Control of Histamine Formation by *Morganilla morganii* in Synthetic Media and Mackerel Fish Homogenate Using Blue Green Alga, *Spirulina platensis*

Sultan, Y. Y.* and Marrez, D. A.

Marine Toxins Lab., Food toxins and contaminants Dept., National Research Centre, Egypt.

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ABSTRACT

Histamine, a chemical health hazard, is formed from the amino acid histidine by bacterial histidine decarboxylase. The effect of *Spirulina platensis* alga extracts on the population of *Morganella morganii* and histamine formation were studied. Among many solvents, diethyl ether extract gave the best inhibition even at the lowest concentration (1000 ppm) using disc diffusion method. Concentrations of 50-1000 ppm of algal extract were tested against *Morganella morganii* in histidine decarboxylase broth (HDB). Significant reduction in the population was noticed at all the tested concentrations notably at the highest one (1.7 log units). Around 50% reduction in histamine content was obtained at the concentration of 500 and 1000 ppm algal extract. The highest reduction (>2.5 log units) in *M. morganii* count in stored sterilized and raw mackerel (incubated for 48 hrs at 25°C) was noticed at 1000 and 2000 ppm of algal extract. The dose 2000 ppm significantly controlled histamine formation (>60%) in stored mackerel. These results open new horizons to control histamine formation at a large scale of fish industry.

Keywords: M. morganii, histamine; S. platensis; histidine decarboxylase broth; stored mackerel

INTRODUCTION

Histamine is formed from the amino acid histidine by bacterial histidine decarboxylase (Fig.1) (Rawles *et al*., 1996, Hungerford, 2010). It does not have odour or colour to indicate its presence in food (Stratton *et al*., 1991). A toxic level of histamine, 50ppm, may be found before the fish appears spoiled or is organoleptically unacceptable (López-Sabter *et al*., 1996).

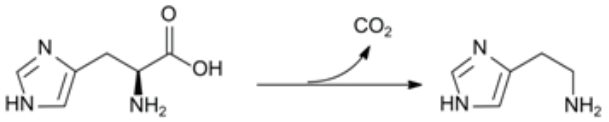

Fig. 1: Decarboxylation reaction of histidine to histamine by microorganisms

Although many different species of bacteria can form histamine, their histamine-producing capacities vary greatly. Only three species, *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei*, have been isolated from fish incriminated in scombroid poisoning (Taylor & Speckard, 1983). *M. morganii* is the most prolific histamine former capable of producing >1000 ppm histamine in culture broth (Rawles *et al*., 1996, López-Sabater *et al*., 1996). Thus, it has been proposed that *M. morganii* plays an important role in histamine production due to its prevalence and histamine-producing capacity (Kim *et al*., 2001).

Fish that commonly cause histamine fish poisoning (HFP) include scombroid fish like mackerel (*Scomber* spp.), tuna (*Thunnus* spp.), and non scombroid fish like sardines (*Sardinells* spp.), sockeye salmon (*Oncorhynchus nerka*), amberjack (*Seriola* spp.) (Taylor, 1986, Gessner *et al*., 1996).

Suppression of histidine-decarboxylating bacteria is a more appropriate approach to avoid histamine production (Joosten & Nunez, 1996). Several studies used food additives and herbs to control histamine production by such bacteria (Ayesh, 1993, Shakila *et al*., 1996, Thadhani *et al*., 2002). However, there were no available studies used algae to control of histamine production in media or fish.

Microalgae are a very diverse group of phototrophic organisms that consists of both prokaryotic and eukaryotic forms. Microalgae have various bioactive substances with antibacterial, antiviral, fungicide, enzyme inhibiting, immunosuppressive and cytotoxic activity (Gerwork *et al*., 1994, Jaki

* **Corresponding author:** sultanay18@hotmail.com

et al., 1999). *Spirulina* is a photosynthetic, filamentous, spiral shaped, multicellular blue green microalgae. *S. platensis* is the most common species of *Spirulina* widely available. It is used as human food and a complementary dietary ingredient of poultry and aquafeed due to its high content of proteins, poly unsaturated fatty acids, vitamins and minerals (Belay, 2008, Mani *et al*., 2008). *Spirulina* has been recognized as GRAS (generally recognized as safe) (Habib *et al.*, 2008). *Spirulina* is also useful as a functional ingredient as it is incorporated into various food products to enhance their nutritional qualities and for therapeutic management of chronic disorders such as diabetes, hypertension and heart disease (Lyer *et al*., 2008). *Spirulina* is also well-known for its antibacterial, antifungal, antiviral and antioxidant compounds such as beta-carotene, γ-linolenic acid, phycocyanin and vitamin E (Chu *et al*., 2010, Chu, 2011).

The present study is considered the first one that used algae to biocontrol histamine production by *M. morganii*. Control of this bacterium and subsequently histamine formation in broth media and mackerel fish homogenate using different extracts of *S. platensis* were the objectives of the present study.

