Proceedings of the AFSA Conference 2016 on

FOOD SAFETY AND FOOD SECURITY

held at KIIT UNIVERSITY, BHUBANESWAR, INDIA ON SEPTEMBER 15-17, 2016

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PREFACE

The passion of some scientists in ASIA who believe that bio-science and biotechnology contribute to improvement in the quality of life and welfare of human beings of the world and know the importance of co-laboratory works and direct exchange of idea and information made this series of conference start. The first AFSSA Conference was held in Osaka on September, 2012 hosted by Osaka Prefecture University, JAPAN. The 2nd AFSA conference was held on August 15 – 18, 2014 at Dong Nai University of Technology, Bien Hoa City, Vietnam. This is the 3rd AFSA conferences which was held on September 15-17, 2016 at KIIT University, Bhubaneswar, India.

Sustaining of our passion, by holding the conference bi-annually, has been supported by private and public institutes/organizations of Asia Pacific Institute of Food Professionals (APIFP), International Committee on Food Microbiology and Hygiene (ICFMH), University of Dhaka, and KIIT University at **Bhubaneswar,** India as the host, as well as by students, volunteers and financial supporters, and many distinguished guests have strongly encouraged us. we express our sincere thanks to all those hosts and supporters. Our endless voyage for obtaining tomorrow's higher QOL and welfare of human beings by bio-science and biotechnology necessitates continuous supply of fresh and innovative wisdom.

This Regional Conference is qualitatively improving as an international meeting; researchers from 13 different countries joined in this 3rd conference, where 170 higher level of research works was presented and hot discussions were made in achieving food safety in this area.

This proceeding contains selected papers submitted by the speakers of different countries based on the presentation made in the conferences. The views expressed in the manuscripts are those of the authors, and the editorial members take no responsibilities for the content or comments, nor the views of Asian Food Safety and Security Association. However, it is believed that the book will act as a good source for relevant information in relation to food safety and food security.

The editorial committee is thankful to Asian Food Safety and Security Association for giving permission and financial support to publish the proceedings.

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CONTENT

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ISOLATION, CHARACTERIZATION AND BIO-CONTROL ACTIVITIES OF BACILLUS SUBTILIS FROM IN FERMENTED SOYBEAN IN CAMBODIA

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Abstract

The objective of this study were to isolate *Bacillus subtilis* produced *Bacteriocin* from Khmer traditional fermented soybean to control the growth of pathogenic bacteria from 120 *SIENG* samples from 9 different local open markets in Phnom Penh, 1 local open market and 2 local producers in Kandal province in Cambodia. 119 out of 120 samples were positive with *Bacillus subtilis* strains. Fifteen of these isolated strains exhibited antimicrobial activity to *Lactobacillus plantarum* by agar well diffusion assay and one of them (*CeM6-7*strain) exhibited strongest antimicrobial activity. The filtered supernatant of this strain also suppressed the growth of several Gram-positive pathogens (such as *Staphylococcus aureus*) or lactic acid bacteria in broth. The filtrate could also suppress the growth of *L. plantarum* inoculated into soymilk. Antimicrobial *B. subtilis* strain *CeM6-7* are thought to be applicable for making a starter culture to produce much safe fermented soybean foods by suppressing the growth of naturally contaminated *B. cereus* or *S. aureus* during production. In addition, the use of this starter culture may contribute to prevent spoilage or quality loss of soybean products caused by the over growth lactic acid bacteria or other Gram-positive bacteria.

Keywords: *B. subtilis*, Fermented soybean, *Bacteriocin*, Food poisoning, Spoilage bacterial

1. Introduction

Fermented foods are commonly produced in Cambodia and other South-Eastern courtiers since they are cheap, high nutrient values and benefit for health. In Cambodia, soybean is categorized in the fourth main agricultural commodities list which is actively generated yearly income for rural farmers (11) and certain amount of soybean are used for fermented soybean production, commonly called *SIENG* in Cambodian which famous and healthy foods among other Cambodian's soybean processed products. There are 2 kinds of fermented *SIENG* in Cambodia, *SIENG PRAI* (salted Soybean), and *SIENG PA-AEM* (Soybean which made from salts and sugar). Not only in Cambodia but also Japan, Korea, Taiwan, and China are also produced similar fermented soybean products [18].

Bacillus subtilis is a gram-positive bacterium in genus *Bacillus* which is naturally found in upper layer of the soil [7]. *Bacillus subtilis* may present on the soybean seed during cultivation or contaminate in *SIENG* during process according to Cambodia production context which *SIENG* is fermented using starter *Bacillus subtilis*, naturally on soybeans in other Asian countries [17]. Up to date research report that antibacterial substance produced by an isolated *Bacillus subtilis* strain LFB112 from Chinese herbs, was identified as *bacteriocins* and effective against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases, including *Escherichia coli, Salmonella pullorum, Pseudomonas aeruginosa, Pasteurella multocida, Clostridium perfingens, Micrococcus luteus, Streptococcus bovis* and *Staphylococcus aureus* [9]. In recent years, considerable effort has been made to develop food applications for many different *bacteriocins* using *bacteriocinogenic* strains [1, 2, 12 and 13]. *Bacteriocins* produced by industrially important *Bacillus subtilis*, which have a history of safe use in food and industry [14]. The *bacteriocins* produced by these strains are thought to be potent food preservative that are applicable and useful for Cambodian food industry.

However, limited research has been done concerning with *bacteriocins* produced by *Bacillus subtilis* compared to LAB species as food preservatives. Hence, the objective of this study was to isolate, characterize and optimize the condition antimicrobial *Bacillus subtilis* strains from fermented soybeans to control Gram-positive pathogenic bacteria (*Bacillus cereus, Staphylococcus aureus*, etc.) or spoilage lactic acid bacteria in foods.

2. Material and Methods

2.1 Isolation and Screening *bacteriocin* producing *Bacillus subtilis* strains

One hundred and twenty fermented soybeans samples were purchased from local market in Cambodia. Each of samples were mixed with 10 times volume of nutrient broth and incubated at 35^{o}C for 18 hr. The culture was streaked on nutrient agar plate to isolate typical colonies of *B. subtilis*. All *Bacillus* strains that have ability to inhibit the grow of indicators from the previous step were streaked into GSP and Mannitol salt agar plate, incubated at 35^{0}C for 24- 48 hrs., and isolated bacteria were identified by gram staining and biochemical tests using catalase /OF test.

Antimicrobial activity against indicator organisms was determined by using a well diffusion assay. There were 3 steps conducted in well diffusion assay. The first and second were used exactly the same testing method. However, either the first or second testing is minor different with the third one specifically on (1) with filtration, and (2) without filtration. One loop of each of the spore suspension of isolated *B. subtilis* strains was inoculated into 5 mL of LB broth and incubated at 35ºC for 18 hr. Filtered culture supernatant was subjected to agar well diffusion assay. *Lactobacillus plantarum ATCC 8014* was used for indicating bacteria and *B. subtilis, Miyagino* strain (used for *Natto* production) were used for negative control, respectively.

2.3 Characterize the *bacteriocin* produced by isolated strain

- ***Incubator Temperature and timing treatment:***

The spore culture of *bacteriocin* produced by *B. subtilis* strain was determined. Transferred to 5ml of LB broth of *B. subtilis* strain and kept on incubator shaker at 35^0C, 72 hrs., and then were heated and cooled it down. There were 2 steps conducted in well diffusion assay as following:

0.5 ml was transferred into 100 ml aliquots flasks of sterile composed LB broth. Then, the flasks were incubated in 2 deference incubator shakers. In both were started at 7:00 AM, and 17:00PM the flasks were incubated at 30^0C and 35^0C, then take 1ml of individual sample respectively at each different timing 6, 8, 10, and 12 hrs. and 12, 14, 16, 18, 20, 22 and 24 hrs. to transfer into micro-centrifuge tubes.

L. plantarum was inoculated into the mixture (1:1 volume) of MRS broth contained supplement agar and keep it till become solid and make wells, and then filtered culture supernatant of *B. subtilis* or *Miyagino* strain and incubated 24 hrs., 30^0C. Growth of inoculated *L. plantarum* in this MRS broth was measured and observed length of wells by mm/ml.

- ***Soy Milk treatment:***

The sensitivity of the *bacteriocin* to enzymes was checked by using a well diffusion assay. *B. subtilis* and *Miyagino* strain for control were inoculated into a 30ml of LB broth and placed on the incubator shaker at 30^0C on 250 rpm for overnight.

5ml *L. plantum*, *Levconostoc mesentanides Jen 6129*, *Micrococcus luceus Spi-flams IFo 13867* and *Staphylococcus aureus. IFo 13276* were transferred to MRS and LB broth and incubated at 30^0C and 35^0C, overnight. After dilution all strains were inoculated shaker from 3, 6 and 9 hrs at 35^0C into the mixture (1:1 volume) of soymilk and filtered culture supernatant of *B. subtilis* or *Miyagino* strains.

L. plantum, *Lev. mesentanides*, *M. luceus* and *S.aureus* plates, the MRS broth contained agar purified powder and TSA agar, aimed at asses viable bacteria count over time is used spiral plate systems, are kept in incubator at 35^0C for 24 hrs. Growth of inoculated all indicator strains in this soymilk was measured by plate counting method and calculated the number of CFU/g or ml of test sample.

Eventually, to evaluate whether all incubated plates are appropriate for the organism sought, we compared by using inoculating culture supernatant against indicator organism. For this purpose, four kinds of bacterial inoculate were prepared in two groups such as MRS broth and LB broth contains 5ml *L. plantum*, *Lev. mesentanides*, *M. luceus* and *S. aureus*, incubated at 30^0C and 35^0C. Two groups of bacterial inoculate was inoculated into the mixture (1:1 volume) of MRS broth. After that make wells, and filtered culture supernatant of *B. subtilis* or *Miyagino* strain and incubated 24 hrs. at 30^0C. Growth of inoculated *L. plantarum* in this MRS broth was measured and observed length of wells by arbitrary unit/ml (AU/ml) was calculated [3].

2.4 Optimize the *bacteriocin* production of the *Bacillus subtilis* strain by cultivating in different media and under different growth condition

The optimize the *bacteriocin* production of the *B. subtilis* strain by cultivating in different media and under different growth condition ware determined by using the spore culture and well diffusion assay. *B. subtilis* and *Miyagino* strain for control. Each strain was separately inoculated into a 5ml of LB broth and placed in the incubator shaker at 37^0C, 24-72 hrs., heated and cool down, and then store at -20^0C, 24 hrs.

Next step, spore culture from two strains was series dilution (10^2,10^4, and 10^6) and then spread on TSA agar plats, incubated at 35^0C for 24 hrs. Growth of inoculated all indicator strains in this TSA was measured by plate counting method and calculated the number of CFU/g or ml of test sample. Following counting colonies, 10^4 dilutions were used spore culture into 30ml LB broth in flasks incubator shaker at 30^0C for 16, 20 and 23 hrs.

Four kind of bacterial inoculate were prepared in two groups such as MRS broth and LB broth contains 5ml *L. plantum*, *Lev. mesentanides*, *M. luceus* and *S.aureus*, incubated at 30^0C and 35^0C. Two groups of bacterial inoculate was inoculated into the mixture (1:1volume) of MRS broth contained supplement agar and keep it till become solid. After that make wells, and filtered culture supernatant of *B. subtilis* and *Miyagino* strains and incubated 24 hrs at 30^0C. Growth of inoculated *L. plantarum* in this MRS broth was measured and observed length of wells by arbitrary unit/ml (AU/ml) [3].

2.5 Apply the *bacteriocin* to control the growth of Gram positive pathogenic or spoilage bacteria in foods

Bacteriocin to control the growth of Gram positive pathogenic or spoilage bacteria in foods was applied by using a well diffusion assay. There were 2 kinds *Bacillus subtilis* strains, (1) *B. subtilis* (Cambodia) and (2) *Miyagino* for control (Japan). Each strain were inoculated into a 20ml of LB broth centrifuge tubes and placed on the incubator shaker at 35^0C, 16 hrs. Gram positive group (*S. avereus, Listeria, and Lactbacillus)* and Lactic acid group (*L.plantum, L.brevis,* and *Lb.lactis*) were inoculated separately to BHI and MRS in incubator shaker at 350C for 18 to 24hr. After shaker measure turbidness at OD 650 nm and dilute until OD 0.1 by PBS all of strains.

Filtered culture supernatant of antimicrobial *B. subtilis* or *Miyagino* strains was mixed with same volume of MRS or BHI broth for lactic acid bacteria (*L.plantum, L.brevis,* and *Lb.lactis*) or other Gram-positive bacteria, respectively. Each of the pre-cultures of indicator strains was inoculated into this mixture and cultivated at 35ºC. Change of the optical density at 650 nm was recorded automatically.

2.6 Data Processing and Analysis

The results were analyzed by Excel, AU, ANOVA and OD. In analyzing data, both quantitative and qualitative methods were used, and other appropriate methods such as Preference Ranking and Indexing were also used.

3. Results and Discussion

3.1. Isolating *Bacillus* strains from Cambodian fermented soybean (*SIENG*) samples

120 raw *SIENG* were isolated for *Bacillus* strains. As the result, only a total of 119 samples of *Bacillus* strains were isolated from 120 raw *SIENG*. *Bacillus subtilis* strains are important for seed fermentation because of their enzymatic activities contributing to desirable texture, flavor and pH development [4]. In general, *Bacillus Subtilus* is the most dominant bacterium in fermented soybean [15]. And it was resistant to heat which showed growth in 70 g dm^{-3} NaCl, nitrate reductase positive reaction [18]. These isolated among 119 *Bacillus* strains, there were found only 15 *Bacillus* strains that have ability to fight against the indicator microorganisms. Furthermore, among 15 samples, only one stain (*Bacillus CeM6-7*) have shown highest level antimicrobial compound of *L. plantum* at 12% of 15.00 mm/ml total zone activity compare to others. One of the main characteristics shared among *Bacillus* strains is the ability to produce a wide range of antimicrobial compounds active against bacteria. Even though microbial control exerted by these metabolites was demonstrated in plant environments, few reports focused their attention on how these compounds can interact with food microbiota [8]. The rank based on per mm/ml and percentage total area of level antimicrobial compound of *L. plantum* showed the trend of all type *Bacillus* strains.

3.2 Identification from isolated *bacteriocin* producing by *Bacillus subtilis strains*:

From the result of previous step, isolating *Bacillus* strains, the 15 colonies of *Bacillus* activities strains were developed to have another four additionally. We therefore obtained 19 purified of *Bacillus* activities strains finally and they were used in this step.

3.2.1 Biochemical and Morphological of *Bacillus strains*

Bacillus is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum *Firmicutes* [6]. The samples were spread plated and streaked on GSP agar plate to get the pure culture and the morphology of the colony is observed. As a result, colonies were found to be circular in shape, white to creamish in colour and measured to be 1-3 mm in size mostly, and the optimum temperature of *Bacillus* strains in order to growth was between 30-37^{0}C [19], but, a well growth condition is 35^{0}C.

The result revealed that in aerobe production *Bacillus* strains were growth all strains. In opposite, in anaerobe production, there were only four *Bacillus* strains grown and had taken a very longer time for growing but stay for only a short life compare to aerobe production because of *Bacillus* species including *B. subtilis* can be obligated in both condition aerobes (mostly) and anaerobes (rarely and stay in short life) according to the existing publications [6]. Nonetheless, according to another publication [18], a good condition that *Bacillus* strain can growth well is in aerobes production and its typical characteristics of the *B. subtilis* is a facultative aerobic, Gram-positive rod, and spore formation. There are three kinds of test for biological counting VPOF test, Manito Salt, and Catalase test. *Bacillus* species will be positive for the enzyme catalase when there has been oxygen. The result in has shown that all *Bacillus* strains are fermented in OF test and all positive in Manito Salt, VP and Catalase test, including *B. subtilis*, and they were catalase-positive reaction, mannitol, and maltose [18].

3.2.2 Subject to assay of *Bacillus subtilis* strains

Selected 19 strains of *B. subtilis* has been taken to determine antimicrobial activity against indicator organisms and was determined by using a well diffusion assay method. There was a significant difference among the zone activities *B. subtilis* strain classifications. As an overview on the result, *B. subtilis* strains had antimicrobial activity against indicator organisms of *L. plantum* bacteria and they mostly had activity at 35^{o}C much more than at 30^{0}C. Further, the *B. subtilis* strains most likely had antimicrobial activity when using un-filtration method. As detail in the result, 6 strains named *B. BTM8-9,B.TaM2-4(1), B. BTM8-10, B. TSM5-10, B. TSM5-3, B.CKM9-2* didn't show any antimicrobial activity, meanwhile, other 4 strains had total zone activities between 6-11mm/ml, including *B. BTM8-4(2), B. TaM2-4(2),B. TSM5-8(1), B. TSM5-7*, shown less antimicrobial activity, and other 9 remaining between 12-64 mm/ml, *B. CeM6-7, B. BTM8-4(1), BTM8-1, CKM9-1, DM1-14(2), DM1-14(1), CKM9-7* and *DM1-17* strains have strong activities against indicator organisms in both temperature condition, and both methods (filtration, and without filtration). These 9 *Bacillus* strains that had strong activities are predicted to be able to suppress the growth of *L. plantum* bacteria, and they were the samples (culture supernatant) in MRS broth with supplement 1% agar mixed. Finally, only 9 *Bacillus* strains that had strong activity were selected for the next experiment.

Table 3.4: *Average zone activity (mm/ml) for Lactobacilus planum by type of Bacillus strain*

S.N	Type of Bacillus strains	Incubator Time (hrs)	Incubator Temperature	Incubator Time (hrs)	Incubator Temperature	N° respondents of B. activates	Diameter of zone activity (mm/ml) for *Lactobacilus planum* bacteria			
							MIN	MAX	Total	Average
1	CeM6-7	17	35^{0}C	24	30^{0}C	6	5	15	64	10.67
2	BTM8-4(1)	17	35^{0}C	24	30^{0}C	4	5	10	36	6.00
3	BTM8-1	17	35^{0}C	24	30^{0}C	5	5	10	35	5.83
4	TSM5-8(2)	17	35^{0}C	24	30^{0}C	4	5	8	25	4.17
5	CKM9-1	17	35^{0}C	24	30^{0}C	3	6	9	22	3.67
6	DM1-14(2)	17	35^{0}C	24	30^{0}C	3	3	7	17	2.83
7	DM1-14(1)	17	35^{0}C	24	30^{0}C	2	0	8	16	2.67
8	CKM9-7	17	35^{0}C	24	30^{0}C	3	0	8	15	2.50
9	DM1-17	17	35^{0}C	24	30^{0}C	3	3	9	12	2.00
	Total	-	-	-	-	33	32	242	391	97.75

Table 3.4 exposed that in average size, *Bacillus CeM6-7* strain had the largest zone activities presented for 10.67mm/ml *L. planum* bacteria and the *Bacillus BTM8-4(1), TSM5-8(2), CKM9-1, DM1-14(2), DM1-14(1), CKM9-7* and *DM1-17* strains had the average zone activities 6.00, 5.83, 4.17, 3.67, 2.83, 2.67, 2.50 and 2.00 mm/ml, correspondingly.

3.2.3 Bacteriocin producing *Bacillus subtilis* strain

From the previous step, subject to assay of *Bacillus* strains, one of *Bacillus* activity strain namely *(CKM9-7(1))* was developed to have one additional strain. We finally obtained purified 10 of *Bacillus* activities strains.

After receiving high Bacteriocin producing *Bacillus* strains, those 10 strains were gone for further test to isolate the strongest *Bacillus subtilis* strain which could produce bacteriocin the best in comparing with other strains. The total diameter of zone activity (mm/ml) for *Lactobacilus planum* and API50 CH were used in the context of "measure of the rank and classify Bacteriocin productions on the *Bacillus subtilis* strain" for final selection of Bacteriocin. The categories, rank and selection of Bacteriocin production analysis of all types of *Bacillus* are presented in the below table (Table 3.5).

S.N	Type of Bacillus strains	Incubator Time (hrs)	Incubator Temperature(^{0}C)	Total N^0 zone activity(mm/ml) at 30^{0}C	Incubator Temperature (^{0}C)	Total N^0 zone activity(mm/ml) at 30^{0}C	Total N^0 respondents of B. activates	Diameter of zone activity (mm/ml) for *Lactobacilus planum*		
								Total	Mean	Percentage
1	CeM6-7	overnight	35	28	30	6	4	34	8.50	71
2	DM1-14(2)			ND		ND	2	5	1.25	10
3	DM1-14(1)			4		ND	2	5	1.25	10
4	CKM9-7(2)			ND		ND	2	4	1.00	9
5	CKM9-7(1)			ND		ND	ND	ND	ND	ND
6	BTM8-1			ND		ND	ND	ND	ND	ND
7	BTM8-4(1)			ND		ND	ND	ND	ND	ND
8	TSM5-8(2)			3		2	ND	ND	ND	ND
9	CKM9-1			3		2	ND	ND	ND	ND
10	DM1-17			ND		ND	ND	ND	ND	ND
	Total		-	38	-	10	10	48	-	100.00

Note: Not detected (ND)

Table 3.5: Rank and selection of Bacteriocin produced by Bacillus subtilis strain category by total diameter of zone activity (mm/ml) for Lactobacilus planum

Table 3.5 revealed that mean of CeM6-7 strain was 8.50 mm/ml, 71%, which was the highest proportion of total area of zone activity for *Lactobacilus planum* likened to other strains that we were measured and the following order of Bacillus strain was DM1-14(2), DM1-14(1), CKM9-7(2), CKM9-7(1), BTM8-1, BTM8-4(1), TSM5-8(2), CKM9-1 and DM1-17 strains respectively. The rank and selection of B. subtilis strain were based on its size (mm/ml) and percentage of zone activity that shown the trend for all type B. subtilis strains.

Accordingly, only one strain *CeM6-7* which had very strong zone activities was selected for further test since it was able to suppress the growth of *L. plantum* bacteria which it was in parallel with existing researches stated that, some *B. Subtilis* have reported to produce *bacteriocins* which suppress the growth of Gram positive spoilage and pathogenic bacteria [10 and 16]. In addition, we also found that antimicrobial activity against indicator organism of *CeM6-7* was more actively at 35^{0}C compared to 30^{0}C. There are also some strains such as *DM1-14(1)*, *DM1-14(2)*, and *CKM9-7(2)* which shown antimicrobial activity against indicator organism. However, these strains are half-strong compared to *B. CeM6-7* strain. The strains *CeM6-7* was identified as *B. subtilis* by 96.2 % and a T-value (0.76) homology correspondingly counted on API 50 CH V4.1 (BioMérieux) profiles (not shown) and their physiological characteristics.

3.3 Characterize the bacteriocin produced by isolated strain

The result obtained from our test shown *B. CeM6-7* strain had ability resistant to heat. Similarly, *B. subtilis* strains may produce other antimicrobial substance, which have been characterized to a much lesser extent [6].

