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Experimental biodegradation of histamine by *Bacillus polymyxa* in fish fillet

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Abstract
Background:*seafoods provide a healthy source of high-quality proteins, Omega-3, vitamins and minerals. Unfortunately, combined high free amino-acids with improper storage and transportation conditions results in formation of serious biogenic amines (BAs) by means of bacteriological decarboxylation mainly, leading to severe health hazards especially allergic reactions caused by histamine poisoning; therefore, trials for safe and rapid reduction of histamine in seafoods is essential.*
Material and Methods:*One-hundred and twenty samples of some commercially sold fishes and shellfishes in Qalubiya governorate markets, Egypt; represented by Oreochromis niloticus, Mullussurmuletus, silver carb, shrimp, crab, and oyster (20 of each) for determination of some biogenic amines including histamine, cadaverine and putrescineby HPLC; in addition, experimental trial was conducted for biodegradation of experimentally inoculated histamine (50 mg/100g) by Bacillus polymxa (10^7 CFU/ml) during cold storage (4^OC).*
Results: *Results revealed significant differences between all the examined samples, where oyster and Oreochromis niloticushad the highest BAs levels among shellfish and fish samples, respectively. In addition, histamine was the highest detected BAs among the detected amines. Regarding with the degradation effect of B. polymyxa, results revealed gradual reduction in histamine concentrations in fish musculature to be 16.3, 10.5 and 9.1 mg/100g with reduction percent 67.4, 79.0 and 81.8% after 8, 16 and 24h of cold storage.*
Conclusion:*Referring to the obtained results, raw seafoods including fish and shellfish may possess a significant source of health hazards up on its BAs concentrations; additionally,biocontrol of histamine by means of probiotic biodegradation showed promising safe way to preclude BAs health hazards. Furthermore, proper storage and transportation have been strongly recommended, especially in high proteinaceous fish species, to avoid formation of significant biogenic amines.*
Keywords: *Biogenic amines; Egypt; HPLC; Fresh seafood*

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I. Introduction

Fish, as well as shellfish,have been considered as essential category of human protein sources that have been related to a lot of health benefits due to its high content of easily digestible and high-quality proteins, omega-3 and many vitamins and minerals. However, many foodborne troubles associated with consumption of seafoods were recorded, which mainly referred to high levels of biogenic amines intake[1].

Seafood may harbor a number of biological, chemical and physical hazards including biogenic amines (BAs), biotoxins,heavy metals, and pathogenic bacteria and parasites, where BAs were recorded to have the most severe cases of seafood borne poisoning cases [2].

Biogenic amines (BAs) are organic nitrogenous bases that have been produced during amino acid decarboxylation or transamination of aldehydes and ketones in high protein content foods especially during storage at elevated temperatures (>8°C) depending on the fish species, whereit usually results from the presence of decarboxylating microorganisms such as Enterobacteriaceae, clostridium, and lactobacillus spp. that able of decarboxylating some amino acids (AAs) such as histidine to histamine and so on[3,4].

Histamine poisoning is one of the most common form of intoxication caused by the ingestion of fish and fishery products. Different heat treatment and cooking methods cannot affect histamine level because of its heat stability. All consumers can be affected by histamine intolerance or intoxication regarding with the

symptom's severity which mainly characterized by dermatitis with skin rashes, decrease in blood pressure, headaches and edemas typical tohypersensitivity reactions[5, 6].

Although,many experimental trials were conducted aiming to reduce histamine levels in many food samples, the use of histamine degrading bacteria has recently become an emerging promising strategyforreducing histamine levels,especiallyinfermented foods[7].

Therefore, this study aimed to determine the levels of some BAs (histamine, putrescine and cadaverine) in six fish and shellfish species commercially sold in Egypt; additionally, experimental histamine reduction by addition of *Bacillus polymyxa* to experimentally histamine inoculated fish fillet sample.

II. Material and methods

2.1. Sample's collection

A total of 120 samples of fresh fishes and shellfish represented by *Oreochromis niloticus*, *Mullussurmuletus*, silver crab, shrimp, crab, and oyster (20 of each) were collected from different fish markets at different localities in Qalubiya governorate, Egypt. Samples were collected during the period of Summer and Autumn seasons, 2020.