MATERIALS AND METHODS

Bacterial and algal strains

Morganella morganii (10466) strain was acquired from The National Collection of Industrial and Marine Bacteria Ltd. (NCIMB), Aberdeen, UK.

The cyanobacterial strain, *S. platensis* was collected from Al-Khadra Lake, Wadi Al-Natroon, Baheira region, Egypt. Isolation and purification of *S. platensis* was carried by streaking plate method of Stein (1973) using BG-11 agar medium (Allen, 1973). Morphological identification of *S. platensis* was carried out using a phase contrast microscope (Carl zeiss, Jena) according to Desikachary (1959), Prescott (1978) and Hinhak (1988 & 1990).

Media

Zarrouk's medium (Zarrouk, 1966) was used for propagation of *S. platensis*. It composed of 18.0 g $NaHCO_3$; 2.5 g $NaNO_3$; 0.5 g K_2HPO_4; 1.0 g K_2SO_4; 1.0 g NaCl; 0.04 g $CaCl_2$; 0.08 g Na_2EDTA; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.01 g $FeSO_4 \cdot 7H_2O$ and 1.0 ml trace elements (TE) in 1000 ml distilled water. TE (gl^{-1}) incorporated 2.86 g H_3BO_3; 0.02 g $(NH_4)_6Mo_7O_{24}$; 1.8g $MnCl_2 \cdot 4H_2O$; 0.08g Cu_2SO_4 and 0.22 g $ZnSO_4 \cdot 7H_2O$. The culture medium pH was adjusted to 8.2 by 1 molar NaOH solution.

Nutrient agar (NA, Oxoid) was used to prepare fresh inocula of *M. morganii*. Two synthetic media were prepared to enhance histamine formation by the bacterial strain. The first one was 1% histidine-tryptic soy broth (1% TSBH, Behling & Taylor ,1982). It composed of 30g tryptic soy broth (Bacto) and 10g L-histidine (sigma) in 1-L distilled water. The second medium was histidine decarboxylase broth (HDB, Klausen & Huss,1987). It composed of 1g tryptone, 5 g NaCl, 2.5 g K_2HPO_4, 6.67 mg pyridoxalphosphate, and 3.5 g l-histidine hydrochloride (sigma) in 1-L distilledwater.

Efficacy of different *Spirulina platensis* extracts against *Morganella morganii*

Preparation of algal extract

(A) *Spirulina platensis* was propagated for 21 days at 30°C ±2 in Zarrouk's medium. Then, the culture was centrifuged and the pellet was spread on a metal tray to dry at room temperature with the support of a big fan. Known weight of the dry mass was rehydrated with distilled water at a ratio of 1:9. Algal solution was sonicated using probe of Ultrasonic processor (DAIGGER GEX 750, USA) for 20 min.

(B) Each of chloroform, hexane, ethyl acetate, diethyl ether and acetone (HPLC grade) were tested separately for their efficiency to extract antibacterial compounds from *S. platensis*. The extraction was performed three times with an equal volume of each solvent in a separating funnel. The collected solvent extracts were evaporated to dryness at 40°C using a rotary evaporator. Regarding to aqueous extract, *Spirulina* solution was centrifuged and the supernatant was left to dry overnight at room temperature. Same procedures were followed for methanol and ethanol but directly from the dried algae. The resulting dry films were dissolved with 50% ethanol to get 1, 5, 10 mg ml^{-1} as a final concentration each.

Antibacterial activity

Antibacterial activity was determined using disc diffusion method (Okigbo *et al.*, 2005). Watman filter paper discs, saturated with different concentrations of algal extracts, were placed on Nutrient agar medium supplemented with *M. morganii*. Disc fed with solvent alone serve as control. The plates were incubated at 35°C and were observed for zone of inhibition (mm) after 24 hr.

Propagation of *M. morganii*

Bacterial strain was inoculated into NA medium and incubated for 24 hr, after which a colony was picked and put into 1% TSBH medium and incubated at 30°C for 24 hr. After that, 1 ml pure culture was removed from 1%TSBH and inoculated into 9 ml of HDB to allow them decarboxylate histidine at its growth temperature for 72 hr.

Effect of metabolites of *S. platensis* on the number of *M. morganii* and histamine formation

In synthetic medium

Autoclaved HDB was supplemented with different concentrations of diethyl ether algal extract between 50 and 1000mg l^{-1} medium. For each concentration, 3 sets of flasks with basal broth were separately inoculated with the bacterial strain. The flasks were kept at 35°C for 72 hr.

Serial dilutions (up to 10^{-6}) were made and 100 µL from each dilution was plated on nutrient agar in triplicate. The plates were incubated at 35°C for 24hr. Colonies of bacteria were counted at each concentration. Two ml from each flask was kept in 2 ml eppendorf at -20°C for later histamine analysis.