3.3.1. Incubator temperature and timing

In this test, we used two variables of temperature (30^{0}C and 37^{0}C) and different timing (0h, 6h, 8h and 12h for first day and 0h, 14h, 16h, 18h, 20h and 22h for second day), and the *L. plantum (LP)* bacteria was used as the indicator strain. The test was conducted for 2 days.

As seen in Figure 3.1, in the first day of test, the antimicrobial activities of bacteriocin was activated at 12h (10mm/ml) at 37^{0}C only, beside there was no antimicrobial activities at all. The antimicrobial activities were actively happened in the second day of test. In the same figure and table revealed that the antimicrobial activities of bacteriocin started to be active after 16h of incubation at 30^{0}C. The antimicrobial activities kept increasing consistently for four hrs, and gradually decrease after 20h of incubation at 30^{0}C. At 37^{0}C, the bacteriocin started increasing its antimicrobial activities after 14h of incubation which was faster compared with 30^{0}C of incubation temperature. Though, its antimicrobial activity was kept increasing slightly for just only two hrs, and started decreasing sharply for the next four hrs until became inactivated after 20h of incubation at 37^{0}C. It demonstrated that bacteriocin produced by *CeM6-7* strain is more likely to be active at 37^{0}C for a longer period compared with its activities at 30^{0}C for two tested days. The finding was similar to previous researches that indicated the behavior of bacteriocin towards heat-resistant pathogens also varies, [12]. Additional finding, at 37^{0}C, *Bacillus CeM6-7* strain could tolerate with heat up to 16h, which was higher toleration than at 30^{0}C, and it still had ability to fight against indicator strain *LP*.

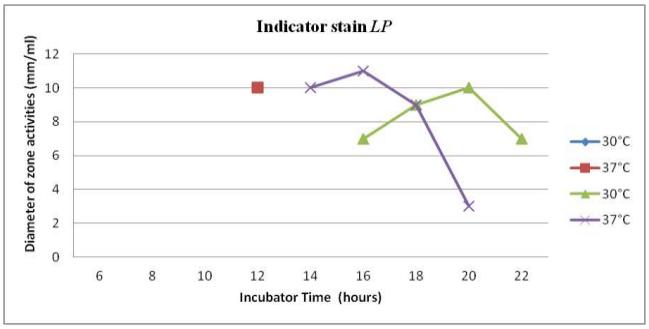

Figure 3.1: Effect of difference incubator temperature and timing on the activity of bacteriocin produced by B. subtilis CeM6-7 strain

3.3.2. Soybean Milk Treatment

In this test, we used the enzyme contained in Soybean Milk as the treatment, and used four types of indicators including L. plantum (LP), Lev. mesentanides (LM), S.aureus. (SA), and M. luceus Spi-flams (ML). We also used other five of bacteriocin producing by Bacillus subtilis strains including, B. CeM6-7, and Miyagino (control strain) for comparing the result.

A. Indicator Strain LP

In the Figure 3.2, presented the characteristics of antimicrobial activity against LP by B. subtilis stains such as B. CeM6-7((isolated from traditional fermented soybean (SEING)), and Miyagino after cultured in Bacillus-Soybean milk broth contained against LP (dilution of 103 times). The result shown that the stain B. CeM6-7 produced the strongest antimicrobial activity against Bacillus-Soybean milk broth contained LP in compare with Miyagino, but the other remaining strains B. CeM6-7 and Miyagino, were found to be no antimicrobial activity at all. At 0h, all strains were counted to be 3.33 log10 CFU/ml equally. At 3h and 6h, the B. CeM6-7 was calculated to be 2.643 log10 CFU/ml to 3.204 log10 CFU/ml and at 9h was calculated 2.623 log10 CFU/ml respectively, which in comparison were less than Miyagino that presented at 3.794 log10 CFU/ml to 4.549 log10 CFU/ml and 4.853 log10 CFU/ml at 9h correspondingly.

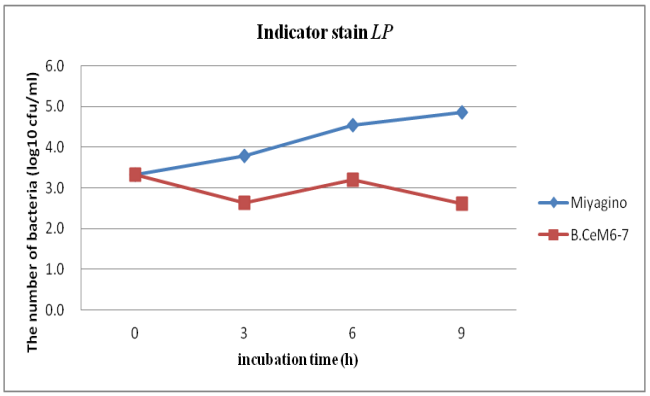

Figure 3.2: Enzymes of Soybean Milk resistant for strain Bacillus CeM6-7 compare with Miyagino strains against indicator LP

B. Indicator Strain *LM*

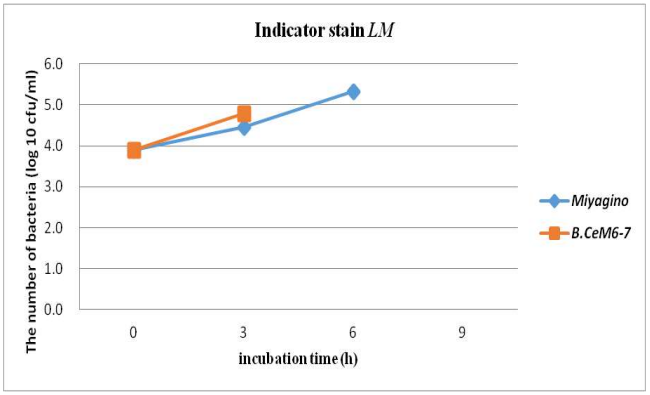

Figure 3.3: Enzymes of Soybean Milk resistant for strain Bacillus CeM6-7 compare with Miyagino strains against indicator LM

In the Figure 3.3 introduced the characteristics of antimicrobial activity of *Bacillus subtilis CeM6-7* strains, and *Miyagino* against *LM* indicator strain after cultured in *Bacillus-Soybean milk* broth contained against *LM* (dilution of 10^3 times). As the result, the *B. Ce6-7* stains no powerful antimicrobial activity with against *LM* at 3h to 9h incubation timing, since it was counted to be 4.795 log10 CFU/ml to MPN, which were less than *Miyagino* (4.461 log10 CFU/ml to 5.336 log10 CFU/ml) at 3h to 6h.

C. Indicator Strain *SA*

The characteristics of antimicrobial activity against *SA* of five strains *B. subtilis* and Miyagino after cultured in *Bacillus-Soybean milk* broth contained against *SA* (dilution of 10^3 times) were discussed in figure 3.4.

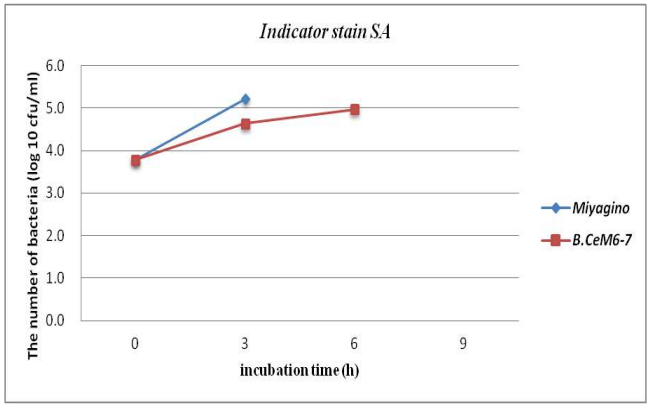

Figure 3.4: Enzymes of Soybean Milk resistant for Bacillus CeM6-7 strain compare with other strains against indicator SA

The figure indicated that at 3h to 6h *B. CeM6-7* strains had antimicrobial activity able to inactivate *Bacillus-Soybean milk* broth contained against *SA.* At these two incubation timing, 3h and 6h, *B. CeM6-7* strain showed the most powerful antimicrobial action compare to *Miyagino* strains. At 9h, there was no antimicrobial activity shown. The *B. CeM6-7* and *Miyagino* strains cultured growing between 3.785 log10 CFU/ml at 0h. At 3h, the antimicrobial produced by *B. CeM6-7* strains were developed between 4.641 to 4.641 log10 CFU/ml which less than Miyagino strain was calculated to be 5.220 log10 CFU/ml. At 6h inoculation, Miyagino strain was counted at MPN that higher than the *B. CeM6-7* strains existed 4.973 log10 CFU/ml.

D. Indicator Strain *ML*

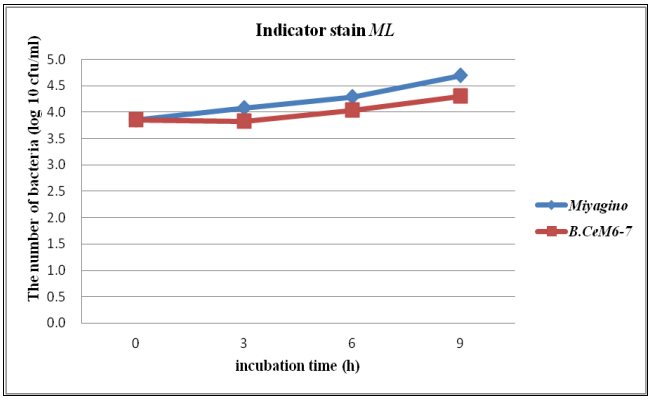

Figure 3.5: Enzymes of Soybean Milk resistant for Bacillus CeM6-7 strain compare with Miyagino strains against indicator ML

In the Figure 3.5, the characteristics of antimicrobial activity against *ML* of Bacillus *subtilis CeM6-7* and *Miyagino* strains after culturing in *Bacillus-Soybean milk* broth contained against *ML* (dilution of 10^3 times) were presented. It was indicated that, at different timing (3h, 6h, and 9h), The *B. CeM6-7* strain had enough ability to fight against *Bacillus-Soybean milk* broth contained *ML* compare to strain Miyagino, but it's more likely be less action. The

B. CeM6-7 strain cultured and grew the same amount at 3.854 log10 CFU/ml at 0h inoculation and it was increased 3.830 – 4.407 log10 CFU/ml at 3h to 9h incubation time. The amount of *B. CeM6-7* strain cultured and grew in each timing, 3h, 6h and 9h were automatically smaller than Miyagino strain that was 4.079, 4.290, and 4.699 log10 CFU/ml respectively.

In short, the *Bacillus CeM6-7* strain performed very well when treated with enzyme, especially for *Bacillus-Soybean milk* broth contained against *SA*, while were the most powerful to fight against *Bacillus-Soybean milk* broth contained against *LP*, but the antimicrobial activity of *B. CeM6-7* strain against *ML* indicator were found to be in lees power (not much different), although they were significant different from *Miyagino* strain.

3.3.3. Evaluation of characteristic of the *bacteriocin* producing by *Bacillus subtilis*

Table 3.11 revealed that *B. CeM6-7* strains had powerful antimicrobial activities zone which presented for 375 AU/ml with against *SA*, but they were very higher activities zone than indicator against *PL* and *ML* and *LM*, which *B. CeM6-7* strain that was existed 275 AU/ml and 75 AU/ml and for *LM* indication, *B. CeM6-7* strain shown none bio-control activity, correspondingly. Meanwhile, in *SA* indication *B. CeM6-7* was most powerful to fight against indicator. Nonetheless, other remaining strains didn't show any action with indicator against *LM*. Eventually, in *ML* indicator, *B. CeM6-7* had a weak positive antimicrobial activity against the indicator strain comparing of the *Miyagino* strain (Not detected). Therefore, *B. CeM6-7* had its powerful antimicrobial activities against uniquely *SA* indicator strain at a very significant level.

Table 3.6: Evaluation of Characteristic of Isolated Bacteriocin Activity by Using Bacteriocin Bioassay by Arbitrary unit (AU)

Type of Bacillus strains	Incubator Temperature	Incubator Time	Diameter of the inhibition-zone for *LP*		Diameter of the inhibition-zone for *LM*		Diameter of the inhibition-zone for *SA*		Diameter of the inhibition-zone for *ML*	
			mm/ml	AU/ml	mm/ml	AU/ml	mm/ml	AU/ml	mm/ml	AU/ml
Miyagino	35^0C	Overnight	ND	ND	ND	ND	ND	ND	ND	ND
B.CeM6-7			11.00	275.00	0.00	0.00	15.00	375.00	3.00	75.00

Note: Not detected (ND)

On the other hand, the existed results also shown the complexity of microbiological flora in the products which was reflected wisely biological diversity of the natural environments used in the fermentation process, suggesting the potential value of traditional fermented foods as natural resources of bacteria and enzymes for industrial application. Likewise, Cambodian fermented soybean (*SIENG*) is similar to Thua nao in Thailand that make from fermented soybean. In fact, strains in the Thua nao (*Bacillus* strains) produced in northern Thailand exhibited complex microflora and molecular diversity [18].

3.4 Optimize the condition of the *bacteriocin* production

The optimization the condition of the *bactoriocin* production was conducted to be fully understand about the whole process of antimicrobial activity against four indicator strains (*LP*, *LM*, *SA* and *ML*) of all our five tested strains comparing with *Miyagino* strain after culturing in MRS broth with 1% agar purified powder contained against *LP* and *LM*; and TSA agar containing against *SA* and *ML* (dilution of 10^4 times). All five tested strains and *Miyagino* strain were diluted in three differences times (10^2, 10^4, and 10^6) and counted spore culture prior to start experiment.

3.4.1 Counting the colony of two *B. subtilis*

Table 3.7 indicated that there was non-significant different between the colony of spore culture of *B. CeM6-7* and *Miyagino* strain (Sig=0.263) in the three different timing of dilution (10^2, 10^4, and 10^6) on its *bacillus subtilis* growth.

Table 3.7 Comparison of average colony counts obtained from Petri films between 2 kinds of *Bacillus subtilis* using One Way ANOVA

Type of Bacillus strains	Parameters of *Bacillus subtilis*					
	Dilution of 10^2 times (log10CFU/ml)	Dilution of 10^4 times (log10CFU/ml)	Dilution of 10^6 times (log10CFU/ml)	n	$\bar{X}$	S±
Miyagino	5.556	5.292	5.230	3	5.359	0.173
B.CeM6-7	5.497	4.870	4.630	3	4.999	0.447
F-value					1.694	
Sig					0.263	

3.4.2 Production of *bacteriocin* at different incubation timing

Incubation time and temperature play a vital role in *bacteriocin* production. Therefore, the modification test was conducted using LB broth and incubation at 30ºC with different timing (0h, 16h, 20h and 23h) and diluted 10^4 times only. Within the expectation, *Bacillus subtilis* is capable to produce *bacteriocin* to fight against four indicator strains finally. The details are presented in figure 3.6.

The results were shown the antimicrobial production was begun at 16h incubation with *B. CeM6-7* strain that was able to fight against MRS broth contained *LP* indicator strain. The *bacteriocin* production of *B. CeM6-7* strains, were very active to kill all four indicator strains at 20h counted between 175 – 325 AU/ml. At 23h incubation, the antimicrobial production of tested strains had the most power to kill four indicator strains and its *bacteriocin* productive volume was listed respectively from strong to low, *B. CeM6-7,* and calculated between 163 – 275 AU/ml. Controlled strain, Miyagino, was found no action in both MRS broths and TSA agar containing *LP*, *LM*, *SA* and *ML* against indicator.

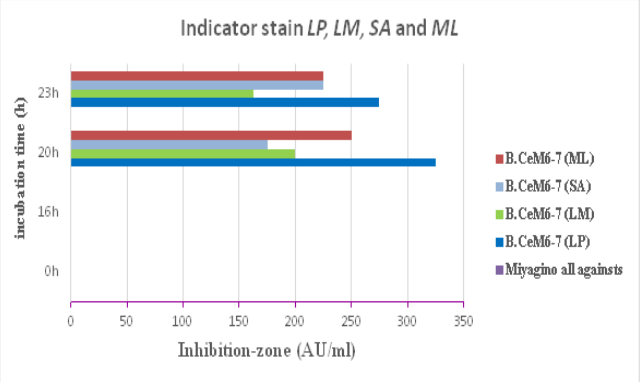

Figure 3.6: Production of bacteriocin at different incubation time for Bacillus CeM6-7 strain compare with Miyagino strains non-producing bacteriocin against different indicators

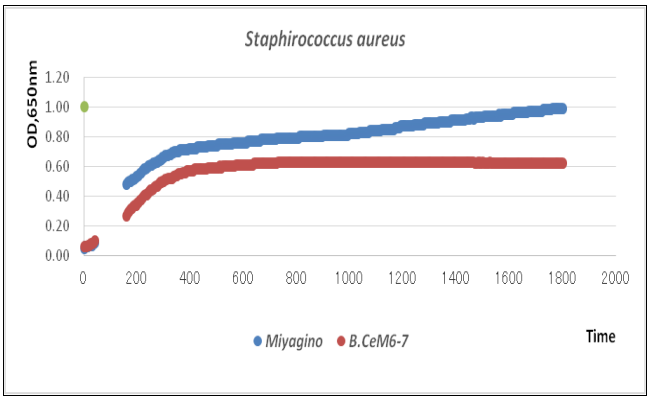

Figure 3.7: Effect of bacteriocin produced by B. CeM6-7 strain on the growth of S. aureus compare to Miyagino at different time in BHI broth at 35^{0}C for 30h

In similar finding to the prior test, at 30 ^{o}C, *B. CeM6-7* stain had highest ability to fight again *LP* indicator strain beginning from 20h incubation to 23h incubation and it was best to kill *LM* and *ML* indicator at 20h incubation as well. However, *B. CeM6-7* stain have similar a powerful to fight against *SA* and *ML* indicator strain at the incubated time (23h). At the meantime, *B. CeM6-7* stain produced the largest amount of *bateriocin* to be inactivating *SA* and *ML* indicator at 23h incubation as well. We therefore found that, the productivity of antimicrobial production of tested strains including *B. CeM6-7*, seem to be in parallel direction with incubation timing. It means that, the longer incubation time (in specific incubation timing) produce the higher *bacteriocin*.

3.5 Apply the *bacteriocin* to control the growth of Gram positive pathogenic or spoilage bacteria in foods

Applying bacteriocin to control the growth of Gram positive pathogenic or spoilage bacteria and *Lactic acid* group in foods was conducted against three indicator strains such as *S. aureus*, *Listeria* and *Lactococcus lactis subsp.lactis* (produced Nisin A) and *Lactic acid* group (*L. plantum, L. brevis, and Lb. lactis*) contained *B. CeM6-7* comparing with *Miyagino* strain after culturing in HBI contained against Gram positive; and MRS containing against *Lactic acid* group (O.D = 0.1, 650nm), and volume measurement culture prior to start experiment. Similarity searches with sequences in the bacteriocin produced by *Bacillus* subtilis strain *LFB112* from Chinese herbs, was effective against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases [8].

3.5.1 Gram positive group

In order to study the growth controlling of Gram positive pathogenic or spoilage bacteria in foods of antimicrobial compound of the two kind of *B. subtilis* group *B. CeM6-7* and Miyagino growth, the inhibitory activity present in cell-free samples taken at different time intervals was measured. Antibacterial activity could be detected at the mid-log growth phase and quickly extended a maximum at the early inactive phase, subsequently, the antagonistic activity declined.

A. Indicator strain *Staphylococcus aureus*

As shown in Figure 3.7, presented the control bacteriocin activity against *S.aureus* by *B.CeM6-7* (reference strains producing bacteriocin) and *Miyagino* after cultured in HBI broth contained against *S.aureus* (O.D=0.1, 650nm) at 35^{0}C for 30h. At 0h, B. CeM6-7 was calculated to be 0.06 O.D. higher than *Miyagino* (0.05 O. D). Until 160 min (2:40h) and 1800 min (30h), *B. CeM6-7* was calculated to be 0.27 and 0.62 O. D respectively, which were less than *Miyagino* that presented at 0.48 and 0.99 O. D correspondingly. In general, from 160mn (2:40h) to 1800mn (30h), the result shown that the stain *B. CeM6-7* produced the strongest antimicrobial activity against HBI broth contained *S.aureus* in compare with *Miyagino* strain.

B. Indicator strain *Listeria*

The control of antimicrobial activity against *Listeria* of two strains *B. subtilis* and *Miyagino* after cultured in BHI broth contained against *Listeria* O. D=0.1, 650nm) at 35^{0}C for 30h, were discussed in figure 3.8. The figure indicated that at 0 min (0h), both strains did not have antimicrobial activity and their O.D. was equally 0.03. Nevertheless, at 185min (3h), the strains *B. CeM6-7* was able to inactivate BHI broth contained against *Listeria and it* was developed to 0.13 O. D less than *Miyagino* strain at 0.15 O.D. At 1150 min (19h) and 1800 min (30h), the *B. CeM6-7* was calculated 0.07 O. D and 0.03 O. D respectively, which were less than *Miyagino* that presented for 0.09 to 0.05 O. D correspondingly. In short, from 160 min (2:40h) to 1800 min (30h), *B. CeM6-7* strain shown the most powerful antimicrobial action compare to Miyagino (Figure 3.8).

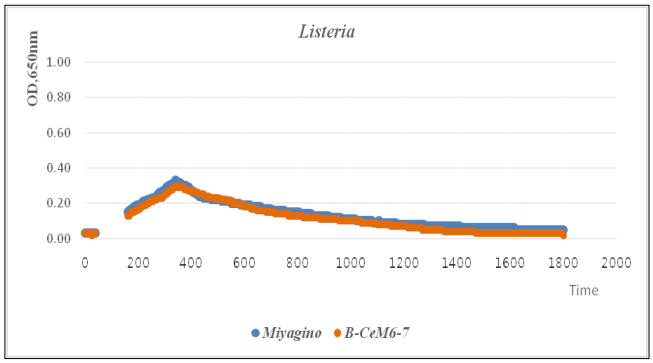

Figure 3.8: Effect of bacteriocin produced by B. CeM6-7 strain on the growth of Listeria compare with Miyagino at different time in BHI broth at 35^{0}C for 30h

C. Indicator Strain *Lactococcus lactis subsp.lactis* (produce Nisin A)

The control bacteriocin activity against *L. lactis subsp.lactis* (produce Nisin A) by *B.CeM6-7* and *Miyagino* after cultured in MRS broth contained against *Lactococcus lactis subsp.lactis* (produce M6-7 Nisin A), O.D=0.1, 650nm at 35^{0}C for 30h. The result shown that the stain *B. CeM6-7* produced the strongest antimicrobial activity against MRS broth contained *L. lactis subsp.lactis* (produce Nisin A) in compare with *Miyagino* strain

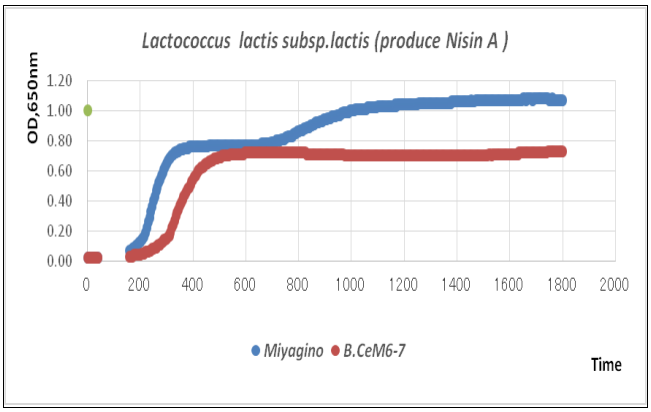

Figure 3.9: Effect of bacteriocin produced by B. CeM6-7 strain on the growth of L. lactis subsp.lactis (produce Nisin A) compare with Miyagino at different time in MRS broth at 35^{0}C for 30h.