2.2. Determination of BAs in fish samples using HPLC

Histamine, cadaverine and putrescine were determined in all examined samples according to [8].

2.2.1. samples' Extraction according to [8].

Twenty-five gm of each sample musculature were blended with 125 ml of 5% Tri chloro-acetic acid (TCA) for 3 min, followed by filtration using filter paper (Whatman, No. 1). Thus, 10 ml of the filtrate were transferred into test tube containing 4 gm sodium chloride and 1 ml of 50% sodium hydroxide. The filtrate was extracted 3 times using 5 ml of mixed solution of n-butanol and chloroform (1:1 v/v), and the upper clear layer was transferred to 100 ml separating funnel by using disposable Pasteur pipette. To combine the organic extracts (upper layer), 15 ml of n-heptane was added in separating funnel and extracted three times with 1.0 ml portions of 0.2 n-HCl, the HCl layer was collected in a glass Stoppard tube. Solution was evaporated just to dryness using water bath at 95°C with aid of a gentle current of air.

2.2.2. Formation of dansyl-amines

One hundred µl of each stock standard solution (or sample extract) were transferred to 50ml vial and dried under vacuum. About 0.5 ml of saturated NaHCO3 solution was added to the residue of the sample extract (or the standard). Vial was stoppered and carefully mixed to prevent loss-due to spattering. Carefully, 1.0 ml dansyl chloride solution was added and mixed thoroughly using vortex mixer. The reaction mixture was incubated at 55°C for 45 min. Actually, 10 ml of distilled water was added to the reaction mixture, then vial was stoppered and shake vigorously using vortex mixer, the extraction of dansylated biogenic amines was carried out using 5ml of diethyl ether for 3 times again, vial was stoppered, shake for 10 min. and the ether layers were collected in a glass tube using disposable Pasteur pipette. The combined ether extracts were carefully evaporated at 35°C in dry bath with aid of current air. The obtained dry material was dissolved in 1ml methanol and 10µl were injected in HPLC.

2.2.3. Apparatus HPLC conditions:

High performance liquid chromatography (HPLC) used for dansylamines determination was an Agilent 1100 HPLC system (Agilen Technologies, Germany) equipped with UV detector (Model G 1314A) set at 254nm wavelength. Data were integrated and recorded using Chemstation Software program.

2.3. Effect of *B. polymyxa* on experimentallu inoculated histamine concentrations in fish fillets

2.3.1. Preparation of bacterial suspension[9]

Bacillus polymyxa strain was obtained adjusted for direct use of 10^7 CFU/ml from Microbiology Unit, Faculty of Agriculture, Ain Shams University, Egypt.

2.3.2. Binding assay [10]

The bacterial pellets were suspended in 1 Kg fish fillets. The mixture was adjusted to reach a final concentration of 1×10^7 bacteria and 50 mg/Kg histamine level. Bacterial pellets and biogenic amine solution were vortexed for 5 seconds, and incubated for 24h on a Finemixer SH2000 orbital shaker (Finepcr, Seoul, Korea) with soft agitation.

2.3.3. Experimental grouping

Fish fillet contaminated with histamine was served as a control assay (G1), while the test group represented fish fillets contaminated with histamine (50 mg/kg) and treated with *B. polymyxa*were served as treated group (G2).

The samples were acidified with ultrapure HNO_3 and examined at zero, 8, 16, and 24hr time points for measuring the histamine using HPLC.

Histamine concentration was investigated at zero time (2h after inoculation), 8, 16 and 24h of incubation.

2.4.Statistical Analysis: Analysis of Variance (ANOVA) test was applied for statistical evaluation of the obtained results of the examined samples of fish and shellfish according to [11].

