
Normal group	1	3.28	3.11	3.31	2.39	<2.0	6.21	0.79
	3	4.22	3.30	4.35	3.40	2.30	6.50	2.73
	5	4.94	3.40	5.27	4.40	3.37	6.72	7.05
	7	5.37	3.59	6.20	5.43	4.44	6.79	14.40
	9	6.29	3.66	6.82	6.16	5.96	7.15	21.23

*: TVB: total viable count; Enterobact.:Enterobacteriaceae

1 to 3 mg/100 g: good freshness. Third grade: TMA of 3 to 5 mg/100 g: intermediate freshness. The results of this study were found to be similar to those of our analysis on whiting fishes.

Ben-Gigirey *et al.*[11] reported fresh albacore tuna were frozen and stored at –18 and –25°C for 1 year. Chemical analyses were carried out at 1, 3, 6, 9 and 12 month of storage. The pH value at the TMA level did not change significantly with period of storage. This result may be related to the limited TMA reduction from TMAO because of the bacteria activity's inhibition at storage.

On the other hand, Reppond and Collins[12] reported that the limit levels of TMA were reached at day 6 of *Gadus macrocephalus*' (cod) storage in ice. This result shows that the freshness of the fish cannot be adequately determined by TMA level. Nevertheless, the study reported also that the detection of TMA level was an appropriate method due to its speed in determining the freshness of the fish.

The results of histamine and trimethylamine determination in three fish species (cod, herring, scomber) of various freshness are presented by Gajewska and Ganoviak[13]. The highest TMA amount was reported in cod flesh-the lowest in scomber. In a similar way to the results of this study, the whiting fish was reported to be more sensitive to spoilage than other species due to the highest level of TMAO detected during the analysis.

To conclude, it was determined that the test group, stored in ice (0°C) was shown more rapid spoilage indication from the control and normal group. pH and TMA amounts were not be exist an important change during 6 months in frozen storage (-18°C) because of microbiological growth was limited. It was observed that the frozen-storage, used in extended storage period, was an important process for preventing the bacteriological spoilage of fish.

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