In figure 3.9 show, at 0h, all strains were calculated to be 0.02 O. D equally. In 170 min (2:50h), *B. CeM6-7* was presented 0.03 O. D which was less than *Miyagino* that presented at 0.08 O.D. At 600min (10h) to 825min (14:15h) the bacteriocin of *B. CeM6-7* increased turbines (*Miyagino* was counted at 0.77-0.88 O. D and *B. CeM6-7* presented at 0.72-0.72 O.D). At 840 min (14h) to 1530 min (25:50h) the antibacterial activity produced by *B. CeM6-7* were 0.71 and 0.70 O. D respectively less than *Miyagino which counted for* 0.89 and 1.07 correspondingly. After that, at 1560 min (26h) to 1800min (30h) bacteriocin of *B. CeM6-7* increase turbines because of the OD of *Miyagino was stable at* 1.07 higher than *B. CeM6-7* counted for 0.71 and 0.73 O. D correspondingly (See also Table 3.14 in appendix). This was indicated that *B. CeM6-7* shown higher ability to fight against *L. lactis subsp.lactis* (produce Nisin A) compare with *Miyagino*.

In short, the *Bacillus CeM6-7* strain performed very well when treated with Gram positive group, especially for HBI broth contained against *S. aureus*, and was the most powerful to fight against MRS broth contained against *L. lactis subsp.lactis* (produce Nisin A), but the antimicrobial activity of *B. subtilis* strains against *Listeria* indicator were found to be no power to suppress, although it was significant different from *Miyagino* strain, which it was in parallel with existing researches stated that, some *Bacillus Subtilis* have reported to produce bacteriocins which suppress the growth of Gram positive spoilage and pathogenic bacteria [10 and 16].

3.5.2 Lactic acid groups

In this test, we used two variables of temperature 35^{0}C and incubator timing 25h, and the three kinds of antibacterial produced by *B. subtilis CeM6-7* of the tested strains comparing with *Miyagino* strain after culturing in MRS containing against *Lactic acid* group (*L.plantum, L.brevis, and Lb.lactis* with O.D = 0.1, 650nm), bacteria was used as the indicator strain. However, the test was conducted to measure pH value of each indicators of bacteriocin produced by *B. subtilis* strains culture prior to start experiment and the effects of heat, pH, nutritional composition and incubation time at 35^{0}C and the antibacterial substances were stable within OD, 650nm and wide range pH value from 3.858 to 3.923 as shown by the isolates strain against on inhibitory by the bacteriocin producing form *B.CeM6-7* strains was compared with *Miyagino* strain to be using control strain in cell-free samples taken at different time intervals was measured. Antibacterial activity could be detected at the mid-log growth phase and quickly extended a maximum at the early inactive phase, subsequently, the antagonistic activity declined.

A. Indicator Strain *L.plantum*

In generally, the antibacterial produced by *B. CeM6-7* strains agents were stable within wide range of pH values from 3.923 to 4.058 (Table 3.15, Appendix). The effects of heat, pH, nutritional composition and incubation time at 35^{0}C and the antibacterial substances were stable within OD, 650nm of *B. CeM6-7*. There were exceptions in the case of the *Miyagino* strain. This strain did not inhibit *L.plantum* at a temperature of 35°C, 25h and it was using as control strain.

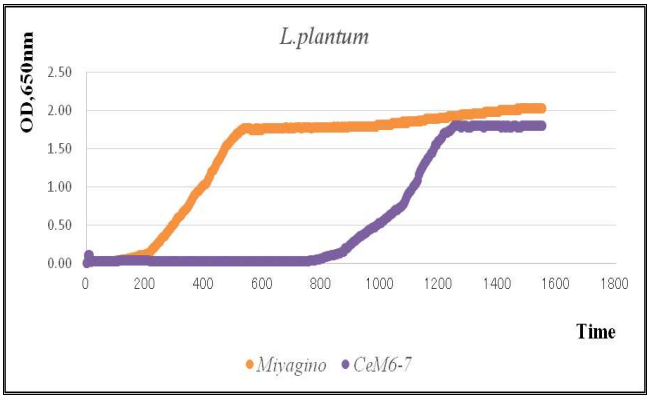

Figure 3.10: Effect of bacteriocin produced by B. CeM6-7 strain on the growth of L. plantum compare with Miyagino at different time in MRS broth at 35^{0}C for 25h

In the Figure 3.5, show that the control of antibacterial activity against *L.plantum of B.CeM6-7* and *Miyagino* strains after culturing in MRS broth contained against *L.plantum* were presented. It was indicated that, at different timing (0h to 25h), *B. CeM6-7* had enough ability to fight against MRS broth contained *L.plantum* compare to strain Miyagino. *B. CeM6-7* were found to be the most powerful antimicrobial activity at 800 to 860min (12h to 13h). At 0h, all strains were counted to be 0 O.D equally. At 5min to 800min (12h), the *B. CeM6-7* was calculated to be 0.02 and 0.06 O.D. Subsequently, 800min (12h) to 1500 min (25h), the *B. CeM6-7* was calculated to be 0.06 to 1.80 O. D, which in comparison were less than *Miyagino* from 300min (5h) to 1500min (25h) which presented at 0.53 to 2.02 O.D. At

1500min (25h), the result has shown so much different turbines of *B. CeM6-7* from starting point.

B. Indicator Strain *L.brevis*

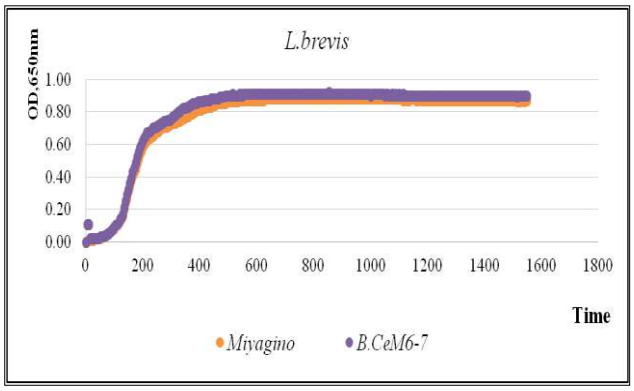

Figure 3.11: *Effect of bacteriocin produced by B. CeM6-7 strain on the growth of L.brevis compare between Miyagino at different time in MRS broth at 35^{0}C for 25h.*

In commonly, the bacteriocin produced by *Bacillus strains* against were stable within wide range of pH values from 3.923 to 4.058, and non-significant different between the pH values. The effects of heat, pH, nutritional composition and incubation time at 35^{0}C and the antibacterial substances were stable within OD, 650nm (0), the *B. CeM6-7* against. There were exceptions in the case of the *Miyagino* strain. This strain did not inhibit *L.brevis* at a temperature of 35°C, 25h and it was using as control strain.

In figure 3.11 indicated that at 0 min (0h) to 60min (1h) all of strains, did not had antimicrobial activity. Nevertheless, at 60min (1h) to 800 min (13:33h), *B. CeM6-7* cultured growing between 0.03 to 0.91 O.D. higher than *Miyagino* 0.03 to 0.88 O.D. After that, *CeM6-7*strain was powerless in antibacterial against *L.brevis* at 800min (13:33h) to 1500min (25h) the antimicrobial activity was almost the same with *Miyagino* (0.91- 0.90 O.D.) compare to (0.87-0.90 O.D). Consequently, *B. CeM6-7* did not inhibit *L.brevis* at a temperature of 35°C, 25h.

C. Indicator Strain *Lb.lactis*

In normally, the bacteriocin produced by three *Bacillus strains* agents were stable within wide range of pH values from 4.05 to 4.17, and nonsignificant different between the pH values. The effects of heat, pH, nutritional composition and incubation time at 35^{0}C and the antibacterial substances were stable within OD, 650nm (0), *B. CeM6-7* against. There were exceptions in the case of the *Miyagino* strain. This strain did not inhibit *Lb.lactis* at a temperature of 35°C, 25h and it was using as control strain.

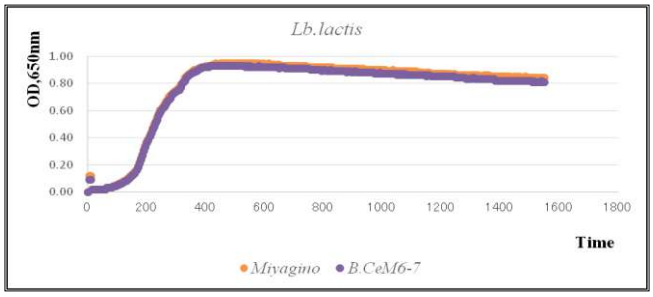

Figure 3.12: Effect of bacteriocin produced by B. CeM6-7 strain on the growth of Lb.lactis compare with Miyagino strains at different time in MRS broth at 35^{0}C for 25h

The control of antimicrobial activity against *Lb.lactis* of three strains *B. subtilis* and *Miyagino* after cultured in MRS broth contained against *Lb.lactis* (O.D = 0.1) were discussed in figure 3.12. The figure indicated that at 0h all of strains were started at 0.00 O. D, and at 180min (3h) they both were almost had equal O.D (*CeM6-7*, 0.24 O. D) and (Miyagino, 0.25 O. D). Nevertheless, at 600min (10h) *B. CeM6-7* strains was counted to be 0.92 O. D and be able to inactivate MRS broth contained against *Lb.lactis, compare* with *Miyagino* (0.94 O.D). From 720min (12h) to 1500min (25h), *B. CeM6-7* strain (0.91 – 0.82 O. D) shown the most powerful antimicrobial action compare to *Miyagino* strains. Therefore, *B. CeM6-7* strain had higher antibacterial action against *Lb.lactis* from 12h to 25h, at 35^{0}C unlike *Miyagino*.

In short, the *B. CeM6-7* strain performed very well when treated with *Lb.lactis*, especially for MRS broth contained against *Lb.lactis*, and were the most powerful to fight against MRS broth contained against *L.planum*. However, the antimicrobial activity of *Bacillus subtilis* strains against *L.brevis* indicator was impossible, but it was somewhat higher antimicrobial activity compare to *Miyagino* to fight against *Lb.lactis*, though the action is limited. This result was aligned with existing researches stated that, the effect of different pH on the antimicrobial potentials of the *Lactobacillus* strains indicates highest inhibitory activities between pH5.5 to pH7.5 and the survival rates of the pathogenic indicator bacteria in the fermented food sources were between 8 and 14 days [1].

Applying bacteriocin to control the growth of Gram positive pathogenic or spoilage bacteria of Cambodian strain *CeM6-7* indicated that Cambodian strain has similar strength with *Miyagino* strains (considered to be the strongest one of Gram positive and Lactic acid groups) since it actively produced bateriocin fighting against all three indicator strains of Gram positive and Lactic acid groups from 0h - 30h and 0-25h incubation times. Thus, this property of bacteriocins produced by *B. CeM6-7* strain can be used as additive in food processing industries to avoid food spoilage even in 35^{0}C temperatures and longer time. Many researches on *Bacillus* bacteriocins are becoming more intensive and important due to their inhibition activity, which may include Gram-negative bacteria in addition to Gram-positive species, some of which are known to be pathogenic to humans and animal [5].

4. CONCLUSION

In this work, we demonstrated the diversity of *B. subtilis* in *SIENG*, a Cambodian's traditional fermented soybean food. 119 samples of *Bacillus* strains were isolated and there were found only 15 of *Bacillus* strains that have ability to fight against the indicator microorganisms *Lactobacillus plantum ATCC 8014* by agar well diffusion assay. Furthermore, among 15 samples, only one stain (*Bacillus CeM6-7*) has shown a great active zone against *Lactobacillus plantum ATCC 8014*.

Additional finding, *Bacillus CeM6-7* strain could tolerate with heat up to 20h at 30^{0}C temperature and 16h at 37^{0}C. Besides, bacteriocin produced by *Bacillus CeM6-7* strain has more ability to suppress all the indicators starts

from 20 hrs compare with *Miyagino* stains. Further, it has strength compare with *Miyagino* originated in Japan, but it has a special unique ability to fight against indicator strain *SA* at a very significant level.

Moreover, *Bacillus CeM6-7* strain performed very well when treated with Gram positive group, against *S. aureus* and Lactic acid groups, against *Lactobacillus plantum* at a very extensive level. *Bacillus CeM6-7* strain has higher strength unlike Japanese strains (*Miyagino*), since it actively produced bateriocin fighting against all three indicator strains of Gram positive and Lactic acid groups from 0h - 30h and 0h-25h incubation times.

So, *Bacillus CeM6-7* strain can be used as a biological control agent in fermented soybean products to exclude growth of pathogenic or spoilage bacteria during manufacturing without inhibiting the fermentation of *Bacillus spp*. and may have further uses in the food, agricultural, and pharmaceutical industries.

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BEHAVIOR OF MIXED-CULTURE BIOFILM OF *LISTERIA MONOCYTOGENES, SALMONELLA ENTERICA* AND *PSEUDOMONAS AERUGINOSA* IN A SIMULATED FOOD PROCESSING ENVIRONMENT

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Abstract

The behavior of mixed cultures of bacteria forming biofilm on three types of food contact surfaces in a simulated food processing (SFP) environment was investigated. The model for biofilm formation was investigated on coupons packed in a stainless steel (SS) biofilm reactor. Medium was released and refreshed daily to allow growth for 8 h; after rinsing, coupons were dried for about 15 h to simulate the processing conditions in a food plant. Three types of coupon materials (SS, Teflon and rubber), three incubation temperatures (4, 18 and 30 °C) and three concentrations of limited-nutrient medium, tryptic soy broth (TSB) (0.03, 0.3 and 3.0 g/L), were examined. In the mixed culture, *Listeria monocytogenes* was the dominant species and formed biofilm faster than the other two cultures, while *Pseudomonas aeruginosa* was found to be a comparatively weak competitor. At 4 °C, only *Listeria* could survive, attach and grow on SS and rubber coupons at a nutrient content of 0.3 and 3.0 g/L TSB. At 18 or 30 °C, the behaviors of *Listeria* and *Salmonella* adhered to SS, Teflon and rubber coupons were similar for the same material and at the same nutrient level. At 18 and 30 °C, strong biofilm of *Listeria*, *Salmonella* and *Pseudomonas* (approximately 7.0, 6.5 and 6.3 log CFU/cm^2, respectively) was formed in all tested nutrient-limitation conditions. However, adhesion of *Pseudomonas* on coupons at 18 and 30 °C did not depend on the amount of nutrient, except at level of TSB 0.03 g/L. These findings may contribute to a better understanding of the effects of different environmental conditions on mixed-species biofilm of *Listeria*, *Salmonella* and *Pseudomonas* adhering to contact surfaces of food processing equipment, and thus may provide valuable information in order to predict and control biofilm formation in a food processing plant.

Keywords: Biofilm; *Listeria monocytogenes*; *Pseudomonas aeruginosa*; Rubber; *Salmonella enterica*; Stainless steel; Teflon

1. Introduction

Improper cleaning leads to microbial cells and soil remaining on food contact surfaces of food processing facilities. Bacterial cells have the ability to adhere to a surface, then multiply, even with a small amount of nutrient, and form biofilms where microbial cells will be enclosed in extracellular polymeric substances (EPS). EPS has been reported to enhance resistance to cleaning and sanitation (Joseph et al., 2001; Pan et al., 2006; Sauer et al., 2007). Therefore, the biofilm could become a persistent source of contamination in food products. Biofilms not only present a considerable hygiene risk in the food industry, but also cause economic losses (Pan et al., 2006). Bacterial contamination may lead to food spoilage, reduced shelf life of products, or transmission of diseases (Bonaventura et al., 2008).

Listeria and *Salmonella* are well-known foodborne pathogens which have the potential to cause harm to health (Black, 1984; Chmielewski & Frank, 2003). Both bacterial pathogens have been found in many food processing environments, including meat, dairy, poultry and seafood facilities (Bagge-Ravn et al., 2003; Guðbjörnsdóttir et al., 2004; Gunduz & Tuncel, 2006; Jessen & Lammert, 2003). These bacteria have also contaminated many types of food products due to unsanitary conditions in food plants (Guðbjörnsdóttir et al., 2004; Kanarat et al., 2011; Norhana et al., 2010). Contamination, particularly in ready-to-eat foods, has caused many outbreaks, since the product may not be heated again before consumption (Black, 1984; Guðbjörnsdóttir et al., 2004; Kanarat et al., 2011; Kuda et al., 2011; Norhana et al., 2010).

Pseudomonas spp. are ubiquitous gram-negative environmental bacteria (Guaglianone et al., 2010) often associated with the spoilage of perishable foods (Marchand et al., 2012; Theel et al., 2011). A large proportion of *Pseudomonas* spp. have been found on food and non-food contact surfaces in meat, dairy, fish and shrimp processing plants (Guðbjörnsdóttir et al., 2005; Sparbier et al., 2012). In addition, *Pseudomonas* spp., especially *P. aeruginosa*, which

has been studied extensively, possess a dynamic process of biofilm development. Commonly, the development begins with attachment, then micro-colony and macro-colony formation, and finally the dispersal stage (Fuster-Valls et al., 2008; Guaglianone et al., 2010; Lehane & Olley, 2000; Patange et al., 2005; Poimenidou et al., 2009; Poli, 2004; Rochex & Lebeault, 2007). The dispersed cells cause heavy loads of bacteria and may compromise further heat treatment during food processing.

In a food processing environment, food spoilage and pathogenic microorganisms can form multicellular surface-associated complex communities (a so-called "slime city") as prevalent lifestyles in nature (Dallal et al., 2010; Guaglianone et al., 2010; Poli, 2004). Mixed species or mixed cultures could equally or competitively adhere and then grow simultaneously on food and equipment surfaces in food processing environments to form biofilms (Bower et al., 1996; Kumar & Anand, 1998; Pan et al., 2006; Theel et al., 2011; Thévenot et al., 2006). *Pseudomonas* spp. have been documented as good producers of EPS (Bae et al., 2012; Fuster-Valls et al., 2008; Guaglianone et al., 2010; Lehane & Olley, 2000; Rochex & Lebeault, 2007; Ryu & Beuchat, 2005). *Pseudomonas* spp. have the ability to facilitate the attachment of different pathogenic microorganisms, including *Listeria monocytogenes* and *Salmonella* spp. (Guaglianone et al., 2010; Theel et al., 2011; Vlková et al., 2008). Many reports have indicated that these three species are strongly capable of adhering and forming biofilms on different surfaces (Auawithoothij & Noomhorm, 2012; Baylis et al., 2011; Jørgensen & Huss, 1989; Kanki et al., 2004; Pratheepa & Vasconcelos, 2013; Rodríguez-Sánchez et al., 2014).

Understanding biofilm behavior in food processing environments will facilitate the development of methods for controlling and preventing bacterial biofilms; this may decrease the risk of food contamination caused by foodborne spoilage and pathogenic bacteria. There are many factors involved in the attachment and detachment of bacterial cells, including the medium in which they are grown, motility, growth phase of the cells, type and properties of material, presence of organic material (or food residue), temperature, pH, length of contact time, production of extracellular polysaccharides, and cell-to-cell communication (Black, 1984; Chmielewski & Frank, 2003; Gram & Huss, 1996; Guaglianone et al., 2010; Pratheepa & Vasconcelos, 2013; Rodríguez-Sánchez et al., 2014; Simões et al., 2010). Numerous research studies on biofilms have investigated the effects of nutrient limitation and growth temperature on biofilm formation in a laboratory; but those study conditions are unlikely to occur in an actual food processing facility (Chmielewski & Frank, 2003). Due to the lack of a model that closely simulates the environment in a food processing facility, the behavior of bacterial biofilm formation under such conditions is not well understood.

Therefore, this study investigated the biofilm formation of a mixed culture of *Pseudomonas aeruginosa*, *Listeria monocytogenes* (3 strains) and *Salmonella enterica* (5 serovars) in a simulated food processing (SFP) environment. The continuous 8 h working conditions in a typical food processing facility, followed by cleaning and then drying overnight for 12–15 h, was simulated under the effects of three different temperatures, nutrient concentrations, and types of food contact materials. The results of this study may contribute to controlling mixed-culture biofilm formation by undesirable bacteria in a food processing environment.

2. Materials and methods

2.1. Bacterial cultures and stock preparation

Pseudomonas aeruginosa (1 strain); *Listeria monocytogenes* (3 strains: 101, 108 and 310) and 5 serovars of *Salmonella enterica* (*S.* Aberdeen, *S.* Poona, *S.* Salamae, *S.* Enteritidis and *S.* Typhimurium) were obtained from the Department of Food Science and Technology, Kasetsart University, Bangkok, Thailand.

All bacterial species were cultured (37 °C, 24 h) and subcultured (37 °C, 18 h) individually in 10 mL tryptic soy broth (TSB; Difco™, BD Diagnostics, Sparks, MD, USA). Cells of the individual cultures were then harvested by centrifugation (10000 × *g* at 4 °C for 10 min; centrifugation tube 15 mL), followed by washing twice in 1 mL phosphate buffered saline (PBS; pH 7.4). Washed cell pellets of each strain or serovar were resuspended in 1 mL TSB. A combination of each culture was done for 3 strains of *Listeria monocytogenes* and 5 serovars of *Salmonella enterica*. *P. aeruginosa*, *L. monocytogenes* and *S. enterica* were combined in a ratio of 80:10:10 to obtain a mixed-culture stock, which was maintained in TSB and 18% glycerol at –20 °C (Stewart & Costerton, 2001). The bacterial population of the mixed-culture stock was enumerated on tryptic soy agar (TSA) after 24 h at 37 °C, obtaining an initial load of about 10^9 CFU/mL.

2.2. Mixed-species biofilm formation in a SFP ecosystem

A CDC (Centers for Disease Control and Prevention) biofilm reactor (BioSurface Technologies, Bozeman, MT, USA), modified from Hadi et al. (2010), was designed to create SFP conditions.

The bioreactor was made of stainless steel (type 304, 2B finish) and included two main components, namely a bioreactor and a frame for holding 48 coupons. Each set of 48 coupons was exposed to a particular temperature/nutrient condition. The volume of medium in the bioreactor was 4000 mL. Individual bioreactors were used to simulate the various temperature and nutrient conditions.