In homogenized mackerel fish

Mackerel fish was chosen for this study as a common type containing histamine. Flesh of 1 Kg fish was well homogenized using warning blender. Impact of *S. platensis* extract on the population of *M. morganii* and histamine formation was studied during storage at 25°C. The experiment started with 2 types of flesh, raw and sterilized (autoclaved). Algal extracts dissolved in 50% ethanol used to prepare concentrations of 500, 1000 and 2000 ppm in sterilized bottles' containing 6 g flesh each. In triplicate, samples of raw and sterilized flesh homogenate were left 2 hr to absorb extracts. Bottles were inoculated with 1ml of *M. morganii* at $2.6x10^8$ cell ml^{-1} and incubated at 25°C for 2 days.

Populations of *M. morganii* after incubation at each concentration were enumerated using serial dilution. Four grams sample from each replicate was kept at -20°C for later histamine analysis.

Histamine extraction

In media

Histamine was determined according to Pircher *et al*. (2007) with some modifications. HDB culture was centrifuged in eppendorf (1000xg for 10 min). 200µl of the supernatant or histamine standard (Sigma-Aldrich), 800µl saturated $NaHCO_3$ solution and 1000µl dansylchloride solution (5 mg dissolved in 1 ml acetone, Fisher) were mixed in a screw-cap glass tube and vortexed for 30 sec. The vial was closed tightly and kept at 70◦C for 10 min. Two milliliter of distilled water was added to the reaction mixture, stoppered and shacked vigorously using vortex mixer. The extraction of dansylated histamine was carried out using 5.0 ml diethylether, stoppered, shacked carefully for 1.0 min and the ether layers were collected in culture tube using disposable Pasteur pipette. The ether extracts were carefully evaporated at 35°C in dry bath with aid of current air.

In homogenized fish

Four grams homogenized mackerel (raw and sterilized) were blended with 20 ml of 5% TCA for 3 min using a warning blender. Filtration was achieved using filter paper Whatman No.1. Ten millilitres of the extracts were transferred into a culture tube with 4g NaCl and 1 ml of 50 % NaOH and extracted three times by 5 ml n-butanol / chloroform (1: 1 v/v) by shaking 3 min each time. Mixture was centrifuged for 5.0 min. at 3000 rpm and the upper layer was transferred to 50 ml separating funnel using disposable Pasteur pipette. Fifteen ml of n-heptane were added in separating funnel and extracted three times with 1.0 ml portions of 0.2N HC1, the HCl layers were collected in a glass stoppered tube. Solution was evaporated just to dryness using water bath at 95°C with the aid of a gentle current of air.

One hundred µl of histamine standard solution (1mg ml^{-1} distilled water) were dried under vacuum. Half milliliter of saturated $NaHCO_3$ solution was added to the residue of the sample extract (or the standard). 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**.** About 10 ml of distilled water were added to the reaction mixture, stoppered and shaked vigorously using vortex mixer. The extraction of dansylated histamine was carried out using three times of 5.0 mI portions of diethylether, stoppered, shacked carefully for 1.0 min and the ether layers were collected in culture tube using disposable Pasteur pipette. The combined ether extracts were carefully evaporated at 35°C in dry bath with the aid of current air. The

obtained dry film was dissolved in 1ml methanol, and then 10 µl spotted on TLC.

Histamine determination

One-dimensional TLC carried to separate the histamine. Ten microliters of standard dansylated histamine (Sigma-Aldrich, St. Louis, MO, USA) and the treatments were spotted and developed using chloroform: benzene: triethylamine (6: 4.5: 1) as mobile phase. The presence of histamine in the samples was detected using a TLC scanner (CAMAG, Switzerland) at a UV wave length of 254 nm). Concentration of histamine was estimated from a calibration curve created by measuring the absorbance at 254 nm of known concentrations of histamine standards (1, 2 and 4 µg per spot). This system was found to give compact spot for histamine at R_f value of 0.43±0.02 (Fig. 2).

Statistical analyses

Data on the populations of *M. morganii* were logarithmically transformed before statistical analysis. This was required because of the wide range of variability (Clewer & Scarisbrick 2001). Statistical significance was determined using Statistica Version 9 (StateSoft, Tulsa, Okla., USA). The means of log CFUs of *M. morganii* and histamine concentrations were determined by analysis of variance (ANOVA, one way analysis) ($P<0.05$). Fisher's LSD Method ($\alpha=0.05$) was applied to compare significant differences between treatments and controls.

RESULTS AND DISCUSSION

Strain of *Morganella morganii* (10466) used in this study showed a high ability to form histamine in HDB medium (0.39 mg ml^{-1}). This result was in agreement with Özoğul (2004) and *Kim et al.*, (2002) who found that histamine formation by *M. morganii* can exceed 3 mg ml^{-1}. Many studies have been done to control histamine formation by bacteria (Ayesh, 1993, Shakila *et al.*, 1996, Thadhani *et al*. 2002). Kim *et al.*, (2002) studied temperature factor to minimize histamine formation. None of these studies used algae or its metabolites to control histamine formation by bacteria, especially by *M. morganii*.