III. Results

The recorded results in Table no 1, BAs concentrations in the examined fishes revealed that *O. niloticus* recorded the highest BAs mean concentrations for histamine, putrescine and cadaverine, followed by silver carp and *Mullussurmuletus*, respectively. On the other hand, oyster samples recorded the highest BAs levels among the examined shellfish samples, followed by crab and shrimp, respectively. Additionally, histamine was the most prominent detected BAs in the examined samples. Thus, the obtained results of the detected BAs showed statistic significant differences ($p \leq 0.05$) between the examined fish and shellfish species.

Table no. 1: Mean values of different biogenic amines levels "mg/Kg" in the examined fish and shellfish samples (n=20).

Fish and shellfish	Histamine (mg/Kg)	Putrescine (mg/Kg)	Cadaverine (mg/Kg)
Mullussurmuletus	10.19 ± 0.93^{f}	7.68 ± 0.59^{f}	6.05 ± 0.43^{f}
Silver Carb	14.58 ± 1.05^{e}	12.05 ± 0.97^{e}	10.28 ± 0.79^{e}
Oreochromis niloticus	23.94 ± 1.82^{c}	19.73 ± 1.40^{c}	16.62 ± 1.08^{c}
Shrimp	17.45 ± 1.34^{d}	13.82 ± 1.15^{d}	12.17 ± 1.21^{d}
Crab	30.21 ± 2.09^{b}	22.47 ± 1.81^{b}	18.55 ± 1.64^{b}
Oyster	38.63 ± 2.71^{a}	31.26 ± 2.12^{a}	25.35 ± 2.05^{a}

Mean values with different superscripts were significantly differed ($p<0.05$)

Regarding with the fitness of the examined samples for human consumption, in referring to histamine levels, as recommended by the Egyptian standards no. 889-1/2009 (≤10mg/100g in fish meat), and 5021/2005 (≤20mg/100g in shellfish meat), the obtained results showed that all the examined samples (100%) were fit for human consumption reflecting the hygienic quality and proper storage and transportation conditions.

Demonstrated results in Table no 2, showed the influence of *B.polymyxa* culture (10^7CFU/ml) on the levels of experimentally inoculated histamine in fish fillets (50 mg/kg). Results revealed promising rapid degradation of histamine concentrations, where histamine was reduced to 9.1 mg/kg after 24h of cold incubation with reduction % of 81.8%.

Table no 2: Effect of *B. polymyxa* culture (10^7CFU/g) on the levels of histamine experimentally inoculated to fish fillets (50 mg/Kg).

Group Storage time	Control (mg/Kg)	*B. polymyxa* Treated group (mg/Kg)	Reduction %
Zero time	50	50	-------
8 hours	50	16.3	67.4
16 hours	50	10.5	79.0
24 hours	50	9.1	81.8

Levels of histamine levels (mg/Kg) in the control and *B. polymyxa* treated fish fillet samples.

IV. Discussion

A. Detection of BAs in fish and shellfish samples

The study was carried out to survey and investigate the concentrations (mg/kg) of BAs (histamine, cadaverine and putrescine) in some commercially sold fresh fishes and shellfishes in Egypt, represented by*M.surmuletus*, silver carb,*O.niloticus*, shrimp, crab, and oyster.

Biogenic amines (BAs) have been widely associated with food quality and safety [12].Although, they are naturally occurring in animals and humans, their presence in food is mainly referred to bacterial decarboxylation of free amino-acids [13].

According to Table no 1,*O.niloticus*and oyster samples recorded the highest levels of BAs, especially histamine, which may be attributed to their higher content of free histidine that consequently affected by improper storage and transportation conditions resulted in high conversion to histamine [14].

It was clearly confirmed in relation to the recommended Egyptian standards for the maximum permissible limit (MPL) of histamine in fish and shellfishes, that all (100%) the examined samples were in safe margins and considered as fit for human consumption.

In comparison with the previous studiesof histamine levels, Elshafey et al.[15] (13.46, 31.52 and 44.96 mg/kg in *O. niloticus*, crab and shrimp, respectively); Helmy et al.[16] (18.31, 41.75, 33.08 and 19.92 mg/Kgin *O. niloticus*, oyster, shrimp and crab, respectively); Refai et al.[17] (89.7 mg/Kg in *O. niloticus*); Arulkumaret al.[18] (8.6, 10.6and 12.5 mg/kg in shrimp, crab and oyster samples, respectively).