Three types of surfaces were tested. Before conducting the experiment, stainless steel (SS) coupons (type 304, 2B finish), Teflon® coupons and rubber coupons (2 × 5 × 0.08 cm) were soaked overnight in commercial detergent solution, degreased with 70% ethanol, and then thoroughly rinsed with distilled water to detach any soil and dust on the surfaces (Hoa et al., 2015a). The clean and dry coupons were placed in individual gaps in the coupon-holding frame. SS, Teflon and rubber coupons were sequentially placed vertically in the gaps in the frame, which was then inserted into the bioreactor. Bioreactors were autoclaved at 121 °C for 15 min prior to use (Pan, 2005; Stewart & Costerton, 2001).

To allow biofilm formation, 2000 mL of sterile medium with different TSB concentrations (0.03, 0.3 and 3.0 g/L) was aseptically poured into the bioreactors until all coupons were completely submerged. One mL of mixed-culture stock was inoculated in the remaining 2000 mL of medium, and then slowly poured into each bioreactor to obtain initial equal bacterial populations of about 10^6 CFU/mL.

Under the SFP system, the coupons were subjected to a 24 h cycle over 30 days. The 24 h cycle consisted of three sequential steps: (i) incubation in growth medium for 8 h; (ii) release of the growth medium and rinsing with 200 mL sterilized distilled water for 30 min; and (iii) storage without liquid media (starvation) for 15 h 30 min. The bioreactors were incubated in different incubators at 30 °C, 18 °C and 4 °C. Duplicate coupon samples were taken at selected intervals for each bioreactor for measurement of adherent cells in biofilms. *Listeria*, *Salmonella* and *Pseudomonas* counts were performed using different selective media: PALCAM agar

(Merck) for *Listeria*; xylose lysine deoxycholate (XLD) agar (Difco) for *Salmonella*; and Cetrimide agar (Difco) for *Pseudomonas*.

2.3. Cell enumeration

Biofilm cells were enumerated at sampling intervals of 1, 3, 5, 7, 10, 15, 20 and 30 days after inoculation. At each sampling interval, coupons were aseptically withdrawn from the reactor using sterile forceps and placed separately in a sterile glass Petri dish. Coupons were rinsed twice in 10 mL of distilled water with gentle agitation for 10 s to remove non-attached bacteria (Rochex & Lebeault, 2007); then each coupon was placed in a fresh sterile glass Petri dish.

Cells attached to each sample coupon were detached by swab method. An area (2 × 5 cm) on both sides of each coupon was carefully swabbed. Prior to swabbing, a pair of cotton swabs was soaked in a capped glass tube containing 10 mL of 0.1% peptone–saline solution (Pan, 2005). Each coupon was swabbed thoroughly and sequentially by a pair of cotton swabs to detach as many cells as possible from the surfaces. The heads of the cotton swabs were broken off aseptically and put back into the tube previously used to soak the swabs. The bacteria present on swabs were re-suspended by vortexing for 60 s at high speed (speed 8) (Vortex-Genie® 2 G-560E; Scientific Industries, Bohemia, NY, USA) (Hoa et al., 2015b).

After vortexing, a cell suspension of each sample was prepared by serial decimal dilution and then spiral plated on PALCAM, Cetrimide or XLD agar (Autoplate® 4000 spiral plater; Spiral Biotech, Norwood, MA, USA). Plates were incubated at 37 °C for 24 h. Bacteria were then counted by a colony-counting machine (Quebec® Darkfield Colony Counter; Reichert, Buffalo, NY, USA). Results were expressed as log CFU/cm^2.

2.4. Scanning electronic microscopy analysis

Microbes adhering to specimens (5 × 5 mm) were rinsed with 0.1 M sterilized PBS (pH 7.4) at 4 °C and pre-fixed in 2% glutaraldehyde with 0.1 M PBS for at least 2 h at 4 °C. Specimens were then removed from the fixing solution, washed two times with PBS (10 min, 4 °C), post-fixed in 2% osmium tetraoxide with 0.2 M PBS, and washed in 0.1 M PBS. Next, samples were dehydrated with a series of ethanol concentrations (50, 70, 80, 90, 95 and 100%), two times for 10 min for every ethanol concentration, and then subjected to freeze drying. Finally, samples were coated with gold and observed using a scanning electron microscope (JSM-6610LV; JEOL, Tokyo, Japan) at 5000× magnification.

2.5. Data analysis

Mean values and standard deviations were determined from the results of four samples. With respect to calculation of means and standard deviations, a value of 0.69 was assigned to sample outcomes below the lower limit of detection (<10 CFU/mL) of the spread method. Data analysis was carried out using SPSS version 18 software. Analysis of variance (ANOVA), expressed at a 95% confidence level, was used to determine significant differences between the means for types of surfaces, bacterial cultures, nutrient concentrations and incubation temperatures.

3. Results and discussion

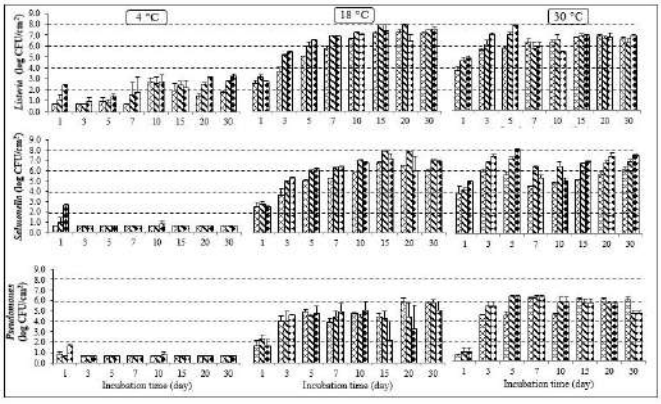
3.1 Effects of bacterial characteristics and composition in mixed-culture biofilm on bacterial growth and biofilm formation

The attachment to surfaces and subsequent biofilm formation is influenced not only by surface characteristics but also by the bacterial cell properties (Giaouris et al., 2014). The bacterial population growth under different limited nutrient contents (TSB 0.03, 0.3 and 3.0 g/L), temperatures (4, 18 and 30 °C) and incubation times (intervals over a 30-day period) on three types of material (stainless steel, Teflon and rubber) in mixed-culture biofilm of *L. monocytogenes*, *S. enterica* and *P. aeruginosa* is shown in Figs. 1–3. Our results showed that the numbers of adherent *L. monocytogenes*, *S. enterica* and *P. aeruginosa* in mixed-culture biofilm on the same material (i.e. SS, Teflon or rubber) were not similar. Among the three tested cultures, the presence of *P. aeruginosa* was the lowest and *L. monocytogenes* was the highest in mixed-culture biofilm for all testing conditions. In fact, during 30-day experimental set-up, the highest of *P. aeruginosa* adherence reached only about 7.0 log CFU/cm^2, meanwhile *Listeria* spp. and *Salmonella* spp. populations were approximately 8.0 log CFU/cm^2. In addition, after 1-day incubation, the highest *Pseudomonas* cells attachment was above 2.2 log CFU/cm^2 to rubber and stainless steel, whereas above 5.0 log CFU/cm^2 of *Listeria* spp. and *Salmonella* spp. in TSB 3.0 g/L. Moreover, at the same period, the lowest *Listeria* spp. and *Salmonella* spp. adherence to Teflon were approximately 2.0 log CFU/cm^2; there was no *P. aeruginosa*, otherwise, presented to both Teflon and stainless steel in TSB 0.03 g/L. Particularly, in mixed stock used to conduct this experiment, *P. aeruginosa* occupied in 80% amount of suspension, while *Listeria* spp. and *Salmonella* spp. were only in 10%, respectively. There were some explanations for this phenomenon that associated with bacterial characteristics such as cell surface hydrophobicity, motility, nutrient competition, generation time of each species (Gram and Huss, 1996; Bagge-Ravna et al., 2003; Chmielewski and Frank, 2003).

First, regarding to the competitive interactions among cultures in a mixed-cultures biofilm may often occur by competing for limited nutrient sources or by production of antimicrobial compounds (e.g. bacteriocins) which inhibit the growth of other species (Giaouris et al., 2014). The quantity of each bacterium in multi-species biofilm is affected by the simultaneous co-culture phenomenon. Normally, in a mixed-species biofilm the attached cell population of each culture is lower than in a monoculture (Giaouris et al., 2013; Jeong & Frank, 1994a, 1994b) due to the effects of competition for nutrients (specific or non-specific Jameson effect) (Aase et al., 2000; Ammor et al., 2004; Fatemi & Frank, 1999). For example, fewer adhesive cells were found in mixed *L. monocytogenes* and *P. putida* biofilm compared with a monoculture *L. monocytogenes* biofilm (Chmielewski & Frank, 2003). However, this phenomenon could also depend on the bacterial species co-cultured in a biofilm. For example, Teh et al. (2014) reported that single-species biofilms of either *Klebsiella pneumonia* or *P. aeruginosa* were thinner than the corresponding mixed-species biofilms; while Giaouris et al. (2014) reported that *L. monocytogenes* may increase its adherence and biofilm formation in the case of surfaces already colonized by other bacteria. In our study, the results showed that the biofilm population of *P. aeruginosa* cells was lowest for all testing conditions compared with the biofilm population of *Listeria* and *Salmonella*. In other words, *Listeria* was the dominant species in mixed-culture biofilm. These results were consistent with the findings reported by other researchers (Giaouris et al., 2013; Jeong & Frank, 1994a, 1994b). In a study of co-culture of *L. monocytogenes* and *P. putida* to develop a dual-species biofilm community on SS, the results indicated that *L. monocytogenes* accounted for 91.6% of the total biofilm cells after 1 day of incubation. Similarly, the results of other studies on co-culture of *L.*

monocytogenes and *Pseudomonas* spp. (M21, isolated from a meat plant) at 10 and 21 °C showed that the population of *Listeria* reached a level of 6.0 and 6.5 log CFU/cm^2 after 21 days and 8 days of incubation, respectively (Jeong & Frank, 1994a, 1994b). This result may be due to the increasing hydrophobicity of *L. monocytogenes* after freezing at –20 °C (Slama et al., 2012; Teh et al., 2014). In the present experiment, freezing conditions were used to store the mixed-culture stock; hence, this may increase the ability of *Listeria* to adhere to various surfaces. Besides, according to Churchill et al. (2006), *L. monocytogenes* may produce listeriolysin O (LLO) toxin that permeates the surrounding environment, leading to inhibition of the growth of other cultures in a mixed-culture biofilm. In addition, Simões et al. (2007) reported that the dominant species in a competitive culture biofilm is the species with the highest growth rate. Overall, based on its rapid growth (Zameer et al., 2009), quick attachment (Takahashi et al., 2010) and production of biocin, *Listeria* can become the dominant species in a mixed-culture biofilm.

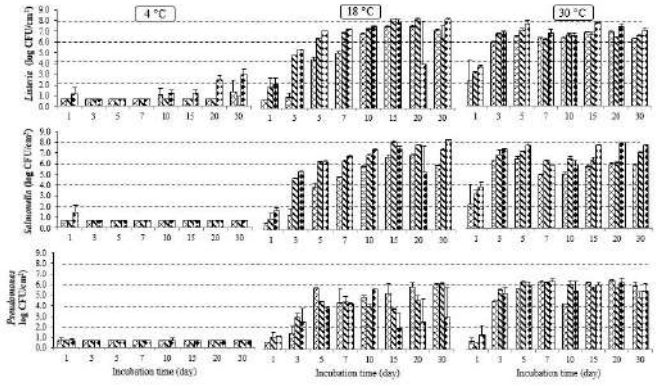
In addition, microbial cell surface charge and hydrophobicity play an important role in the initial steps of microbial adhesion (Bonaventura et al., 2008). For *L. monocytogenes*, the presence of particular surface appendages such as flagella and fimbriae also affected the adherence capacity of cells (Van Houdt, 2010; Yu et al., 2011). Flagellum-mediated motility is required for initial cell attachment during biofilm formation, which helps bacteria overcome any repulsive interfacial forces (Bonaventura et al., 2008; Langsrud et al., 2003; O'Toole & Kolter, 1998; Theel et al., 2011). Several studies have indicated that the motility of *L. monocytogenes* is correlated with biofilm formation (Takahashi et al., 2010; Van Houdt, 2010). Moreover, the hydrophobicity level of *Listeria* was found to increase with increasing temperature (Bonaventura et al., 2008; Takahashi et al., 2010). These phenomena could explain how *L. monocytogenes* is able to quickly attach and build up a biofilm. Thus, our results indicated that *L. monocytogenes* adhered approximately 5.0 log CFU/cm^2 on rubber and stainless steel after 1-day incubation. The maximum adherence of *L. monocytogenes* on rubber, stainless steel and Teflon happened on day 5 approximately 8.3, 8.1 and 7.9 log CFU/cm^2 in TSB 3.0 g/L, respectively.



[Fig. 1]

Fig. 1. Population of *Listeria*., *Salmonella* and *Pseudomonas* (log CFU/cm^2) of mixed species biofilms of *Pseudomonas* spp., *Listeria* spp., and *Salmonella* spp. on stainless steel in different temperature (4; 18 and 30°C) and different TSB concentrations (TSB 0.03 (▨) ;0.3(▧) and 3.0 (▩) g/L) for 30 days sampling intervals. The data expresses as a mean values of two independent tests in duplicate. Value of 0.69 was assigned to sample outcomes that below the lower limit of detection (10 CFU/mL # 100 CFU/1 coupon = 20 cm^2, or 5 CFU/cm^2) of the spread method.

The adherence of *Salmonella* spp. on materials may be associated with thin aggregative fimbriae (agf) and cellulose; both are involved in adhesion to surfaces, cell aggregation, environmental persistence, and biofilm development (Atanassova et al., 2013; Hjelm et al., 2002; Jørgensen & Huss, 1989; Khot & Fisher, 2012). The co-expression of thin agf and cellulose leads to the formation of a highly hydrophobic network with tightly packed cells aligned in parallel in a rigid matrix. Moreover, according to the results of previous studies, in nutrient-limited media *Salmonella* Typhimurium expressed the maximum agfD promoter to produce agf at 28 °C (Hjelm et al., 2002; Jørgensen & Huss, 1989; Rodríguez-Sánchez et al., 2014). Therefore, *Salmonella* spp., especially the *S.* Typhimurium strain which was one of five *Salmonella* strains included in our research, could quickly attach and build multicellular biofilm (Hjelm et al., 2002) on tested materials. In fact, our data showed that the highest overall level of adherent *Salmonella enterica* cells was reached at day 15 on SS coupons at 18 °C: approximately 6.7, 7.8 and 7.1 log CFU/cm^2 in TSB 0.03, 0.3 and 3.0 g/L, respectively.

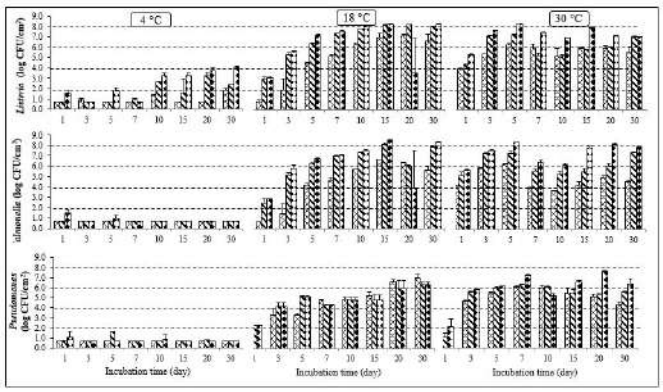


[Fig. 2]

Fig. 2. Population of *Listeria*., *Salmonella* and *Pseudomonas* (log CFU/cm^2) of mixed species biofilms of *Pseudomonas* spp., *Listeria* spp., and *Salmonella* spp. on Teflon in different temperature (4; 18 and 30°C) and different TSB concentrations (TSB 0.03 (▨) ;0.3(▧) and 3.0 (▩) g/L) for 30 days sampling intervals. The data expresses as a mean values of two independent tests in duplicate. Value of 0.69 was assigned to sample outcomes that below the lower limit of detection (10 CFU/mL # 100 CFU/1 coupon = 20 cm^2, or 5 CFU/cm^2) of the spread method.

In terms of *Pseudomonas*, many authors assumed that *Pseudomonas* spp., especially *P.aeruginosa* has play important role in biofilm formation (Bagge-Ravna et al., 2003; Chmielewski and Frank, 2003; Poli, 2004; Guaglianone et al., 2010; Jong et al., 2013). *Pseudomonas aeruginosa* is a metabolically versatile Gram-negative environmental bacterium (Poli, 2004; Guaglianone et al., 2010). The mechanism of initial attachment of *P. aeruginosa* cells to surface was related with cell surface appendages, type IV pili and flagella, (Poli, 2004; Guaglianone et al., 2010). Type IV pilus of P. *aeruginosa* has two components such as Pel (glucose rich Pel polysaccharide) and Psl. (mannose rich Psl polysaccharide). Releasing of Pel and Psl polysaccharides from *Pseudomonas* spp. may help quickly and firmly attachment of *Listeria* spp. and *Salmonella* spp. on the surface of materials. However, after initial attachment, *Pseudomonas* spp. became non-motile in subpopulations. Therefore, the ability adherence of *P. aeruginosa* on coupon should be decreased. In fact, a comparison with *Listeria* spp. and *Salmonella* spp. in mixed culture biofilm, population of *P.*

aeruginosa was lowest in all of testing conditions. It was agreement by Chmielewski and Frank (2003) and Fatemi and Frank (1999), who studied a mixed biofilm formed by *Listeria monocytogenes* and *Pseudomonas putida*. This result was explained by Giaouris et al. (2014) *L. monocytogenes* does not have a high potential for forming mono-species biofilms *in vitro* on food contact materials at relevant food industry temperatures, but surfaces already colonized by other bacteria, such as *Pseudomonas* spp., may increase its adherence and biofilm formation.

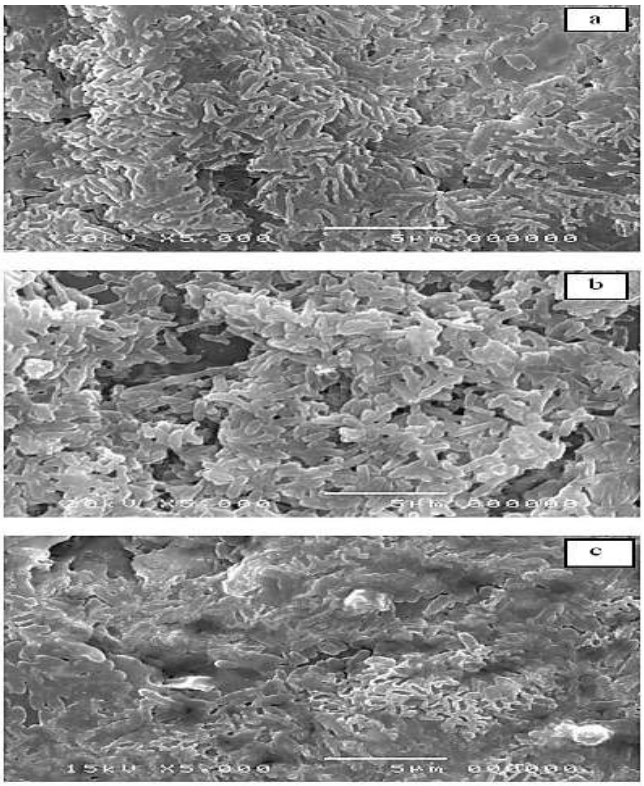


[Fig. 3]

Fig. 3. Population of *Listeria*., *Salmonella* and *Pseudomonas* (log CFU/cm^2) of mixed species biofilms of *Pseudomonas* spp., *Listeria* spp., and *Salmonella* spp. on rubber in different temperature (4; 18 and 30°C) and different TSB concentrations (TSB 0.03 (▨) ;0.3(▧) and 3.0 (▩) g/L) for 30 days sampling intervals. The data expresses as a mean values of two independent tests in duplicate. Value of 0.69 was assigned to sample outcomes that below the lower limit of detection (10 CFU/mL # 100 CFU/1 coupon = 20 cm^2, or 5 CFU/cm^2) of the spread method.

3.2 Effect of food contact surface on adherence of Listeria, Salmonella *and* Pseudomonas

Food contact surface characteristics may also influence the attachment or detachment of bacterial cell populations in biofilm (Bridier et al., 2014; Chmielewski & Frank, 2003; Teh et al., 2014). The results of this experiment indicated that all tested bacterial species (*Listeria monocytogenes*, *Salmonella enterica* and *Pseudomonas aeruginosa*) had the ability to adhere to all tested materials (SS, Teflon and rubber) (Fig. 4). There was no significant difference ($p \geq 0.05$) in the bacterial adherence of each species in mixed-culture biofilm to different types of food contact surfaces (e.g., SS, Teflon or rubber). In fact, on SS, Teflon and rubber, respectively, the average cell attachment values for all testing conditions were: for *L. monocytogenes*, 5.4, 5.1 and 5.2 log CFU/cm^2; for *S. enterica*, 4.9, 4.9 and 4.8 log CFU/cm^2; and for *P. aeruginosa*, 4.0, 4.0 and 3.9 log CFU/cm^2. Similarly, some authors have indicated that food contact surfaces do not affect the attachment or detachment of bacterial cells in biofilm. For example, Yu et al. (2011) found a similar degree of adherence of *L. monocytogenes* and *Salmonella* Typhimurium to SS and rubber (Buna-N); while Bagge-Ravn et al. (2003) noted the same adherence of *E. coli* O157:H7 to SS and high-density polyethylene. The results of this experiment were supported by Hoa et al. (2015b), which found no significant difference in the population of mixed-species biofilm of *Listeria*, *Salmonella* and *Pseudomonas* adhered to SS, Teflon and rubber in 1% TSB after 7 days at 16 °C. In contrast to our results, Sinde and Carballo (2000) demonstrated that *L. monocytogenes* and *Salmonella* spp. produced more biofilm on more hydrophobic materials such as Teflon, followed by rubber and finally SS; while *Salmonella* biofilm formation showed the highest density on plastic, followed by cement and SS (Joseph et al., 2001). Bonaventura et al. (2008) found that less biofilm was produced by *L. monocytogenes* on Teflon than on glass and SS. These contradictory observations can be explained due to the fact that the attachment of cells to surfaces may be influenced by the initial attachment of bacterial cells, which is associated with free surface energy, as well as by the interaction between the cultures in mixed-species biofilms. High free energy and wet surfaces promote bacterial adhesion; in other words, SS allows greater bacterial attachment and biofilm formation than Teflon or rubber (hydrophobic surfaces, low free surface energy) (Chmielewski & Frank, 2003; Shi & Zhu, 2009). Therefore, the results of this experiment (Figs. 3–5) show that the initial cell attachment of *Listeria*, *Salmonella* and *Pseudomonas* to SS was higher than to Teflon after 1 day of incubation. This is in agreement with Chmielewski and Frank (2003), who found that the initial attachment of *L. monocytogenes* to SS was more rapid than to rubber; adhesive bacteria prefer to accumulate at the hydrophilic region of the hydrophilic–hydrophobic interface of a SS surface (Bos et al., 2000). The initial population of attached bacteria can change the surface so that the following species can attach via cell-to-cell association, especially in mixed-culture biofilms (Chmielewski & Frank, 2003).