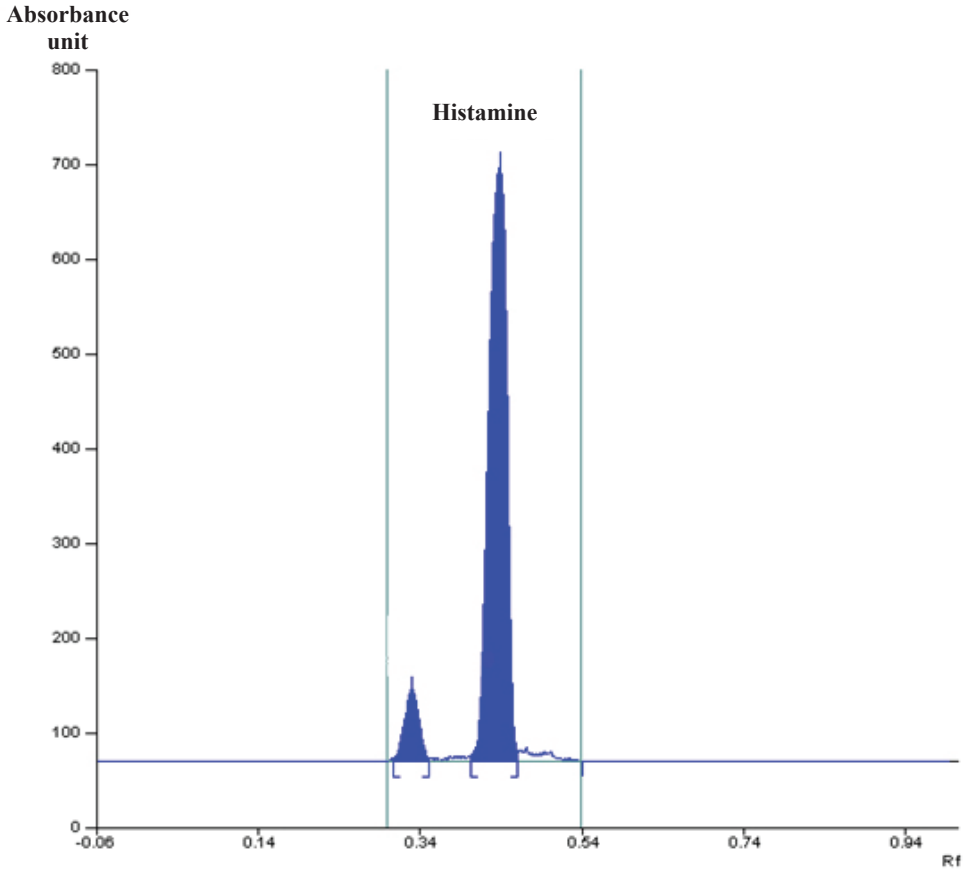

Fig. 2: Chromatogram of histamine (Rf=0.43±0.02)

The efficacy of *Spirulina platensis* extracts against *Morganella morganii*

The antibacterial activity of different extracts of *S. platensis* against *Morganella morganii* was evaluated (Table 1). No inhibition zone was observed by addition 50% methanol alone as a control treatment. Generally, all treatments showed closed inhibition effects (7-8.7mm) except hexane and chloroform extracts. The only extract showed an inhibition at the lowest concentration (1000ppm) was diethyl ether in addition to 5000 and 10000ppm. Similar work was carried out by Kumar *et al.*, (2011) using acetone and methanol extracts of *S. platensis* against other bacteria. He got an inhibition zone in range of 10-19 mm depends on the used concentration (250-7000ppm). Also, Pradhan *et al.*, (2012) found that the ethanolic extract of *S. platensis* showed good activity (15.7mm) against *Aeromonas hydrophila*.

Effect of algal diethyl ether extract on the growth of *Morganella morganii* in HDB medium

Based on the observation of the inhibition zone results, it was decided to use diethyl ether extract to minimize the growth of *Morganella morganii* in HDB culture. In general, the addition of *S. platensis* extract to the media significantly affected the population of *M. morganii* (Fig.3). Comparing with the control (0ppm), the best reduction reached from log 8.3 to log 6.9 and 6.5 CFU g^{-1} using doses of 500 and 1000 ppm of *S.platensis*. Figure (4) illustrates inhibition effect of such concentrations on *M. morganii* when plated on nutrient agar. This result is in contrast with de Caire *et al.*, (2000) who found that the aqueous extract of *S. platensis* promoted the growth of lactic acid bacteria.

Table 1: Effect of different *S. platensis* solvent extracts on inhibition zone (mm) of *M. morganii* using the disc diffusion method

Extract type / Extract Conc.	Aqueous	Ethanol	Methanol	Hexane	Diethyl ether	Ethyl acetate	Acetone	Chloroform
10000 ppm	7.0±0.0	8.3±0.7	7.3±0.3	Nil	8.7±0.3	7.7±0.3	8.7±0.3	Nil
5000 ppm	7.0±0.0	7.7±0.3	7.0±0.0	Nil	7.7±0.3	7.0±0.0	7.0±0.0	Nil
1000 ppm	Nil	Nil	Nil	Nil	7.0±0.0	Nil	Nil	Nil
Negative control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

*Mean values ±SE.