Regarding to Putrescine levels, Kulawik et al.[19] (ranged from 53.8 to 187.6 mg/Kg in *O. niloticus* samples), Al-Ashmawy[20] (12.18 mg/Kg in shrimp samples), Elshafey et al.[15] (6.61, 17.06 and 25.19 mg/kg in *O. niloticus,* crab and shrimp, respectively), and Arulkumaret al.[18] (1.3, 17.3 and 39.0 mg/kg in shrimp, crab and oyster samples, respectively).While for cadaverine, Kulawik et al. [19] (ranged from zero to 15.2 mg/Kg in *O. niloticus*), Al-Ashmawy[20] (ranged from 3.3 to 23.5 with mean value 11.66±0.80 mg/Kg);Helmy et al.[16] who recorded 16.57, 29.16, 21.83 and 13.09 in *O. niloticus,* oyster, shrimp and crab, respectively.

The recorded variations between different studies may be attributed to difference in localities of collection, storage and transportation conditions, and season of collection.

histamine poisoning is a serious public health and food hypersensitivity related with ingestion of improperly stored and processed fish containing high level of histamine (>50 mg/kg). histamine poisoning characterized by headache, tacchy-cardia, hypotension, skin rashes, flushing and edema[21,22].

Also, putrescineis formed in any food item by ornithine decarboxylation. They are considered as potential indicators of fish quality as they early as soon as once bacterial spoilage begins; so,they may be recorded as the most objective quality indicators for histidine poor fishessuch as white muscle fish, shell fish and fermented seafoods[23]. The health impact of putrescine and cadaverine consumption appears as hypotension, brady-cardia, and paresis of extremities;they also can combine with nitrite forming carcinogenic nitrosamines[24]**.** From these results which obtained, it was found that shell fish were higher contaminated with biogenic amines (histamine, putrescine and cadaverine) than fish species can be explained by presence of respective free amino acids and the more potential microbial activity which accelerate their spoilage, and also, due to deterioration fish and shellfishes is temperature and time dependent[25]**.**

B. Experimental part

However, biogenic amines formation in foods can be controlled or prevented by inhibiting bacterial decarboxylation process, many approaches were recorded such to limit microbial growth as making hydrostatic pressures, irradiation, controlled atmospheric packaging (CAP), or by using food additives which were considered non-practical based on fishing[26]; therefore, the other bio-controlling measureshave to be invented to control BAs levels likeby using bacterial amine oxidase or amine negative bacteria.

Current study aimed to study the biodegrading effect of *B. polymyxa*on the artificially inoculated histamine (50 mg/Kg) in fish fillet samples. Its effect as shown in Table no 2 appeared to be potentially good and rapid, where 81.8% of the inoculated histamine declined within 24h in cold storage conditions.

Current results came in line with the previously recorded results of Lee et al.[4; 27] who recorded that overall biogenic amine contents (including histamine, putrescine, cadaverine, and tyramine) in the control samples were markedly higher ($p < 0.05$) than those of the inoculated samples throughout fermentation. After 120 days of fermentation, the histamine and overall biogenic amine contents in the inoculated samples were reduced by 34.0% and 30.0%, respectively.

Potential activity of *B. polymyxa*in controlling of histamine production through its ability to produce histamine oxidase or histamine dehydrogenase which have the ability to catalyze the oxidative deamination of histamine to imidazole acetaldehyde and ammonia. In addition, histamine oxidase can also catalyze the conversion of histamine, in the presence of water and oxygen, to imidazole acetaldehyde, ammonia, and hydrogen peroxide[28].

V. Conclusion

Referring to the obtained results, it can be concluded that sellfishes had more potentiality to rapid decomposition and BAs formation; where oyster samples revealed the highest BAs concentrations. Besides that, *Bacillus polymyxa*showed potential rapid degradation effect on the histamine levels, that make it a good bio-controlling method in cold storage conditions.

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