[Fig. 4]

Fig. 4. Scanning electron microscopic images of multi-species biofilm of *Listeria monocytogenes*, *Salmonella enterica* and *Pseudomonas aeruginosa* at 16 ± 2 °C in tryptic soy broth (TSB) 0.3 g/L for 7 days on (a) stainless steel (SS), (b) Teflon and (c) rubber. The white scale bar indicates 5 μm.

3.3. Effects of nutrient content, temperature, and incubation time on mixed-culture biofilm formation of Listeria, Salmonella *and* Pseudomonas

The bacterial population growth under different limited nutrient contents (TSB 0.03, 0.3 and 3.0 g/L), temperatures (4, 18 and 30 °C) and incubation times (intervals over a 30-day period) in mixed-culture biofilm is shown in

Figs. 3–5. According to Chmielewski and Frank (2003), nutrient availability and growth temperature are the major factors influencing the composition of microbial communities and biofilm populations. Increasing nutrient concentrations and temperatures would therefore increase the number of adherent cells in mixed-culture biofilms. Under real-life conditions in a food processing environment, bacteria may be exposed to various nutrient levels in different locations in a food processing plant. Therefore, in our study the TSB concentrations ranged from 3.0 to 0.03 g/L, representing low nutrient medium to very low nutrient medium, mimicking the amounts of food residue typically found under food processing conditions.

Regarding nutrient content, statistical analysis showed that the highest cell population in biofilm was generated by TSB 3.0 g/L, followed by TSB 0.3 g/L, with the least occurring in TSB 0.03 g/L. Among the three tested cultures, *Listeria* was affected by nutrient availability; there was a significant increase in *Listeria* population ($p<0.05$) with increasing TSB concentration at the same incubation temperature. However, nutrient availability did not influence the population of *Pseudomonas*, except at level of TSB 0.03 g/L. For example, the average number of *Listeria* cells adhering to SS coupons in TSB 0.03; 0.3 and 3.0 g/L after 3 day of incubation at 30 °C ranged from 5.7; 6.3 and 7.2 log CFU/cm^2, respectively; whereas *Pseudomonas* cells ranged from 4.7; 5.5 and 5.5 log CFU/cm^2. Similar effects of nutrient content on the growth of *Listeria* in biofilms were reported by other researchers (Stepanović, S., Cirković, I., Ranin, L., & Švabić-Vlahović, 2004), who concluded that *L. monocytogenes* produced remarkably strong biofilm in nutrient-rich medium. However, the bacterial population in mixed-species biofilm is not only affected by the nutrient content but also by the production of EPS in biofilm. The results of our experiment showed that higher levels of *Listeria* accumulated (8.1 log CFU/cm^2) in TSB 0.3 g/L than in TSB 3.0 g/L (7.6 log CFU/cm^2) in a competitive culture at 18 °C. Similar results were reported by Jeong and Frank (1994a, 1994b), who demonstrated that there was a higher population of *Listeria* in mixed-culture biofilm grown in low-nutrient medium at both 10 and 21 °C. This result may be explained by the greater amount of EPS produced, leading to a higher *Listeria* population in the biofilm (Jeong & Frank, 1994a, 1994b).

Bacteria were incubated at a temperature range of 4 to 30 °C, which represented the temperatures in a chilling room (4 °C), processing room (18 °C) and packaging room (ambient temperature, 30 °C) in a food plant. The incubation temperature significantly influenced the quantity of biofilm produced. For example, the average bacterial counts in mixed-culture biofilm on SS coupons at 4, 18 and 30 °C were, respectively: 1.8, 6.3 and 6.4 log CFU/cm^2 for *Listeria*; 0.8, 5.7 and 6.2 log CFU/cm^2 for *Salmonella*; and 0.7, 4.3 and 5.3 log CFU/cm^2 for *Pseudomonas* (Figs. 1–3). At 4 °C, the presence of *Salmonella* and *Pseudomonas* could not be observed for all tested condition, except on day 1 of incubation in TSB 3.0 g/L. Indeed, only *Listeria* survived and grew slowly at 4 °C in 0.3 and 3.0 g/L TSB (Figs. 1-3). The maximum number of attached cells of *Listeria* was greater than 3.0 log CFU/cm^2 after 30 days of incubation. At 18 °C, the maximum number of attached cells of *Listeria*, *Salmonella* and *Pseudomonas* on SS coupons was 8.1, 7.8 and 6.0 log CFU/cm^2, respectively. At 30 °C, the maximum number of attached cells of *Listeria*, *Salmonella* and *Pseudomonas* on SS coupons was 8.1, 8.2 and 6.5 log CFU/cm^2, respectively. Similar results for *Listeria* were obtained in studies by Zameer et al. (2009) and Bonaventura et al. (2008). Their findings indicated that *Listeria* grew very slightly at 4 °C, even after 7 days, and the biofilm formed was not strong, consisting only of sparse clusters of cells. When the growth temperature was increased, however, *Listeria* formed multilayer biofilm. This may be due to the effects of temperature on *Listeria* surface hydrophobicity, motility (Bonaventura et al., 2008), and co-culture with other species, especially *P. aeruginosa* (Bagge-Ravn et al., 2003; Chmielewski & Frank, 2003; Giaouris et al., 2014; Gram & Huss, 1996).

During prolonged storage for 30 days, cell attachment or biofilm formation was influenced by the incubation time. For example, in TSB 3.0 g/L at 30 °C, the number of *Listeria* cells attached to SS coupons increased from 5.0 log CFU/cm^2 on day 1 to 8.1 log CFU/cm^2 on day 5, and then decreased to 5.6 log CFU/cm^2 on day 10; after that, biofilm accumulation of *Listeria* with the competitive cultures reached 7.1 log CFU/cm^2 on day 15 and remained at this level for the rest of the 30-day incubation period. The high resistance of the remaining adhered cells and less detachment of the cells in biofilm (Figs. 1–3) at 18 and 30 °C could lead to macro-colony biofilm formation in the later stages. This phenomenon was correlated with the cooperative interaction among these cultures in biofilm by production of EPS (Giaouris et al., 2014), as well as the motility and non-motility of *P. aeruginosa* in the biofilm (Guaglianone et al., 2010).

In short, similar to the findings of previous studies working on different bacteria and materials, the differences found in the degree of attachment of cell populations in biofilm not only depend on material surface characteristics, but also on nutrient content, testing conditions (contact time, temperature and pH), and characteristics of microorganisms (Bagge-Ravn et al., 2003; Chmielewski & Frank, 2003; Fatemi & Frank, 1999; Pratheepa & Vasconcelos, 2013).

4. CONCLUSIONS

The attachment and colonization of *Listeria*, *Salmonella* and *Pseudomonas* in mixed-culture biofilm on SS, Teflon and rubber indicated that there was no significant difference in the bacterial population adhered to the various materials. Strong biofilms still formed under very low nutrient condition (TSB 0.03 g/L) at 18 and 30 °C. Among the three tested cultures, *Listeria monocytogenes* was the dominant species under all testing conditions. At 4 °C *Listeria* was able to survive and attach to all tested surfaces, whereas *Salmonella* and *Pseudomonas* may not be present in the mixed-culture biofilm.

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MOLECULAR CHARACTERIZATION AND MTCC SUBMISSION OF LOVASTATIN MAXIMUM YIELDING FUNGI ISOLATED FROM NATURAL SAMPLES

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Abstract

Aspergillus sp. is known to produce cholesterol reducing drug-lovastatin. Lovastatin is a naturally occurring drug found in food such as red yeast rice (red rice *koji* or red fermented rice, red koji rice, red *koji* rice, *anka*, or *ang-kak*). Lovastatin (mevinolin) was the first hypocholesterolemic drug to be approved in 1987 by Food and Drug administration (FDA), USA. In the present study, three lovastatin maximum yielding wild type fungi i.e *Aspergillus terreus*- SSM4, isolated from wild oyster mushroom bed with lovastatin yield (997 µg/g dry matter), *A. terreus*- SSM3 from compost source (900 µg/g dry matter) and *A. flavus,* SSM8 from compost source (643 µg/g dry matter); obtained from the Upendra *et al.,* (2013a) were initially identified using scanning electron microscopy (SEM), further characterized at molecular level by restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing (SSM4) and 18s RNA (ITS1 - 5.8S - ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using *MEGA* version 5 software. Results confirmed the similarity between SSM3 and SSM4 isolates and they were found to be *Aspergillus terreus*, and also inferred that SSM8 is a completely different species, belongs to *Aspergillus flavus species*. Sequence information of the three isolates was submitted in NCBI GenBank with the issued accession numbers: JX419386- *Aspergillus terreus* **(**SSM3), JQ897354- *Aspergillus terreus* (SSM4), and JQ899451- *Aspergillus flavus* (SSM8). Characterized maximum lovastatin yielding wild type fungi were deposited in the MTCC IMTech- Chandigarh, with issued accession number as *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus* NHCEUPBTE 11396 (SSM8). Lovastatin in the SmF extract of *Aspergillus terreus* (SSM4) was confirmed using both ^{1}H NMR and ^{13}C NMR studies. In conclusion the presented study successfully characterized the lovastatin high yielding fungi and deposited to MTCC IMTech-Chandigarh with issued accession numbers.

Keywords: Lovastatin, RFLP, RAPD, β2 Tubulin & 18S rRNA gene sequencing, Phylogenetic analysis.

Introduction

High levels of cholesterol in the blood refer to the condition called Hypercholesterolemia also called dyslipidemia **(Durrington, 2003).** The highest % of global death today is caused by cardiovascular diseases (CVDs) occurs due to the hypercholesterolemic conditions, an estimate of 17.3 million people were died due to CVD, representing 30% of all global deaths in the year 2008 **(WHO, 2010).** An estimate of 80 % of death occurring in the low and middle income countries mainly due to CVD and affects men and women equally **(WHO, 2011).** Hypercholesterolemia can be easily treated with medications by targeting the reduction of the low density lipoprotein (LDL) cholesterol in the blood. Among the fungal polyketide metabolites, statins are in prime importance due to their anti-cholesterol nature **(Praveen and Savitha, 2012).** US Food and Drug administration approved lovastatin as the first hypo-cholesterolemic drug in the year 1987 **(Tobert, 2003).** Lovastatin competitively inhibits the enzyme HMG-CoA reductase (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34) which catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol **(Alberts *et al.*, 1980 & 1988, Upendra *et al.*, 2016).** Lovastatin found to inhibits cellular proliferation, induces apoptosis and necrosis in breast cancer **(Kalwitter *et al.*, 2010)** and also suppress the proliferation and migration of human glioblastoma cancer cells by the inhibition of Ras farnesylation mechanism **(Xia *et al.*, 2001).** Lovastatin therapy was effective in preventing creatinine clearance decline and also suppresses renal function loss in patients with kidney disease **(Buemi *et al.*, 2002).** High doses of lovastatin stimulate bone formation *in vitro* and *in vivo* and, stimulate biomechanical strength to heal fractures **(Garrett *et al.*,**

2007). Lovastatin treatment was observed to reduce the prevalence of Alzheimer's disease (AD) in patients suffering from hypercholesterolemia **(Eckert *et al*., 2005).** Lovastatin therapy improves endothelial function, modulates inflammatory responses, maintain plaque stability and prevent thrombus formation **(Palmer *et al*., 1990, Pickin *et al*., 1999).**

Aspergillus sp **(Upendra *et al*., 2013a & 2013b)** and *Monascus sp* **(Negishi *et al.*, 1986, Upendra *et al*., 2014a)** were known to produce lovastatin through polyketide biosynthatic pathway **(Lai *et al*., 2002).** Lovastatin from *Aspergillus terreus* was the first statin to be approved by FDA in 1987 for therapeutic use **(Tobert, 2003, Upendra *et al*., 2014b).** The colony morphology observations studies and sub-typing studies on the *Aspergillus terreus* species revealed that the species section was found to be more diverse in their genotype (**Balajee, 2009).** Species level identification can be achieved successfully with molecular characterization studies of β-Tubulin gene and 18s RNA (ITS1 - 5.8S - ITS2) region studies **(Henry *et al*., 2000).** Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis **(Martin and Rygiewicz, 2005).** A novel PCR-based assay (ITS1 and ITS2 regions), was developed and introduced to differentiate medically important species of *Aspergillus* not only from other opportunistic species of moulds and yeasts but also from other strains belong to the same species (**Hinrikson *et al*., 2005)**. A new species of *Aspergillus* genus, section *Terrei* was studied *using* a polyphasic approach including sequence analysis of parts of the β-tubulin, calmodulin genes and the ITS region **(Samson *et al*., 2011).** The study of molecular evolutionary process of different microorganism, comparative analysis of molecular sequence data is essential for reconstructing the species and inferring the nature and extent of selective forces shaping the evolution of genes and species **(Tamura *et al.*, 2011)**. MEGA version5 software was used for building sequence alignments and phylogenetic trees, used in evolution analysis. This software is equipped with new tools which were used in studying maximum likelihood (ML) analyses for inferring evolutionary trees, selecting best-fit substitution models (nucleotide or amino acid), inferring ancestral states and sequences (along with probabilities), and estimating evolutionary rates site-by-site **(Tamura *et al.*, 2011).** A novel method of lyophilization for the preservation of fungal strains was investigated and applied for the fungi which were found to possess high industrial value (**Bunse and Steigleder, 1991)**.

With the view of review of literature discussed, the present study aimed at identification of three lovastatin maximum yielding wild type fungi i.e *Aspergillus terreus*-SSM4, isolated from wild oyster mushroom bed with lovastatin yield (997 µg/g dry matter), *A. terreus*- SSM3 from compost source (900 µg/g dry matter) and *A. flavus*, SSM8 from compost source (643 µg/g dry matter); obtained from the Upendra *et al.,* (2013a). Study initially identified all the three lovastatin maximum yielding fungi, using scanning electron microscopy (SEM), further characterized at molecular level by Restriction fragment length polymorphism (RFLP), Random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing (SSM4) and 18s RNA (ITS1 - 5.8S - ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using *MEGA* version 5 software. Results confirmed the similarity between SSM3 and SSM4 isolates and they were found to be *Aspergillus terreus*, and also inferred that SSM8 is a completely different species, belongs to *Aspergillus flavus species*. Sequence information of the three isolates was submitted in NCBI GenBank with the issued accession numbers: JX419386-*Aspergillus terreus* **(**SSM3), JQ897354- *Aspergillus terreus* (SSM4), and JQ899451-*Aspergillus flavus* (SSM8). Characterized maximum lovastatin yielding wild type fungi were deposited in the MTCC IMTech-Chandigarh, with issued accession number as *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus* NHCEUPBTE 11396 (SSM8).

MATERIALS & METHODS

All the chemicals and reagents used in this study were of analytical Grade (Merck and Qualigens).

Analysis of lovastatin by NMR (^{1}H NMR & ^{13}C NMR) Spectroscopy

Aspergillus terreus (SSM4), SmF extract obtained from Upendra *et al*., (2013a) was analyzed by NMR (^{1}H NMR & ^{13}C NMR) spectroscopy to elucidate the srucure of lovastatin**.** The structure of lovastatin was also determined by ^{1}H NMR, ^{13}C NMR. Proton and carbon NMR measurements were performed on a Bruker Avance Ultrashield spectrometer 400.23 MHz instrument in deuterated dimethylsulfoxide and chloroform solvent ($CDCl_3$) system. Instrument was equipped with 5 mm BBO-head (^{1}H-channel, X-channel) probe with Z-gradient coils and an automatic Sample Changer B-ACS 120 (Bruker BioSpin, Rheinstetten, Germany). The data processing was performed using BRUKER X-WIN NMR 3.5 software under Microsoft Windows. All spectra were acquired at 298.0 K and the chemical shift values were reported on the δ scale relative to TMS **(Lankhorst *et al*., 1996 & Holzgrabe *et al*., 1998).**

Microorganism

The three culture of *Aspergillus species* SSM3, SSM4 and SSM8 obtained from Upendra *et al*., (2013a) were revived on the potato dextrose agar (PDA) slants and kept at 28°C in incubator for the period of seven days. After seven days of incubation, fully grown fungal cultures were stored at 4°C for further molecular characterization studies.

Scanning Electron Microscopic (SEM) confirmation of lovastatin maximum yielding fungal isolates

Selected three fungal isolates were initially confirmed by the Scanning Electron Microscopic (SEM) screening. Fungal mycelium was segmented to 0.5 to 1 cm thin sections with the blade; Segments were immersed in centrifuge tubes containing 1.5 ml of modified Karnovisk's solution (Glutaraldehyde 2.5%, 2.5% formaldehyde in cacodilato sodium buffer 0.05 M, pH 7.2, CaCl2 0.001M.) by 24 hours. Samples were than washed 3 times (each of 10 minutes) with aldehyde in 0.05 M cacodylate buffer (0.2M cacodylic acid $(CH_3)_2AsO_2H$ (MW = 138.0) 27.6 gm + ddH_2O to make 1 liter) and were immersed in tetroxide solution of 1% of osmium in 0.05 M cacodilato buffer (pH 7.2), at room temperature, in laminar flow chamber for 4 hours. Samples were washed 3 times in distilled water and were dehydrated in gradient of acetone (25, 50, 75, 90 and 100%) 10 minutes in each concentration, being repeated by 3 times in the concentration of 100%. Subsequently, the samples were submitted to CPD Balzers 030® equipment to complete dehydration. The specimens were pasted using adhesive tapes on the surface of stubs covered with aluminium and submitted to the metallization with gold using SCD

Balzers 050® equipment, in order to increase its conductivity. Finally, the specimens were observed by the scanning electron microscope LEO Evo 40®, interfaced by digital image processing software. Microscopic properties such as conidial head, conidiophores, vesicle and conidia were studied under Scanning Electron microscopy at 2000X - 3000 X magnification for all the three maximum lovastatin yielding fungal isolates **(Gonzalez and Woods, 2002).**

Molecular characterization by Restriction fragment length polymorphism (RFLP)

Fungal DNA was isolated by adopting the method as described previously by Zhao *et al*., (2001). The DNA pellet isolated was resuspended in 200 µl of TE buffer and stored at 4ºC for further analysis. Extracted Fungal DNA was subjected to electrophoresis on 1.2 % agarose gel for purity checking. PCR amplification was carried out for isolated DNA of all the three lovastatin high yielding fungal isolates of the present study. Conserved regions of 18S rRNA (ITS 1) and the 28S rRNA (ITS 4) gene (intervening 5.8S gene under the ITS 1 and ITS 2 noncoding regions) amplification was done using ITS 1 (5'TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers (118,119,167). 50 µl final volume of PCR reaction mixture **(Table-1)** was amplified following the PCR programme conditions **(Table-2)** designed for a total of 35 cycles.

Table-1. PCR reaction Mixture

Slno	Component	Volume(µl)	Concentration
1.	Triple Distilled water	36 µl	--
2.	10X assay buffer	05 µl	Tris-HCl (pH 8.4)- 200mM, KCl-500 mM $MgCl_2$-15 mM
3.	dNTP mix	02 µl	(10 mM)
4.	ITS1 primer	02 µl	50 ng/µl
5.	ITS4 Primer	02 µl	50 ng/µl
6.	Taq DNA Polymerase	01 µl	3u/µl
7.	Template DNA	02 µl	50 ng/µl

Table-2. PCR Programme Table

Segment	Step	Number cycles	Step	Temperature	Time
I		01	Initial denaturation	94ºC	5 Min
	1.		Denaturation	94 ºC	2 min
II	2.	35	Annealing	53 ºC	2 min
	3.		Extension	72 ºC	2 min
III		01	Final Extension	72 ºC	10 Min

Following amplification, 5 µl of PCR products were digested with the restriction enzyme *Hae*III, *Eco*RI and *Tag*I **(Table-3) (Munusamy *et al*., 2010).** After incubation the digested fragments were added with an aliquot of 2.5 µl of 6X gel loading dye and the fragments were separated on 1.2% agarose gel in 1X TAE buffer, stained with ethidium bromide and visualized under UV transillumination. The sizes of the digested products were determined with standard 1 kb or 100 bp molecular markers (Aristogene Laboratories Pvt. Ltd., Bangalore, India).

Table-3. Restriction digestion enzyme

Restriction enzyme	DD H_2O	Assay buffer	Template DNA	Enzyme (µl)	Final volume (µl)
EcoR I	35µl	4µl	10µl	1µl	50 µl
Hae III	35µl	4µl	10µl	1µl	50 µl
Taq I	35µl	4µl	10µl	1µl	50 µl

2.5. Molecular characterization by RAPD techniques

Random amplification was carried out for isolated DNA of all the three selected fungi using R108 random primer (5'GTA TTG CCC T-3') **(Teresa *et al.*, 2000, Raclavsky *et al.*, 2006).** 50 µl final volume of PCR reaction mixture (**Table-4**) was amplified following the PCR programme table (**Table-5**) designed for a total of 40 cycles. After amplification, the products were analyzed by the gel electrophoresis using 2% agarose gel.

Table-4. RAPD reaction Mixture

Slno	Component	Volume(µl)	Concentration
1.	Triple Distilled water	36 µl	--
2.	10X assay buffer	05 µl	Tris-HCl (pH 8.4)- 200mM, KCl-500 mM $MgCl_2$-15 mM
3.	dNTP mix	02 µl	(10 mM)
4.	Random primer	04 µl	50 ng/µl
5.	Taq DNA Polymerase	01 µl	3u/µl
6.	Template DNA	02 µl	50 ng/µl

Table-5. RAPD Programme Table

Segment	Step	Number cycles	Step	Temperature	Time
I		01	Initial denaturation	95ºC	5 Min
	1.		Denaturation	95 ºC	1 min
II	2.	35	Annealing	35 ºC	1 min
	3.		Extension	72 ºC	2 min
III		01	Final Extension	72 ºC	10 Min

Following amplification, 10 µl of amplified products were added with an aliquot of 2.5 µl of 6X gel loading dye and the amplified fragments were separated on 2% agarose gel in 1X TAE buffer, stained with ethidium bromide and visualized under UV transillumination. The sizes of the amplified products were determined with standard 1 kb or 100 bp molecular markers (Aristogene Laboratories Pvt. Ltd., Bangalore, India).