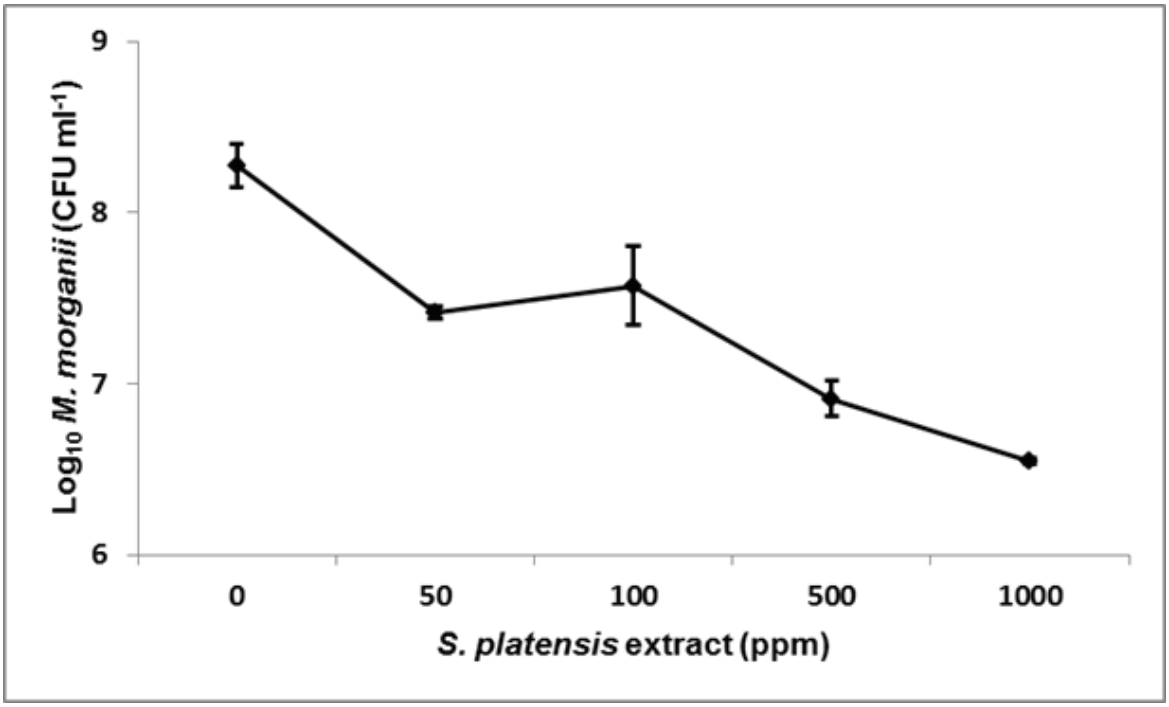

Fig. 3: Effect of diethyl ether extract of *S. platensis* on growth of *M. morganii* in HDB medium

***Mean values ±SE.**

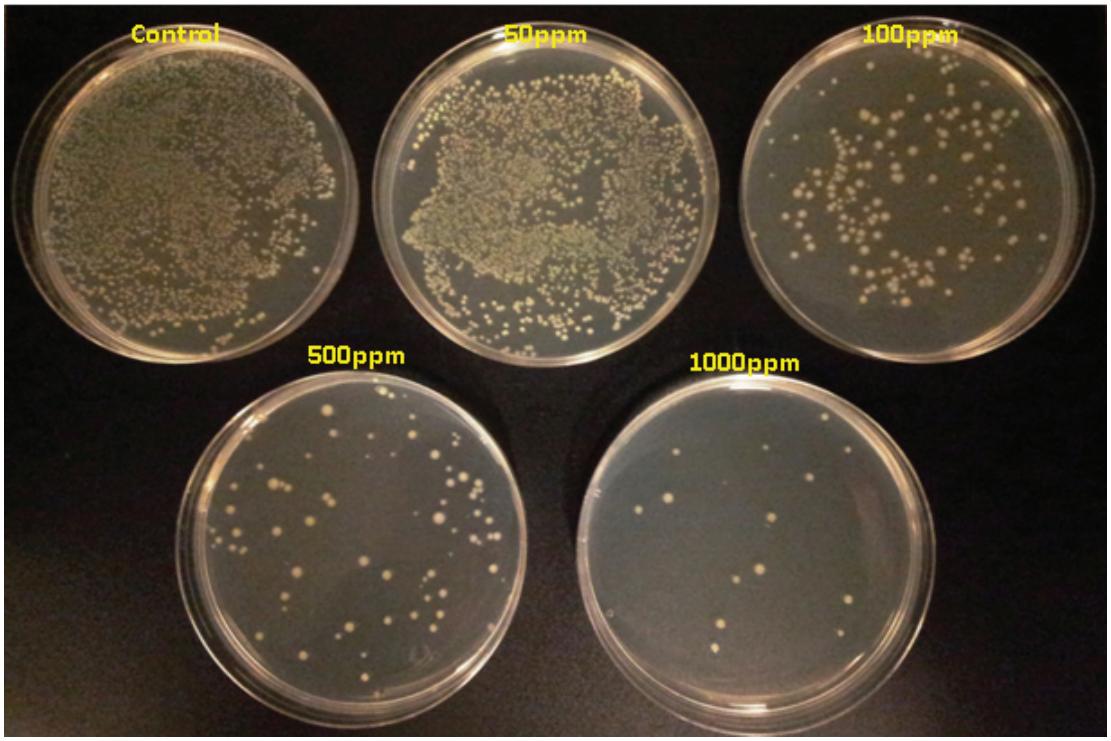

Fig. 4: Growth of *M. morganii* on NA at different concentrations of diethyl ether extract of *S. platensis*