Phylogenetic and molecular evolutionary analyses selected fungal isolates

Fungal DNA was extracted with phenol-chloroform protein extraction, precipitated using ethanol and checked on 1% agarose gel for purity **(Zhao *et al.*, 2001).** PCR amplification of partial regions of β2-tubulin gene was done by using primer pairs Bt2a (5'GGT AAC CAA ATC GGT GCT GCT TTC 3') (forward) and Bt2b (5' ACC CTC AGT GTA GTG ACC CTT GGC3') (reverse) for *Aspergillus terreus* (SSM4), conserved regions of 18S rRNA amplification was done as discussed in section 2.4 **(Henry *et al.*, 2000).** PCR product obtained was gel purified and taken for sequencing **(Glass and Donaldson, 1995).** PCR product was sequenced with the big dye terminator cycle sequencing ready reaction kit on an ABI3730XL genetic analyzer (Applied Biosystems) instrument model/name: 3730xl /ABI3730XL-15104-028, sequence scanner version 1.0 software **(Balajee *et al.*, 2007).** Forward strand partial β 2 tubulin gene sequence (545 nt) of *Aspergillus terreus* (SSM4) was aligned with maximum identity score sequence *Aspergillus terreus* NRRL 255 strain, forward strand 18S rRNA gene sequence of *Aspergillus terreus* (SSM3) (740 nt), *Aspergillus flavus* (SSM8) (751 nt) were aligned with maximum identity score sequence EF669586-*Aspergillus terreus* NRRL 255 strain and EF661563-*Aspergillus flavus* NRRL 4818 strain respectively through BLAST. Phylogenetic analyses of *Aspergillus terreus* SSM4 was performed using the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbor-joining method **(Saitou and Nei, 1987).** The percentage of replicate trees (500 replicates), associated taxa clustered was done by bootstrap test **(Felsenstein, 1985).** The evolutionary distances computed using Jukes-cantor method **(Jukes and cantor, 1969)** to infer phylogenetic tree. Phylogenetic analyses of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) were performed by the maximum parsimony (MP) method and molecular evolutionary relationship was inferred using neighbor-joining method **(Saitou and Nei, 1987).** The percentage of replicate trees (100 replicates), associated taxa clustered was done by bootstrap test **(Felsenstein, 1985).** The evolutionary distances computed using kimura 2-parameter method **(kimura, 1980)** to infer phylogenetic tree. Evolutionary analysis was conducted using MEGA version 5 software **(Tamura *et al.*, 2011).**

RESULTS & DISCUSSION

Analysis of lovastatin by NMR Spectroscopy

^{1}H-NMR Spectroscopy

^{1}H NMR (400 MHz,$CDCl_3$) determination of [*Aspergillus terreus* (SSM4)] SmF extract obtained from Upendra *et al* ., (2013a) showed resonances at δ 6.004-5.980ppm, δ 5.804-5.764 ppm, δ 5.530-5.523 ppm, δ 5.40-5.337 ppm, δ 4.632-4.579 ppm, δ 4.382-4.352 ppm (mid-field region) and δ 2.7635-2.750 ppm, δ 2.719-2.707 ppm, δ 2.641-2.627 ppm, δ 2.597-2.583 ppm, δ 2.462-2.436 ppm, δ 2.396-2.388 ppm, δ 2.281-2.275 ppm, δ 2.252-2.245 ppm, δ 2.095-2.088 ppm δ 1.998-1.937 ppm, δ 1.64-1.57 ppm, δ 1.117-1.0677 ppm, δ 0.90-0.863 ppm (aliphatic range) (Fig.1a). The ^{1}H NMR (400 MHz, $CDCl_3$) spectral prediction showed that the multiplet at δ 5.400-5.377 ppm clearly belonged to an H-atom at the hexahydronaphthalene moiety, with overlap of the signals of both atoms H6, H4 **(Fig. 1a).** The ^{1}H NMR spectral

data of lovastatin was found to be in concurrence with reported spectral data **(Holzgrabe *et al*., 1998).**

^{13}C-NMR Spectroscopy

^{13}C NMR (400 MHz, $CDCl_3$) determination of [*Aspergillus terreus* (SSM4)] SmF extract showed resonances at δ 133.04-128.33 ppm, δ 77.32-76.24 ppm, δ 41.49-11.72 ppm, **(Fig.1b).** The ^{13}C NMR (400 MHz, CDCl3) spectral prediction showed that the methylene signal at 26.81 ppm of C-3, methyl signal at 11.72 ppm of C-4" and an acetoxymethyl signal at 30.70 ppm indicating the presence of carbon-atom. The ^{13}H NMR spectral data of lovastatin was found to be in concurrence with reported spectral data **(Belwal *et al*., 2013).**

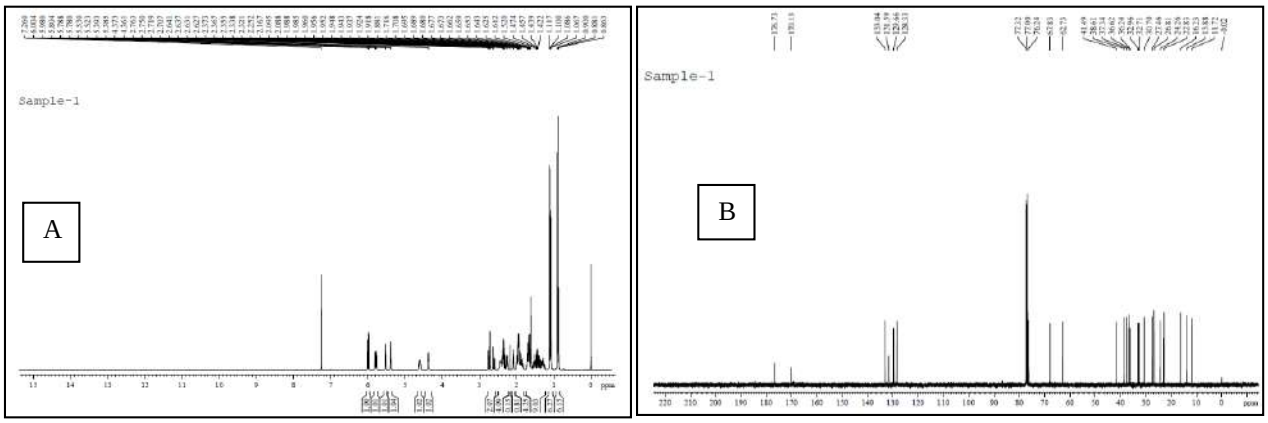

Fig.1: NMR spectrum of lovastatin (*Aspergillus terreus* (SSM4) SmF extract).
A. ^{1}H NMR spectrum of lovastatin, 400.23 MHz, solvent $CDCl_3$. B. ^{13}C NMR spectrum of lovastatin, 400.23 MHz, solvent $CDCl_3$.

Microorganism:

The fully grown cultures of *Aspergillus terreus* (SSM3), *Aspergillus terreus* (SSM4) and *Aspergillus flavus* (SSM8) on PDA plates obtained from Upendra *et al*., (2013a) were shown in **Fig.2.** *A. terreus* colonies are commonly powdery masses of cinnamon-brown spores on the upper surface and reddish-gold on the lower surface. *A. flavus* colonies are commonly powdery masses of yellow-green spores on the upper surface and reddish-gold on the lower surface.

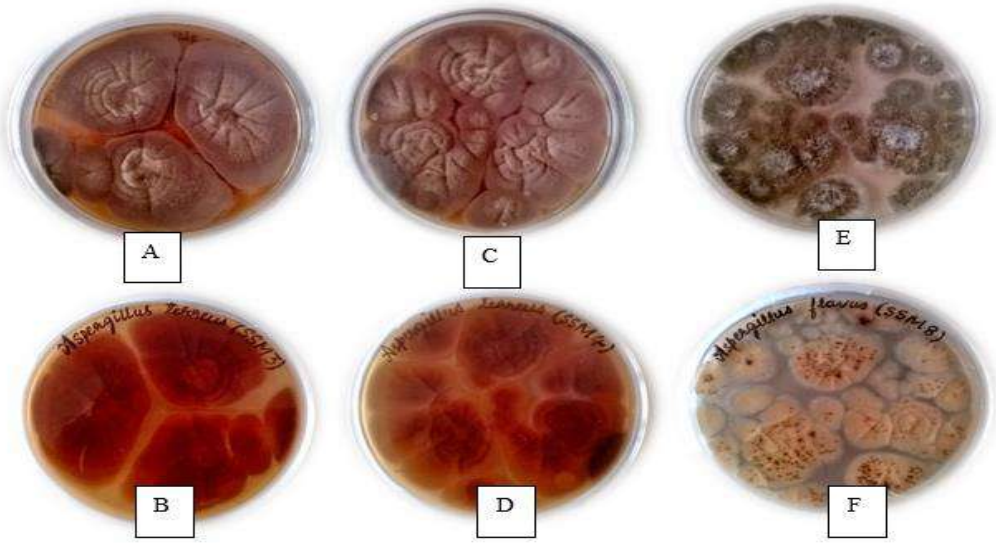

Fig 2: Selected fungal strain for molecular characterization

A. *Aspergillus terreus* (SSM3) on PDA media showing cinnamon brown on upper surface B. *Aspergillus terreus* (SSM3) showing reddish-gold on the lower surface. C. *Aspergillus terreus* (SSM4) on PDA media showing cinnamon brown on upper surface D. *Aspergillus terreus* (SSM4) showing reddish-gold on the lower surface. E. *Aspergillus flavus* (SSM8), on PDA media showing Olive to lime green color colonies upper surface. F. *Aspergillus flavus* (SSM8) showing reddish-gold on the lower surface.

Scanning Electron Microscopic (SEM) confirmation of lovastatin maximum yielding fungal isolates.

Under SEM *A. terreus* colonies were found to show characteristic properties i.e compact, biseriate, and densely columnar conidial heads (Fig 2-A, D). Smooth and hyaline conidiophores (Fig 2-B, E). The conidia are small, about 2 µm in diameter, globose-shaped, smooth-walled, and can vary from light yellow to hyaline (Fig 2-C, F). *Aspergillus flavus* hyphae are septate and hyaline (Fig 2-G). The conidiophores of are rough and colorless (Fig 2-H). Phialides are both uniseriate (arranged in one row) and biseriate. Conidia producing thick mycelial mats are often seen (Fig 2-I).

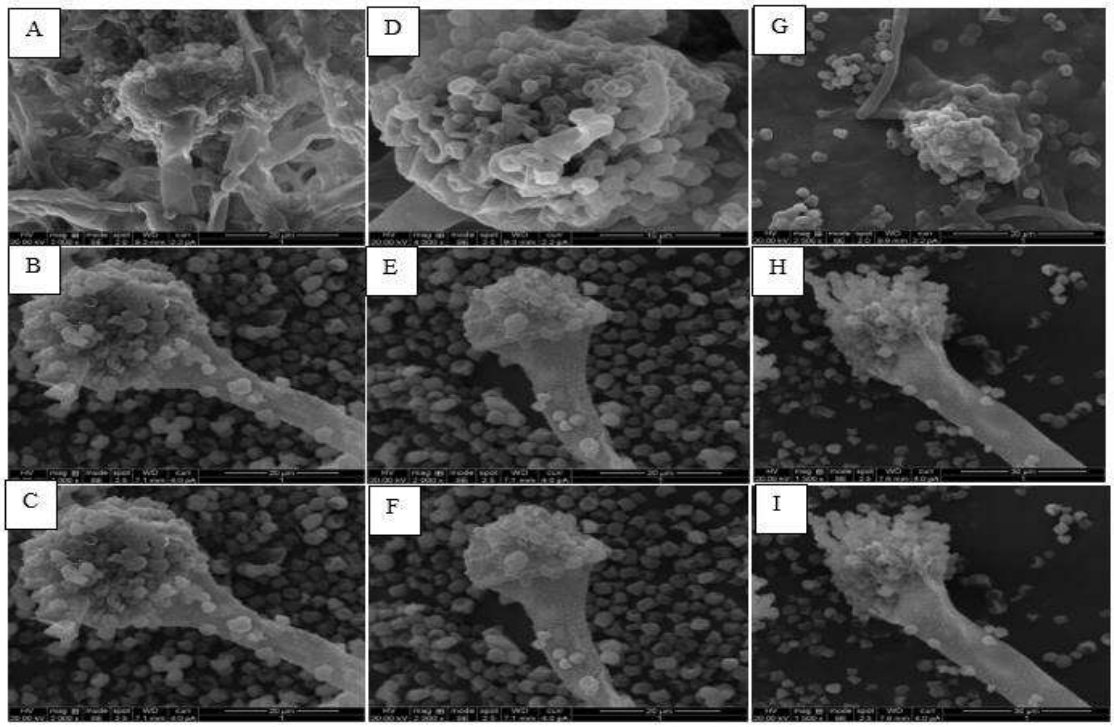

Fig 3: SEM Characterization of isolated fungal strains

A-C. Conidiophores and Conidia of *Aspergillus terreus* (SSM4). D-F. Conidiophores and Conidia of *Aspergillus terreus* (SSM3). G-I. Conidiophores and Conidia of *Aspergillus flavus* (SSM8).

Molecular characterization by Restriction fragment length polymorphism (RFLP)

The isolated and purified (1.2% agarose gel) DNA from the selected fungal cultures was shown in the Fig.4A. PCR amplification (ITS1-ITS4 primers) of genomic DNA extracted from the three selected fungi *A. terreus* (SSM3), *A. terreus* (SSM4) and *A. flavus* (SSM8) isolates with ITS1 and ITS4 primers resulted in the amplification of a product of approximately 600 bp (Fig. 4B). Upon digestion of the PCR products with the enzymes *Eco*RI, *Hae*III and *Taq*I indicated genetic variability among the isolates which varied in size of RFLP fragments and number of fragments (Fig. 4C).

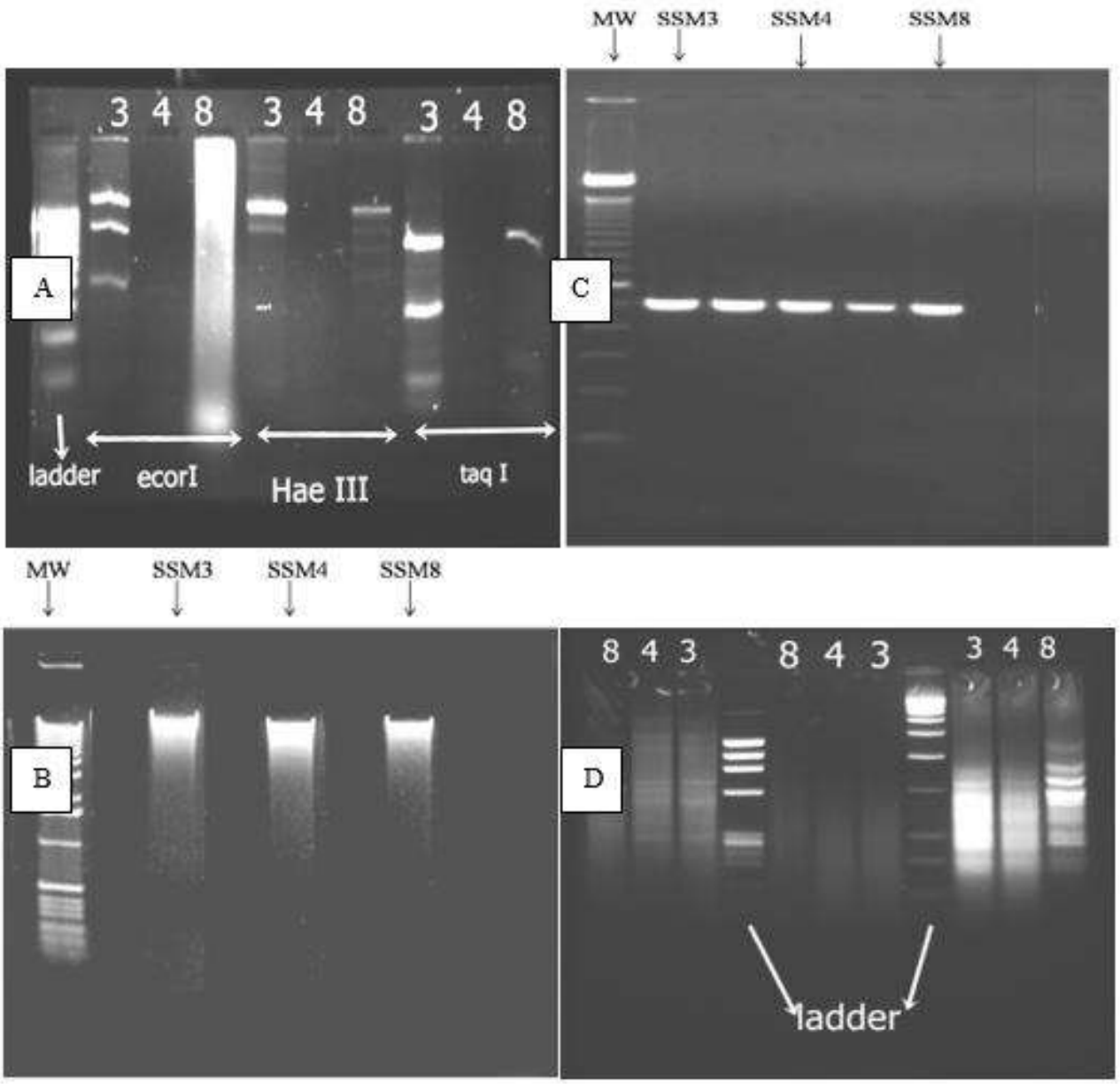

Fig. 4. Molecular characterization by RFLP and RAPD

Isolated DNA on 1.2 % agarose gel, (B) PCR amplified product (ITS region) on 2 % agarose gel, (C). Restriction patterns of the ITS regions of the ribosomal DNA of *A. terreus* (3), *A. terreus* (4), *A. flavus* (8) isolates digested with *Eco*RI, *Hae*III and *Taq*I, (D) RAPD finger printing profile of the ITS regions of the ribosomal DNA of *A. terreus* (3), *A. terreus* (4), *A. flavus* (8) isolates.

Molecular characterization by Random Amplification of Polymorphic DNA (RAPD)

RAPD-PCR amplification (random primer) product separated on 2% agarose gel of extracted genomic DNA from the three selected fungi *A. terreus* (SSM3), *A. terreus* (SSM4) and *A. flavus* (SSM8) isolates was shown Fig 4D. RAPD-PCR finger printing profiles of SSM3 and SSM4 were same and SSM8 fingerprinting profile is completely different, clearly inferring that SSM3 and SSM4 belong to same species and SSM8 belongs to the different species.

Phylogenetic and molecular evolutionary analyses of selected fungal isolates

Partial β 2 tubulin gene forward sequence information of *Aspergillus terreus* SSM4 was submitted to NCBI genbank with genbank accession number (JQ897354). Forward strand partial β 2 tubulin gene sequence (545 nt) were aligned with maximum identity score sequence *Aspergillus terreus* NRRL 255 strain through BLAST. The evolutionary history was inferred using Neighbor-joining method (Fig. 6A), the optimal tree with sum of branch length = 0.97315489 was shown. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branch. The tree was drawn to scale, with branch length in the same unit as those of the evolutionary distance used to infer the phylogenetic tree. The evolutionary distance was in the units of number base substitutions per site. The analysis involved 16 nucleotide sequences. All ambiguous were removed for each sequence pair. There were a total of 595 positions in the final data set (Adopted from Upendra *et al*., 2013a). 18S rRNA gene forward sequence information of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) were submitted to NCBI genbank with genbank accession numbers JX419386, JQ899451 respectively. *Aspergillus terreus* (SSM3) forward strand 18S rRNA gene sequence (740 nt) was aligned with

maximum identity score sequence EF669586-*Aspergillus terreus* NRRL 255 strain, *Aspergillus flavus* (SSM8) forward strand 18S rRNA gene sequence (751 nt) was aligned with maximum identity score sequence EF661563 *Aspergillus flavus* NRRL 4818 through BLAST. The evolutionary history was inferred using Neighbor-joining method (Fig. 6b), the optimal tree with sum of branch length = 0.10936750 was shown. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (100 replicates) was shown next to the branch. The tree was drawn to scale, with branch length in the same unit as those of the evolutionary distance used to infer the phylogenetic tree. The evolutionary distance was in the units of number base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 388 positions in the final data set.

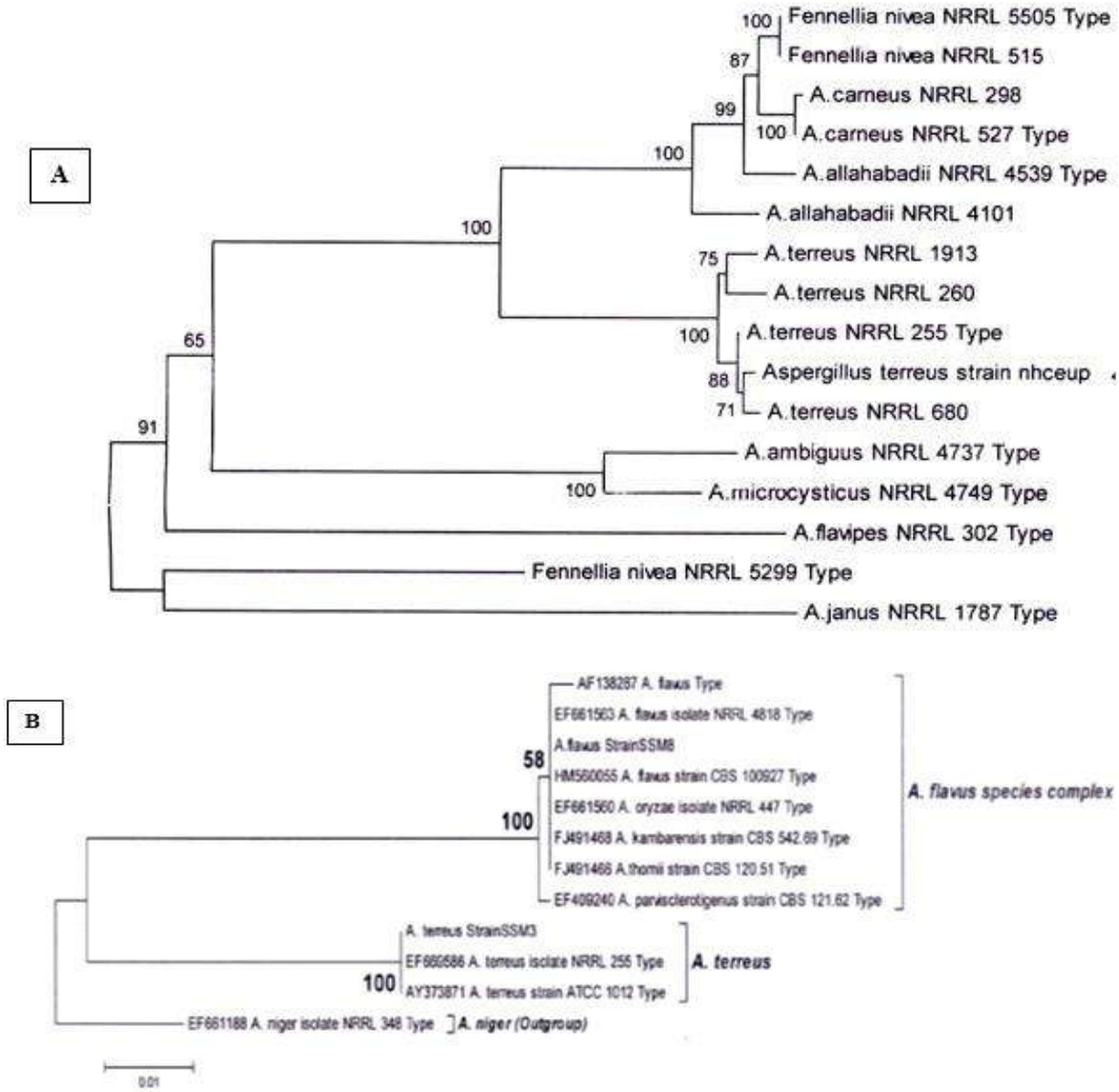

Fig 6: Phylogenetic analysis of isolated high yielding fungal strains

A. Phylogenetic tree of *Aspergillus terreus* strain nhceup (SSM4), inferred from Neighbour-Joining analysis of partial β-tubulin gene sequence (Adopted from Upendra *et al*., 2013a). B. Phylogenetic tree of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) inferred from Neighbour-Joining analysis of ITS1-ITS4 region sequencing

Deposition of lovastatin maximum yielding fungal isolates with MTCC (IMTech) Chandigarh

The maximum lovastatin yielding, molecular characterized and phylogenetically analyzed three new fungal isolates were deposited in Microbial Type Culture Collection, IMTech-Chandigarh, with issued accession number *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus NHCEUPBTE* 11396 (SSM8).

Conclusion

In the present study, attempts were made on molecular charecterization and MTCC registration of three lovastatin maximum yielding fungal cultures (obtained from the Upendra *et al.*, 2013a) isolated from natural samples. Selected fungal cultures were initially identified using scanning electron microscopy (SEM), further characterized at molecular level by Restriction fragment length polymorphism (RFLP), Random amplification of polymorphic DNA (RAPD), β2 tubulin gene sequencing (SSM4) and 18s RNA

(ITS1 - 5.8S - ITS2) sequencing and finally recognized by phylogenetic evolutionary analyses using *MEGA* version 5 software. Sequence information of the three isolates was submitted in NCBI GenBank with the issued accession numbers: JX419386-*Aspergillus terreus* **(**SSM3), JQ897354-*Aspergillus terreus* (SSM4), and JQ899451-*Aspergillus flavus* (SSM8). Characterized maximum lovastatin yielding wild type fungi were deposited in the MTCC IMTech-Chandigarh, with issued accession number as *Aspergillus terreus* nhceup 11045 (SSM4), *Aspergillus terreus* NHCEUPBT 11395 (SSM3) and *Aspergillus flavus* NHCEUPBTE 11396 (SSM8).

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DETECTION OF MULTIDRUG RESISTANCE *AEROMONAS HYDROPHILA* IN MARINE RUPCHANDA FISH SOLD AT LOCAL FISH MARKETS AT MYMENSINGH CITY IN BANGLADESH

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Abstract

This study was conducted for detection of multidrug resistance *Aeromonas hydrophila* in marine Rupchanda (Pampus chinensis) sold at local fish markets at Mymensingh city, Bangladesh. Rupchanda (n=30) were collected from three local fish markets. Intestine (n=30) and gill (n=30) samples were aseptically collected and inoculated into alkaline peptone water for enrichment at 37°C for 8 hrs. Enriched culture was streaked onto thiosalfate bile salt sucrose (TCBS) agar to isolate bacteria. Identification of bacteria was performed by routine bacteriological tests and polymerase chain reaction assay. The antibiogram profile of bacteria was investigated against nine commonly used antibiotics such as: gentammicin, chloramphenicol, ampicillin, erythromycin, streptomycin, tetracycline, cefalexin, azithromycin and ciprofloxacin by disc diffusion method. A total of 10 *Aeromonas hydrophila* were identified and all isolates were found resistant to ampicillin and cefalexin. Data of this study suggest that Rupchanda harbour multidrug resistance *Aeromonas hydrophila* which may cause public health problem if enter into human food chain.

Keywords*:* Multidrug resistance, *Aeromonas hydrophila*, Rupchanda fish, Bangladesh.

INTRODUCTION

Fish carries various types of bacterial flora in gills, gut and skin. *Aeromons hydrophila* is a ubiquitous and opportunistic bacterium that constitutes part of the normal flora of fish. It has been reported both from freshwater and marine environments. It is a Gram negative facultative anaerobic bacterium. It causes diseases in fish at the time of stress (Peters et al., 1988). It has been associated with several disease conditions in fish such as fin rot, tail rot and *haemorrhagic septicaemia*. It causes gastroenteritis and localized wound infection in humans (Nemetz and Shotts, 1993). Fish spoilage is caused by *Aeromonas* spp. due to the action of its extracellular microbial enzymes such as haemolysin, enterotoxins, cytotoxins, lipases and proteases (Farag, 2006). During the last years, the interest to *Aeromonas* spp. extended beyond the boundaries of fish pathology due to the increased incidence of human disease caused by *Aeromonas* after consumption of contaminated foods. Motile aeromonads are emerging food pathogens as some isolates could produce virulence factors not only at optimum temperatures, but also under cold storage conditions (Neyts et al., 2000). Waters receive antimicrobial agents from human and animal waters which results in the emergence of multidrug resistance (MDR) bacterial flora in the aquatic environment (Morita et al., 1994). The multidrug resistance was reported in the genus of *Aeromonas* (Albert et al., 2000; Palu et al., 2006).

A. hydrophila were detected in fresh water fishes and prawns sold at fish markets in Bangladesh (Rahim et al., 1984; Rahim and Aziz, 1994). *Rupchanda* (Pampus chinensis) is a popular and costly sea fish in Bangladesh. It is harvested from the Bay of Bengal of Bangladesh. This fish is regularly sold in the domestic fish markets throughout Bangladesh. Both dried and fresh forms of this fish have a huge demand among the consumer levels including the tourists. Rupchanda is also exported from Bangladesh to the foreign countries. In order to predict the hazard for consumers' health no study has been conducted so far in Bangladesh on the status of *A. hydrophila* in Rupchanda fish sold at the local fish markets. The objectives of this study were (i) Isolation and identification of *A. hydrophila* from Rupchanda sold in the local fish markets at Mymensingh city and (ii) determination of antibiogram profile of *A. hydrophila* against nine commonly used antibiotics.

Materials and Methods

Collection of samples: Rupchanda (n=30) were collected from three local fish markets such as Kewatkhali fish market (n=10), Kamal Ranjit (KR) market (n=10) and Mesuabazar markets (n=10) located at Mymensingh city. The samples were packed into sterile polyethene bags in an ice box and transported to the Department of Microbiology and Hygiene at the Bangladesh Agricultural University (BAU), Mymensingh for bacteriological study.

Processing of samples: Gill (n=30) and intestine (n=30) of Rupchanda were aseptically collected. Intestinal samples were cut into small pieces using sterile scissors and mixed with 4.5 ml APW and grinded by pestle and mortar to prepare homogenous

suspension. Gill swab samples were collected using sterile cotton swabs.

Enrichment of samples: Swab of gill was inoculated into a test tube containing 4.5 ml of Alkaline Peptone Water (APW) and incubated at 37°C for 8 hrs. Intestinal samples (0.5g) were separately inoculated into test tubes containing 4.5 ml APW and incubated at 37°C for 8 hrs.

Isolation of bacteria: One loopful of enrichment culture of gill and intestine was separately streaked duplicate onto thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia, India) and incubated aerobically at 37°C for 24 hrs. Single colony grown onto the TCBS agar was further sub cultured onto TCBS agar until pure cultures were obtained.

Identification of bacteria: Identification of bacteria was conducted by observing cultural characteristics and colony morphology on the TCBS agar and growth of bacteria into nutrient broth containing 0% and 6% sodium chloride (NaCl). Gram's staining method, motility test, sugar fermentation and biochemical tests (oxidase test, catalase test, citrate test, indole test and MR-VP test) were performed to identify bacteria.

Molecular detection of bacteria by PCR: A genus specific PCR assay was performed to identify *Aeromonas* spp. by amplifying 276-bp fragment of lipase gene (Delamare et al., 2012).

Antibiotic sensitivity test: Antibiogram profile of 10 *A. hydrophila* isolates was done against five different antibiotics such as: Gentamicin, Azithromycin, Ciprofloxacin, Ampicillin, Cefalexin, Erythromycin and Chloramphenicol (Himedia, India). The antibiotics sensitivity testing was carried out according to instructions of the Clinical and Laboratory Standard Institute (CLSI, 2011).

RESULTS

Isolation and identification of *A. hydrophila*

Gill (n=30) and intestine (n=30) samples of Rupchanda were streak onto Thiosulfate Citrate Bile salt (TCBS) agar to isolate *Aeromonas* spp. Yellow shin colony was seen on the TCBS agar after 24 hrs incubation at 37°C which are characteristics of the genus *Aeromonas*. All isolates produced β- hemolysis on blood agar media. *Aeromonas* isolates grew in nutrient broth with (6%) or without (0%) addition of NaCl. In Gram's staining *Aeromonas* isolates were found Gram negative, rod-shaped and arranged in single and pair. Sugar fermentation and biochemical tests confirmed *Aeromonas* isolates as *A. hydrophila* (Table 1).

Table 1: Summary of sugar fermentation, motility and biochemical test results for *Aeromonas hydrophila*

Name of tests	Results of this study	Results of Bergey's Manual*	Results of other investigators		Interpretation
			Results	References	
1. Sugar fermentation					*Aeromonas hydrophila*
Dextrose	A	A	A	Kannan *et al. (*2011)	
Sucrose	A	A	A	Popovic *et al.* (2000)	
Lactose	A	NS	A	Chandrakanthi *et al.* (2000)	
Maltose	A	A	A	Popovic *et al.* (2000)	
Mannitol	A	A	A	Chandrakanthi *et al.* (2000)	
2. Motility test using hanging drop method	Motile	Motile	Motile	Chandrakanthi *et al.* (2000)	
3. Biochemical tests					
Oxidase	+	+	+	Kaysner and Depaola (2004)	
Catalase	+	+	+	Kannan *et al.* (2011)	
Citrate	+	NS	+	Kannan *et al.* (2011)	
Indole	+	NS	+	Al- Fatlawy *et al.* (2013)	
MR	+	NS	+	Chandrakanthi *et al.* (2000)	
VP	+	+	+	Kaysner and Depaola (2004)	

A=Acid, + = positive, NS= Not stated, MR= Methyl Red, VP= Voges Proskauer, *= Bergey's Manual of Systematic Bacteriology (Krieg, 1984)

Molecular detection of ***A****eromonas* spp. was performed by PCR a**s**say. *Aeromonas* spp. was confirmed by ampl**i**fication of the expected 276 bp amplic**o**n of lipase genes in all isolates (Fig. 1).

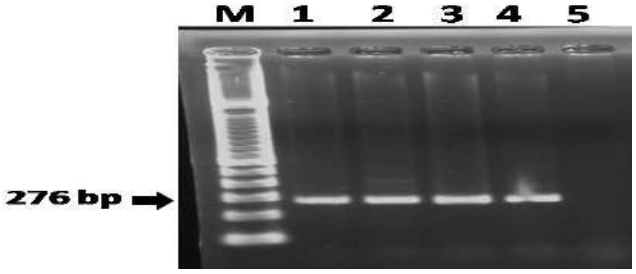

Fig. 1: PCR assay to am**p**lify *lipase* gene of *Aeromonas* spp.of marine Rupchada. Lane M: 1 kb DNA marker (Prom**e**ga, USA); lanes 1, 2, 3 and 4: DNA of *Aer**o**monas* spp. Isolated from marine Rupchada, lane 5: Negative control without DNA.

Prevalence of *Aeromonas hydrophila* in Rupchanda

Aeromonas hydrophila were isolated from gill and intestine samples of marine Rupchada. A **t**otal of 10 *Aeromonas hydrophila* were **i**solated from gill and intestine of marine Rupchada. The prevalence of ***A****. hydrophila* in gill and int**e**stine was 13.33% and 20%, respe**c**tively. The overall prevalence of *A. hydroph**i**la* in marine Rupchada was 33.33% (Ta**b**le 2). Prevalence of *A. hydrophila* was 40% in Kewatkhali fish market, 60% in KR market and 20% in Mesuabazar markets.

Table 2: Prevalence of *Aeromonas hydrophila* in gill and intestine samples of marine Rupchada

Name of samples	No. of Samples tested	No. of culture positive samples	Prevalence (%)	Overall Prevalence (%)
Gill	30	4	13.33	33.33
Intestine	30	6	20	

Antibiotic susceptibility profiles

All 10 isolates of *A. hydrophila* were found sensitive to seven antibiotics such as Gentamicin, Azithromycin, Ciprofloxacin, Tetracycline, Chloramphenicol, Erythromycin & Streptomycin and resistant against two antibiotics such as Ampicilin and Cephalexin (Table 3).

Table 3: Results of antibiotic sensitivity test of *Aeromonas hydrophila* isolated from Rupchada

Name of fish markets	No of isolates tested	No of isolates sensitive to antibiotics							No of isolates resistant to antibiotics	
		E	AZM	GEN	C	CIP	TE	ST	AMP	CN
Kewatkhali	4	4	4	4	4	4	4	4	4	4
KR market	4	4	4	4	4	4	4	4	4	4
Meshua bazar	2	2	2	2	2	2	2	2	2	2

GEN= Gentamicin, AZM= Azithromycin, CIP= Ciprofloxacin, AMP= Ampicillin and CN= Cefalexin, ST= Streptomycin, TE= Tetracycline C= Chloramphenicol, E= Erythromycin.

Discussion

The genus *Aeromonas* is widely distributed in aquatic environment and increasingly reported as a primary pathogen of human and lower vertebrates. Many of these bacteria are capable of causing human infection and intoxication. *Aeromonas* has been reported as an etiological agent in variety of human infections including gastroenteritis and extra intestinal infections. *Aeromonas hydrophila* causes disease in marine Rupchada under stress conditions or in association with infection by other pathogens. Bacterial causal agents of human disease transmitted from fish when used as a food or by handling them. This causes risks for public health, especially for immuno compromised individual children and aged person. Hence, there is need for public enlightment, campaign and general education to assist in curtailing the outbreak of diseases in human through ingestion of the bacteria along with fish. The purpose of the present work was to isolate, identify and determine the antibiotic profile of *A. hydrophila* of marine Rupchanda sold at three different fish markets of Mymensingh.

A total of 30 Rupchanda fishes were collected three local fish markets. Gill (n=30) and intestine (n= 30) were collected aseptically and inoculated into alkaline peptone (APW) water for enrichment at 37℃ for 8 hrs. Enriched cultured was streaked into TCBS agar to isolate bacteria. Identification of bacteria was performed by cultural characteristics, Gram's staining, sugar fermentation and biochemical tests and polymerase chain reaction (PCR) assay. The antibiogram profiles of bacteria were investigated against nine commonly used antibiotics (Gentamicin, Cefalexin, Ampicillin, Azithromycin, Erythromycin, Streptomycin, Tetracycline, Chloramphenicol and Ciprofloxacin) by disc diffusion method (CLSI, 2011).

In the present study, TCBS agar was used for isolation of *Aeromonas* spp. TCBS agar supports the growth of both *Vibrio* spp. and *Aeromonas* spp. (Kaysner and Depaola, 2004). The most frequently isolated species of *Vibrio* in fresh water are *V. cholera* and *V. mimicus* (Fouz *et al.*, 2002). In TCBS agar *V. cholera* produce yellow colour colony and *V. mimicus* produce green colour colony (Kaysner and Depaola, 2004). *Aeromonas* spp. on the other hand also produce yellow colour colony on TCBS agar. In this study, DNA extracted from yellow colour colony grown on TCBS agar successfully amplified 276 bp fragment of lipase gene confirmed bacterial isolates belonged to *Aeromonas* spp. (Delamare *et al.*, 2012). Additionally, differential diagnosis between *Vibrio* spp. and *Aeromonas* spp. were also performed by Gram's staining technique, lactose fermentation test and VP test. *Vibrio* spp. are Gram negative, comma shaped or curved rod, non- lactose fermenter and VP negative (Brooks *et al.*, 2007; Islam *et al.*, 2013; Kaysner and Depaola, 2004; Jayashinghe *et al.*, 2008). In this study, bacteria isolated from Rupchada were Gram negative, rod shaped, lactose fermenter and VP test positive which are characteristics for *A. hydrophila*. Another differential diagnosis carried out to differentiate *Vibrio* spp. from *Aeromonas* spp. by their growth characteristics in nutrient broth containing 0% and 6% NaCl (Kaysner and Depaola, 2004; Jayashinghe *et al.*, 2008). *V. cholera* and *V. mimicus* cannot grow in nutrient broth containing 6% NaCl.

Ashiru *et al.*, (2011) isolated *A. hydrophila*, *A. caviae* and *A. sobria* from Tilapia fish and Catfish. These three species of *Aeromonas* can be differentiated by lactose and sucrose fermentation tests. *A. hydrophila* can ferment lactose and sucrose. On

the contrary, *A. caviae* and *A. sobria* cannot ferment lactose and sucrose (Ashiru *et al.,* 2011). In this study, bacterial isolates of Rupchada fermented lactose and sucrose confirming their identity as *A. hydrophila.* In present study, 10 *A. hydrophila* were isolated and identified from Rupchada. The prevalence of *A. hydrophila* in marine Rupchada in this study was 33.33%. In Iran, 13.89% prevalence of *A. hydrophila* in Rupchanda was reported by Khamesipour *et al.,* (2014). Vivekanandhan, (2005) reported 17.62% prevalence of *A. hydrophila* in Rupchanda in India. In the current study, gill and intestine samples were screened for *Aeromonas* spp. since these samples were also analyzed by other investigators (Lijon *et al.*,2015; Ashiru *et al.,* 2011; Jayasinghe *et al.,* 2008). This study detected the presence of *A. hydrophila* in gill and intestine samples of Rupchada. The prevalence of *A. hydrophila* was the highest in intestine (20%) followed by gill (13.33%). Vivekanandhan (2005) also recorded the highest prevalence of *A. hydrophila* in the intestine (38.43%) as compared to and gill (29.10%).

A. hydrophila are sensitive to ciprofloxacin and gentamicin (Ko *et al.,* 2003; Truong *et al.,* 2008; Overman, 1980) and resistant to ampicilin (Geiss and Freij, 1989 and Overman, 1980) Cefalexin. In this study, all *A. hydrophila* were found to be sensitive to ciprofloxacin, gentamicin and azithromycin and resistant to ampicillin and cefalexin. Similar antibiotic resistance patterns of *A. hydrophila* isolated from fresh water prawn were reported by Lijon *et al.* (2014). Antibiotic resistance frequencies and profile varied according to the source of the strains (Ko *et al.,* 1996). The widespread use of antibiotics in the aquaculture systems and agricultural sectors in Bangladesh may act as the source of antibiotics diffusion into the sediment (Sorum, 2006). Sometimes fishes are treated with some antibiotic solutions to extend their shelf life. The broad spectrum antibiotics, tetracyclines, chloramphenicol etc. have been used to extend the shelf life of fish (Balachandran, 2001).

The results of this study indicate that *A. hydrophila* are prevalent in marine Rupchada. Data of antibiogram profiles suggest that marine Rupchada harbors multidrug resistant *A. hydrophila.*

CONCLUSIONS

The results of the current study indicated that the marine Rupchada sold at the local fish markets of Mymensingh city carry multidrug resistant *A. hydrophila* which may cause health hazard in consumers if enter into the human food chain.

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DETECTION OF FOOD BORNE BACTERIA FROM SALAD VEGETABLE ITEMS OF LOCAL MARKETS AND THEIR ANTIBIOGRAM PROFILE

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Abstract

The study was conducted to evaluate bacterial flora of salad vegetable items and for isolation, identification, characterization and antibiogram studies of the organisms obtained. For this, a total of 90 samples from mixed vegetables which are commonly used for salad such as tomato, lemon, green chili, coriander leaf, carrot and cucumber were collected from five different markets located in Mymensingh city. All the vegetables were highly contaminated with bacterial flora. Range of microbial count of tomato was log 6.276 CFU/ml to 6.543 CFU/ml, lemon was log 5.493 to 6.261 CFU/ml, green chili was log 5.205 to 5.64 CFU/ml, coriander leaf was log 7.055 to 7.759 CFU/ml, carrot was log 6.786 to 7.221 CFU/ml and cucumber was log 5.469 to 6.845 CFU/ml respectively. All these samples were also analyzed by culturing in different selective media such as Salmonella-Shigella (SS) agar, Eosin Methylene Blue (EMB) agar, Acetamide agar, M-Aeromonas agar, Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar and Nutrient agar. Biochemical tests were performed to identify the organisms. Out of 90 salads vegetable samples a total of 234 bacterial isolates of six genera were identified. *Pseudomonas* spp. (28.20%) was the most dominant followed by *Bacillus* spp. (20.94%), *Aeromonas* spp. (20.51%), *Vibrio* spp. (16.66%), *Salmonella* spp. (8.11%) and *E. coli* (5.55%) respectively. Results of antibiotic sensitivity test showed that, out of five antibiotics *Salmonella* sp., *Bacillus* sp., *Pseudomonas* sp., *Aeromonas* sp. and *E. coli* were equally sensitive to three antibiotics such as Chloramphenicol, Ciprofloxacin and Gentamicin and resistant to Ampicillin and Cefalexin respectively. Whereas *Vibrio* sp. was sensitive to Chloramphenicol and Gentamicin, intermediately sensitive to Ciprofloxacin and Ampicillin and resistant to Cefalexin. In this study the bacterial load was highest at Natunbazar and lowest bacterial load was recorded in KR market at BAU campus. These results suggest that the necessity to follow the hygienic practices of handling the salad vegetable items in local markets of Mymensingh city, Bangladesh

Keywords*:* Salad vegetables, Microbiological quality, Multi-drug resistant bacteria, Mymensingh city

INTRODUCTION

Vegetables are considered a good dietary source of nutrients, micronutrients, vitamins and fiber, needed vitally for health and well-being of humans. Well balanced diets, rich in vegetables, are especially valuable for their ability to prevent vitamin C and vitamin A deficiencies are reported to minimize the risk of several diseases (Kalia and Gupta, 2006). Tomato, cucumber, carrot, green chili, lemon and coriander leaf are recognized as some of the salad vegetables that are normally consumed raw. Their traditional use in preparing salads is familiar throughout the world. In recent days' increase in health awareness has led to consumption of minimally processed foods (Wells and Butterfield, 1997). Salad vegetables have thus become popular as it suits the present day necessity and does not need any elaborate preparations (Tournas, 2005).

Vegetables are widely exposed to microbial contamination through contact with soil, dust and water and by handling at harvest or during postharvest processing. Microbial spoilage and contaminating pathogens pose a serious problem in food safety. Several outbreaks of gastroenteritis have been linked to the consumption of contaminated salad vegetables. The world's largest reported vegetables borne outbreak, occurred in Japan in 1996 in which 11,000 people affected and about 6,000 cultures were confirmed. The outbreak involved the death of the children and was caused by *E. coli* (Beuchat, 1996).