Effect of algal diethyl ether extract on the production of histamine by *Morganella morganii* in HDB medium

The inhibition of histamine by *M. morganii* in HDB medium was examined using algal diethyl ether extract. The treatment significantly affected histamine production at P <0.05. No significant effect was observed at 50 and 100 ppm when compared with the control (Fig.5). Around 57% inhibition in the histamine was obtained by the addition of 500 ppm extract to the media followed by 41% at 1000 ppm. As mentioned before, there are no available works to control histamine formation by bacteria using algae.

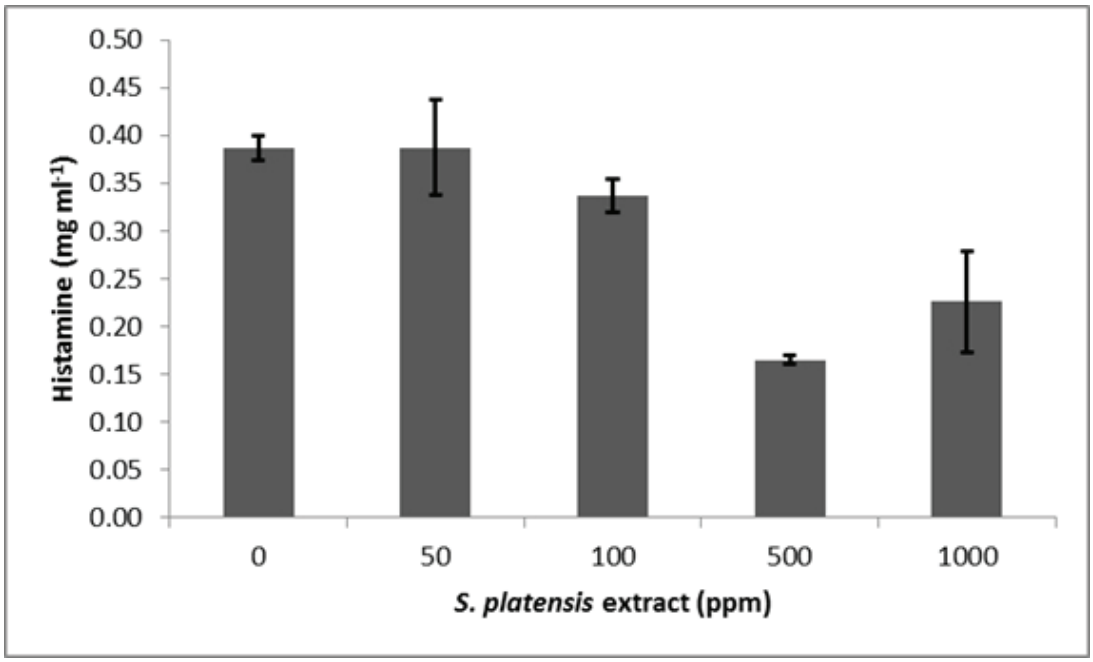

Fig. 5: Effect of the diethyl ether extract of *S. platensis* on histamine formation by *M. morganii* in HDB medium. *Mean values ±SE.

Impact of algal diethyl ether extract on the population of *Morganella morganii* and histamine content in mackerel fish homogenate

Since the tissue of mackerel contained high level of free histidine (600mg $100g^{-1}$) Lukton & Olcott (1958), mackerel fish was chosen for *in vivo* study. According to the results of *In vitro* experiment on HDB media, no reduction in histamine level was observed by the addition of 50 or 100 ppm algal extract. So, the impact of higher doses (500, 1000 and 2000 ppm) on bacterial count in fish was studied (Fig. 6). The addition of *S. platensis* extract significantly affected histamine production at $P<0.05$. In General, sterilized samples showed no spoilage clues like odour, texture and colour when compared with the raw samples after storage. Untreated sterilized samples contained 8.3 log CFU of *M. morganii* whereas it recorded 10.4 log CFU in untreated raw samples. This was due to decrease the available free water resulting from autoclaving process (Oduro *et al.*, 2011). The population of *M. morganii* in both raw and sterilized samples followed the same inhibition trend. The concentration 1000 and 2000 ppm gave the best effect. As mentioned before, no studies on the effect of *S. platensis* on *M. morganii* are available. However in a recent study, Ibrahim *et al.*, (2013) found that feeding Tilapia fish on feed containing 1% Spirulina for 2 months increased the immunity and disease resistance.

The low population of *M. morganii* in sterilized mackerel when compared with raw one reversed a perspective low level of histamine, 5.6 ppm (Fig. 7). Regarding to sterilized samples, all concentrations of algal extract (500-2000ppm) statistically gave the same inhibition effect on histamine formation (53-66%). Raw samples showed spoilage odour especially the control and that one treated with 500 ppm algal extract. This can explained the high content of histamine when compared with sterilized samples. Surprisingly, a significant stimulation was obtained at 500ppm instead of inhibition influence. Significant decrease was noticed only at 2000 ppm algal extract (60% inhibition). Little information is available on the inhibitory or stimulatory effects of food additives in general on histamine formation (Kang & Park, 1984, Mah & Hwang, 2009). However, stimulatory effects can be explained by that low dose of algal extract can be acted as a stress factor on *M. morganii* which increases the decarboxylase activity and subsequently increase histamine formation.

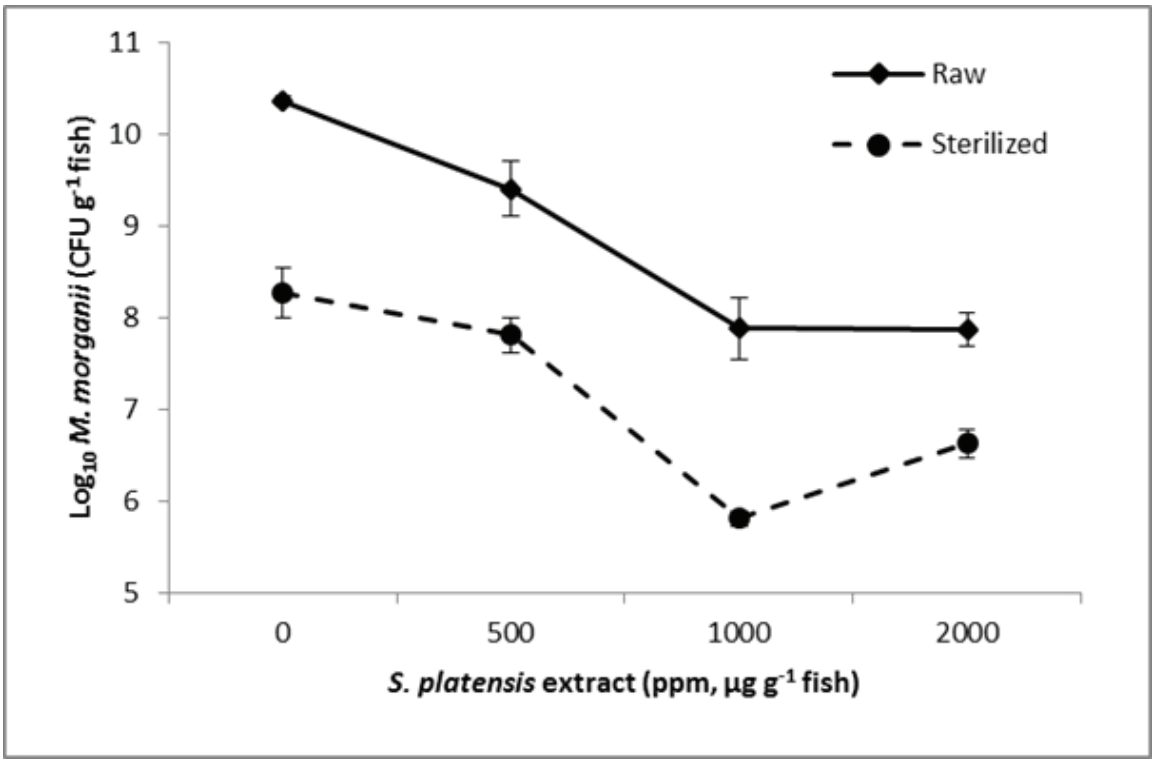

Fig. 6: Effect of *S. platensis* extract on the population of *M. morganii* on raw and sterilized mackerel after storage at 25°C for 48 hr. *Mean values ±SE.

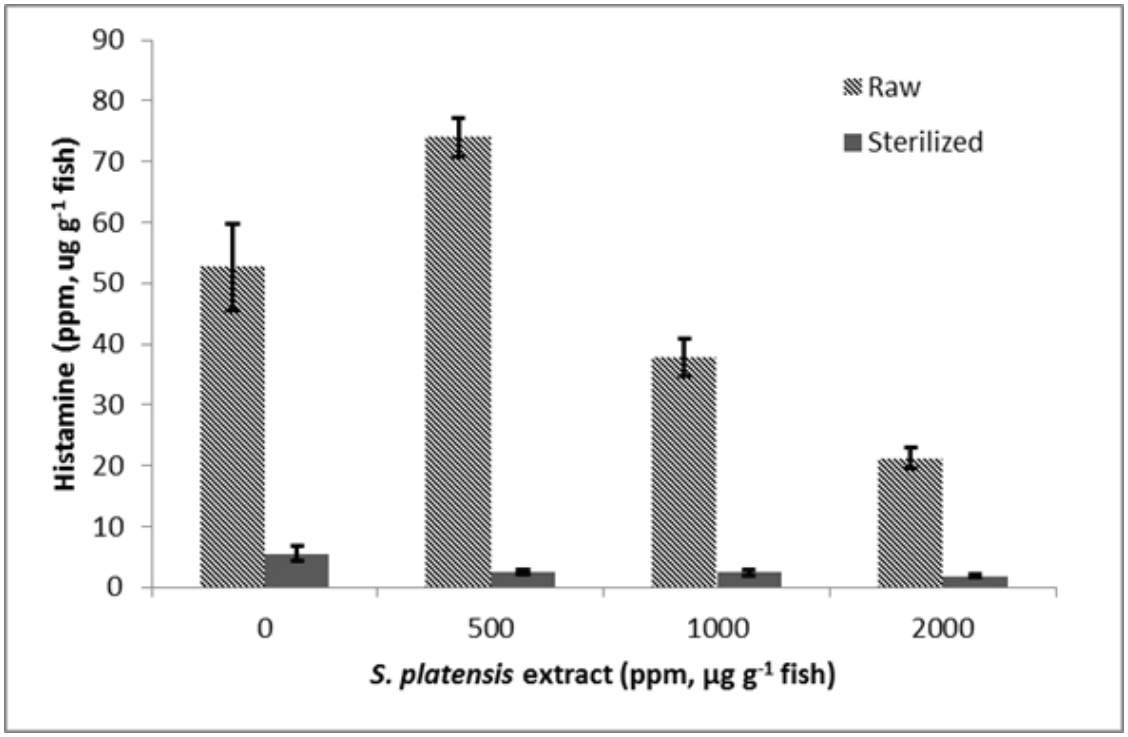

Fig. 7: Effect of *S. platensis* extract on histamine produced by *M. morganii* on raw and sterilized mackerel after storage at 25°C for 48 hr. *Mean values ±SE.

***Bars indicate mean values±SE.**

CONCLUSION

It could be concluded that diethyl ether extract of *S. platensis* decreased the growth of *M. morganii* in HDB medium and consequently decreased the rate of histamine formation effectively notably at 500 and 1000 ppm. Sterilization process changed the nature of protein of mackerel fish and made it poor medium for *M. morganii* growth. The inhibition trend of algal extracts on bacteria was the same for both raw and sterilized mackerel. Histamine formation in sterilized samples was found to decrease when all concentrations of algal extracts were used while it was inhibited only at 2000 ppm of algal extract in raw samples. Our results open a new horizon to minimize histamine formation in fish using algae from the same niche.

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التحكم فى الهستامين المتكون بواسطة بكتريا *M. morganii* فى البيئات الصناعية ومهروس سمك الماكريل بإستخدام طحلب *Spirulina platensis*

يوسف ياسين سلطان- ضياء عطية مريز

معمل السموم البحرية- قسم سموم وملوثات الغذاء- المركز القومي للبحوث

يمثل مركب الهستامين خطراً كيماوياً وهو يتكون من الحامض الأمينى هستدين عن طريق نزع مجموعة الكربوكسيل بواسطة الإنزيم الذي تفرزه البكتريا. تم دراسة تأثير مستخلصات طحلب *Spirulina platensis* على أعداد *M. morganii* وتكوين الهستامين فى كل من البيئات السائلة وعلي سمك الماكريل، من بين العديد من المستخلصات ، اعطى مستخلص الداى إثيل إثير أفضل تثبيط حتى فى أقل تركيز (١٠٠٠ جزء فى المليون). تم دراسة تأثير التركيزات من ٥٠ – ١٠٠٠ جزء فى المليون من مستخلص الداى إثيل إثير على *M. morganii* فى بيئة HDB. كان التأثير المعنوى على أعداد البكتريا كان ملحوظ فى كل التركيزات ولاسيما التركيز العالى (إنخفضت بمعدل ١,٧ وحدة لوغاريتمية مقارنة بالمجموعة الضابطة). كما حدث أنخفاض فى محتوى الهستامين بمعدل ٥٠٪ عند تركيزي ٥٠٠ و ١٠٠٠ جزء فى المليون، وكان أعلى انخفاض فى أعداد *M. morganii* فى سمك الماكريل (الخام أو المعقم)والمخزن لمدة ٤٨ ساعة على ٢٥ °م (< ٢,٥ وحدة لوغاريتمية) عند المعاملة بتركيزي ١٠٠٠ و ٢٠٠٠ جزء فى المليون من المستخلص الطحلبى. تبين أن للتركيز ٢٠٠٠ جزء فى المليون تأثيراً على التحكم فى إنتاج الهستامين (< ٦٠٪ تثبيط) فى أسماك الماكريل المخزنة. تفتح هذه النتائج أفاقاً جديدة للتحكم فى إنتاج الهستامين على نطاق واسع فى صناعة الأسماك.