Usually four categories of microbiological quality have been determined based on standard plate counts, levels of indicator organisms and the number or presence of pathogens. These are satisfactory, marginal, unsatisfactory and potentially hazardous. Satisfactory results indicate good microbiological quality. No action required. Marginal results are border line in that they are within limits of acceptable microbiological quality but may indicate possible hygiene problems in the handling of the salad vegetables. Premises that regularly yield borderline results should have their vegetables handling controls investigated. Unsatisfactory results are outside of acceptable microbiological limits and are indicative of poor hygiene or food handling practices. Potentially hazardous, the levels in this range may cause foodborne illness and immediate remedial action should be initiated. Limited works have been carried out to determine microbial contamination of vegetables in Bangladesh (Nipa *et al*, 2011; Rahman and Noor, 2012) but a comprehensive study needs to be performed to determine the wide range of bacteria associated with contamination of vegetables.

Materials and Methods

Collection of samples

A total of 90 salad vegetable samples such as tomatoes (n=15), lemon (n=15), green chili (n =15), coriander leaf (n=15), carrot (n=15) and cucumber (n=15) were collected from five different markets of Mymenshing Sadar Upazilla. The samples were transported to the bacteriology laboratory at 4°C temperature using an ice box.

Isolation of bacteria

Samples were enriched in nutrient broth at 37°C for 24 hours. The overnight bacterial broths were streaked on SS agar (for *Salmonella* spp*)*, EMB (for *E. coli*), NA (for *Bacillus* spp.), MA agar (for *Aeromonas* spp.), AC agar (for *Pseudomonas* spp), TCBS agar (for *Vibrio* spp.) which were incubated at 37°C for 24 hours. Single colony was further subculture until pure culture was obtained.

Identification of bacteria

Colony characteristics of bacteria such as shape, size, surface texture, edge, elevation and color observed in pure culture, Gram's staining and biochemical tests (sugar fermentation, Methyl red, Voges-Proskauer and Indole production) were used for identification of bacteria (Cheesbrough, 1985).

Motility Profile

E. coli and *Bacillus* spp. were found non motile but *Salmonella* spp., *Pseudomonas* spp., *Aeromonas* spp. and *Vibrio* spp. were found motile when examined their hanging drop slides under microscope.

Biochemical tests

Results of sugar fermentation tests using five basic sugars such as dextrose, maltose, lactose, sucrose and mannitol are measured in Table 4. Acid and gas production was indicated by change of color of Phenol red from red to yellow and presence of gas bubbles in Durham's tube. Negative reaction was indicated by no change of color.

Antibiotic sensitivity test

Six isolates randomly selected from six genera were tested for antimicrobial drug susceptibility against 05 commonly used antibiotics such as Ampicillin (10 µg), Chloramphenicol (30 µg**)**, Ciprofloxacin (5 µg**)**, Gentamicin (10 µg) and Cefalexin (30 µg) by disc diffusion or Kirby-Bauer method (Bauer *et al*., 1966). Results of antibiotic sensitivity tests were recorded as sensitive, intermediate and resistant following the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2007).

RESULTS

Total viable count (TVC) of salad vegetables

The mean Log CFU of bacteria in tomato, lemon, green chili, coriander leaf, carrot and cucumber samples collected from different markets are presented in Table 1.

Table 1: Total viable count (TVC) per ml of total samples from different markets

Markets name	Tomato (mean log CFU±SD)	Lemon (mean log CFU±SD)	Green chili (mean log CFU±SD)	Coriander leaf (mean log CFU±SD)	Carrot (mean log CFU±SD)	Cucumber (mean log CFU±SD)
KR market	6.276 ± 0.177	5.493 ± 0.479	5.205 ± 0.074	7.055± 0.855	6.786 ± 0.543	5.469 ± 0.415
Sheshmore	6.415 ± 0.513	5.559 ± 0.506	5.556 ± 0.693	7.276± 0.100	6.989 ± 0.746	5.585 ± 0.546
Kewatkhali	6.485 ± 0.457	5.607 ± 0.470	5.62 ± 0.618	7.549± 0.394	7.134 ± 0.807	5.973± 0.505
Mesuabazar	6.302 ± 0.298	5.547 ± 0.369	5.545 ± 0.422	7.115± 0.834	6.927 ± 0.720	5.554 ± 0.501
Natunbazar	6.543 ± 0.531	6.261 ± 0.113	5.64 ± 0.571	7.759± 0.499	7.221 ± 0.966	6.845 ± 0.570

Isolation of bacteria

Six genera of bacteria such as: *Salmonella* spp., *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. and *E. coli*, were isolated from different salad vegetables are presented in Table 2.

Table 2: Summary of isolation of bacteria from different salad vegetables

Name of samples	Bacterial genera (Number and percentages)					
	***Salmonella* spp. (n)**	***Bacillus* spp. (n)**	***Pseudomonas* spp. (n)**	***Aeromonas* spp. (n)**	***Vibrio* spp. (n)**	***E. coli* (n)**
Tomato (n=15)	5(33.33%)	6(40%)	9(60%)	7(46.66%)	6(40%)	ND
Lemon (n=15)	ND	8(53.33%)	7(46.66%)	5(33.33%)	5(33.33%)	ND
Green chili (n=15)	ND	9(60%)	13(86.66%)	10(66.66%)	8(53.33%)	4(26.66 %)
Coriander leaf (n=15)	6(40%)	11(73.33%)	12(80%)	9(60%)	9(60%)	3(20%)
Carrot (n=15)	5(33.33%)	9(60%)	14(93.33%)	11(73.33%)	7(46.66%)	6(40%)
Cucumber (n=15)	3(20%)	6(40%)	11(73.33%)	6(40%)	4(26.66)	ND

ND= Not Detected

Identification of bacteria

Identification of bacteria was performed by cultural characteristics, staining methods and biochemical tests. Summary of cultural characteristic, sugar fermentation and biochemical tests of *Salmonella* spp., *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp.and *E. coli* are presented in Table 3 and Table 4.

Table 3: Cultural characteristics of the bacterial isolates

Sl No.	Name of bacteria	Name of selective media	Colony characteristics
1	Salmonella spp.	SS agar	Opaque, smooth, round with black centered
2	Bacillus spp.	Nutrient agar	Thick, grayish-white or cream colored colonies were produced
3	Pseudomonas spp.	Acetamide agar	Purplish red colour colony
4	Aeromonas spp.	M-Aeromonas agar	Yellow brown colony
5	Vibrio spp.	TCBS agar	Yellow colony
6	*E.coli*	EMB agar	Metallic sheen(Greenish black) colony

SS=Salmonella Shigella agar; EMB=Eosin Methylene Blue; TCBS=Thiosulfate Citrate Bile Salts Sucrose

Table 4: Biochemical characteristics of the bacterial isolates

Carbohydrate fermentation test					MR test	VP test	Indole test	Interpretation
DX	ML	L	S	MN				
A	A	-	-	A	+	-	+	Salmonella spp.
AG	A	A	AG	AG	-	+	-	Bacillus spp.
A	A	-	-	-	-	-	+	Pseudomonas spp.
AG	-	-	-	-	+	-	+	Aeromonas spp.
A	A	-	A	A	+	-	+	Vibrio spp.
AG	AG	AG	AG	AG	+	-	+	*E.coli*

DX=Dextrose, ML=Maltose, L=Lactose, S=Sucrose, MN=Mannitol; A=Acid, AG= Acid & Gas; '+'=Positive; '- '=Negative; 'MR'=Methyl red; 'VP'=Voges Proskauer

Overall prevalence of *Salmonella* spp., *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. *and E. coli*

Among 90 samples total number of isolates 234, Prevalence rate of *Salmonella* spp. was 8.11% (19 of 234). Whereas 20.9% (49of 234), 28.20% (66 of 234), 20.5% (48 of 234), 16.66% (39 of 234) and 5.5% (13 of 234) for *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. and *E. coli* are shown in figure 1.

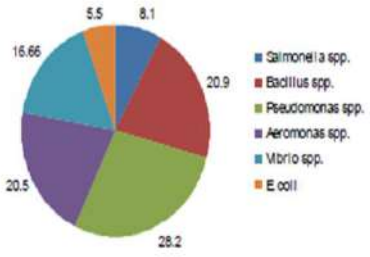

Fig. 1: The prevalence rate of *Salmonella spp., Bacillus spp., Pseudomonas spp., Aeromonas spp., Vibrio spp. and E. coli*

Antibiotics sensitivity tests

Summary of antibiogram profile of *Salmonella* sp., *Bacillus* sp., *Pseudomonas* sp., *Aeromonas* sp., *Vibrio* sp. and *E. coli.* against antibiotics are presented in Fig 2. *Salmonella* sp. was sensitive to 3 and resistant to 2 antibiotics, *Bacillus* sp. was sensitive to 3 and resistant to 2 antibiotics, *Pseudomonas* sp. was sensitive to 3 and resistant to 2 antibiotics, *Aeromonas* sp. was sensitive to 3 and resistant to 2 antibiotics, *Vibrio* sp. was sensitive to 2 and intermediately sensitive to 2 antibiotics but resistant to one antibiotic and *E. coli* was sensitive to 3 and resistant to 2 antibiotics.

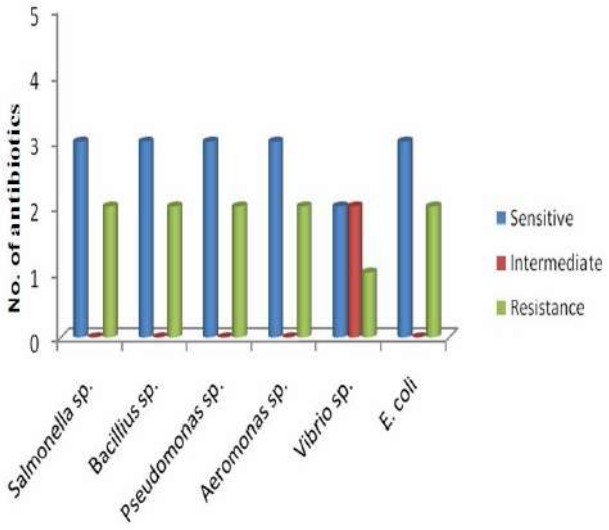

Fig. 2: Summary of antibiogram profile of *Salmonella sp., Bacillus sp., Pseudomonas,* sp., *Aeromonas sp., Vibrio sp. and E. coli against 5 antibiotics.*

Summary of antibiogram profile of Ampicillin (AMP), Chloramphenicol (C), Ciprofloxacin (CIP), Gentamicin (GEN) and Cefalexin (CN) against *Salmonella* sp., *Bacillus* sp., *Pseudomonas* sp., *Aeromonas* sp., *Vibrio* sp. and *E. coli* are presented in Fig. 3. All bacterial isolates (n=6) were sensitive to 2 antibiotics such as Chloramphenicol and Gentamicin. Five isolates were sensitive to Ciprofloxacin. All isolates were resistant to Cefalexin. Five isolates were resistant to Ampicillin. One isolates were intermediately sensitive to Ciprofloxacin and Ampicillin.

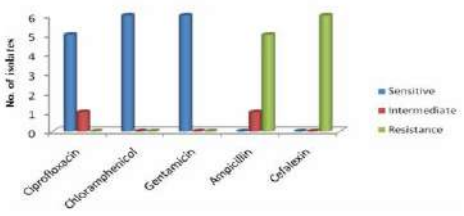

Fig. 3: Antibiogram profiles of bacteria isolated from salad vegetables.

Discussion

The present research work has been chosen to measure microbial loads in salad vegetables items sold in five markets in Mymensingh city and isolate and identify bacteria from salad vegetables items. High total viable count (TVC) indicates unsafe condition and therefore the occurrence of possible contamination.

In case of tomatoes washing sample, the highest TVC mean value was found at Natunbazar where it was log 6.543 CFU/ml and the lowest value was at KR market which was log 6.276 CFU/ml. The TVC value was lower to moderate in the vegetables sample Mesuabazar, Sheshmor and Kewatkhali.

The result of microbial load of lemon washing samples has also shown the similar condition. Lemon collected from Natunbazar with lower hygienic condition was evidenced consistently high mean value (log 6.21 CFU/ml). On the other hand, the lowest bacterial load of lemon washing sample was observed at KR market (log 5.493 CFU/ml). The mean values of TVC of green chili was found highest at Natunbazar where it was log 5.64 CFU/ml and the lowest at KR market which was log 5.205 CFU/ml. The TVC value was lower to moderate in the vegetables sample Mesuabazar (log 5.545), Sheshmor (log 5.556) and Kewatkhali (log 5.62). In case of coriander leaf washing sample, the highest mean values of TVC was found at Natunbazar (log 7.759 CFU/ml) and the lowest value was at KR market where it was log 7.005 CFU/ml.

The obtained TVC mean value of lemon, green chili and coriander leaf washing samples of the present study are in close agreement with the findings of Nipa *et al.* (2011). She also reported that, total bacterial population was log 5.62 CFU/ml, log 4.80 CFU/ml and log 5.5 CFU/ml for lemon, green chili and coriander leaf sample respectively.

Unfortunately, or it might be the real situation that the mean values of TVC of carrot and cucumber washing samples has also depicted the same hygienic level as found in tomatoes, lemon, green chili or coriander leaf washing samples. Carrot collected from Natunbazar with lower hygienic condition was evidently high mean value (log 7.221 CFU/ml) and the lowest at KR market (log 6.786 CFU/ml. In case of cucumber washing sample, the highest TVC mean value was found at Natunbazar where it was log 6.854 CFU/ml and the lowest was at KR market where it was log 5.469 CFU/ml. The TVC value was lower to moderate in the vegetables sample Mesuabazar, Sheshmor and Kewatkhali.

The obtained TVC mean value of carrot and cucumber washing samples of the present study are in close agreement with the findings of Uzeh *et al.* (2009). He also reported that, total bacterial population was log 6.77 CFU/ml and log 3.11 CFU/ml for carrot and cucumber samples respectively. This variation may be due to variation in maintained sanitary, handling and preservation condition of vegetables. Doores (1983) suggested to pay careful attention to maintain a microbiologically stable environment to achieve high quality in raw vegetables and processed products.

In this study, the colonies of *Salmonella* spp. on SS agar plate were opaque, translucent with black centers which were similar to the findings of other authors (Cheesbrough, 1985). In Gram's staining bacteria exhibited short rods, Gram negative, single or paired in arrangement. Similar findings were also reported by Buxton and Fraser (1977). Sugar fermentation tests profile of *Salmonella* spp. in the present study showed similarities with the findings of other researchers (Cowan, 1985). Morphology and staining characteristics of *Bacillus* spp. recorded in this study are in agreement with the finding of the other researchers (Merchant and Packer, 1967). Biochemical test of *Bacillus* spp. was able to ferment the five basic sugars by producing acid (Granum, 2001**)**. All the isolates fermented dextrose, sucrose, lactose, maltose and mannitol with the production of acid within 24h-48h of incubation.

In this study, the colonies of *Pseudomonas* spp. on Acetamide agar plate were purplish red colour which was similar to the findings of Izumi *et al.* (2004**)**. The result of Gram's staining was also in agreement with the report of Izumi *et al.* (2004). Sugar fermentation and catalase test are positive of *Pseudomonas* spp. in the present study. In this study, the colonies of *Aeromonas* spp. on M-Aeromonas agar plate were yellow-brown colour. In Gram's staining bacteria exhibited short rods, Gram negative, single, paired arrangement. Sugar fermentation and catalase test are positive of *Aeromonas* spp. in the present study.

In this study, the colonies of *Vibrio* sp 5 3S agar plate which were similar to the findings of other authors Khan *et al.*, (2007). In Gram's staining bacteria exhibited curved rod shaped appearance which was supported by other researchers (Faruque *et al.*, 2008; Kaper *et al*., 1995). In this study *Vibrio* spp. produced acid in dextrose, maltose, mannitol and sucrose and negative to VP test (Kaper *et al*., 1995).

In this study, different selective and differential agars are used for isolation of *E. coli* from samples. Colony characteristics of *E. coli* observed in EMB agar were similar to the findings of other authors Sharada *et al.* (1999)**.** Morphologically *E. coli* were Gram negative short rod arranged in single or paired and motile. Several researchers also described similar cultural, staining and motility characteristics of *E. coli* Thomas *et al.* (2005). The identified bacteria were re-confirmed through the use of different sugar fermentation and other biochemical tests which were found similar with the findings of other researchers Thomas *et al.* (2005).

In the present study, antibiotic sensitivity or resistance pattern (antibiogram profile) of isolated bacteria was investigated against five commonly used antibiotics (Chloramphenicol, Gentamicin, Ciprofloxacin, Cefalexin and

Ampicillin) using disc diffusion method (Bauer *et al.*, 1966). The results of antibiogram showed that *Salmonella*, *Bacillus*, *Pseudomonas*, *Aeromonas* and *E. coli* were almost sensitive to Chloramphenicol, Gentamicin and Ciprofloxacin, only *Vibrio* spp. were intermediately sensitive to Ciprofloxacin. Whereas, almost all the bacterial isolates showed resistance pattern to Cefalexin and Ampicillin, except *Vibrio*, which showed completely resistance to only Cefalexin, but intermediately sensitive to Ampicillin.

Nawas *et al.* (2012) found multiple drug resistance (MDR) in 39 and 51 isolates of *Salmonella* and *Vibrio* isolates, respectively among a total of 102 isolates, using disc diffusion method. The author reported that *Salmonella* spp. from salad and water showed resistance against Amoxicillin (75%), Cephradine and Cefalexin (68.75%). 85.71% *Vibrio* spp. isolated from salad and water were resistant to Amoxicillin respectively. The antibiogram profile of *Bacillus* spp. was almost similar to the findings of Whong and Kwaga (2007), where the author found that all *B. cereus* isolates were found to be susceptible to Ciprofloxacin, Chloramphenicol and Ofloxacin and overall resistance to Penicillin G (82%), Cefotaxime (56.7%), Ceftriaxone (53.3%) and Ampicillin (44%) were most frequent.

Łaniewska-Trokenheim *et al*. (2006) examined the antibiotic resistance pattern of *E. coli* and found that the highest number of isolated strains were resistant to Ampicillin (81.9%), whereas a lower number of the strains exhibited resistance to the Chloramphenicol (19.8%). *A*ntibiotic sensitivity test of the *Pseudomonas fluorescens* isolates were conducted by Foysal *et al.* (2011) using disc diffusion method for seven antibiotics, where all of the isolates were found to be sensitive only against Streptomycin and Gentamicin but, most of the isolates (80%) were found resistant to Chloramphenicol less similar with the findings of antibiogram profile of *Pseudomonas* spp. conducted in the present study.

CONCLUSIONS

A significantly high bacterial load was recorded in all vegetable samples in the study. Six genera of bacteria were isolated such as *Salmonella* spp., *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. and *E. coli.* However, *E. coli* was not found in tomatoes, lemon & cucumber and *Salmonella* spp. was not found in lemon & green chili. Antibiogram profiles of this study indicated that all six bacterial genera of vegetables are multidrug resistant which may cause human food-born infection and intoxication.

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HYGIENE CONTROL AND EMPLOYEES "HANDS WASHING" EDUCATION IN SUSHI RESTAURANT CHAIN SHOPS

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Abstract

In order to determine major process for hygiene control in Sushi restaurant chain, analysis was made on microbiological testing results from all stores of the chain to extract issues to threaten hygiene control.

According to the data analysis, it turned out that there are two routes of microbiological contamination. One is a parallel contamination through wiping cloth, and the other is vertical contamination from floors to working tables, for instance, by putting cookware on the floor and then onto the working table.

Considering the results, it is shown that cutting off of these two routes is critical. Taking account into consideration above, the authors set 4 control measures: 1) introduction of disposable wiping cloth, 2) prevention of cookware direct contact, 3) routine spraying of alcohol, and 4) education of "hand washing" to employees.

Education item that was carried out with particular care to employees is a hand-washing, such as before starting work. Microbiological efficacy of handwashing was determined using ATP luminescence method. Using the ATP luminescence method to investigate the quality of hand washing to review the education program, we decided to change the hand soap formulation which had been conventionally used, by a result of analyzing the data. New disinfectants contained in the new detergents triclosan have been reported evaluated already at the effect much

To examine the education plan, the employees were stratified due to age and years of service. We find out that since most employees are employed at the time the store opened, then the first education should be held before store open, and re-education should be held at one-year point after the store opened. Furthermore, it was found that there is a special need to re-educate the teens and 60s at after first year from joining our team.

Keywords: Conveyor-belt sushi-restaurant chain, Restaurants, Microbial contamination route, Hand washing and disinfection, Employee education, ATP Luminescence Measurement Method

INTRODUCTION

While compliance to an established manual is the basis of sanitary management in the foodservice industry, especially in businesses handling perishable food products, the majority of on-site workforce is a part-time employee. Education of such employees on sanitation is therefore vital. Recently, many cases have been reported where young part-time employees jokingly take an irrational action and post it on a social network site, which results in such employees being criticized. It is increasingly becoming important to provide training to such employees. Adachi et. al. (2012) has investigated hand washing at home and school for primary school students by each grade level and the relationship thereof with involvement training by their guardian and the like. It is conjectured that not only education provided upon joining the firm, but also age, education and training etc. make meaningful contribution to the effect of hand and finger washing by employees in the food service industry.

The Food Safety Network operating in the Japan Kansai region advocates "Food Hygiene 7S" as means for pursuing cleanliness at food production sites at the microbial level. The Food Safety Network (2006, 2008, 2009, 2013) defines rules for shifting, systematic arrangement, sweep, scrub, and sanitation there among and emphasizes the necessity for having employees properly perform tasks in line with the manual. The Food Safety Network states that "self-discipline" there among is important in order to have employees correctly implement the rules and manuals, and education is at the core of self-discipline.

Although Satsuta et. al. (2011) has published a bacteriological study focusing on sushi toppings of conveyor belt sushi, the study does not discuss sanitation management or employee education at restaurants. Original methods of employee education are developed at each restaurant of restaurant chains, but very few systems are published with the exception of McDonald's examples (H.Yamaguchi 2000, 1998).

The authors (2015) have measured the level of microbial contamination in the environment of conveyor belt sushi chain restaurants among the foodservice industry and studied the microbial contamination route from the results thereof to discuss the need for employee education. And the authors mainly focused on sanitation management and employee education at a conveyor belt sushi chain as in the previous report, examined the timing of hand and finger washing education and plan to be carried out at the restaurants

and conducted a study based on the resulting data, which is reported hereinafter.

Materials and Methods

1. Restaurants and Employees Subjected to Experiment

Akindo Sushiro Co. established in 1984. was selected for this study.

Four restaurants were selected as the restaurants subjected to experiment from over 400 chain restaurants in the Kanto, Kansai, and Shikoku regions in Japan while taking into consideration the restaurant size, conventional state of sanitation management etc. All (total of 262) employees (part-time employees) who have reported for work at the selected 4 restaurants from July to September of 2015 were subjected to tests of hand washing effect by using a swab test method. Since employees reporting to work vary depending on the day of the week, time frame, etc., employees have, although in limited number, participated in hand washing tests of different methods. However, examination and analysis were conducted on the data in this article as independent data.

2. Detergents and Disinfectants Used

Conventional, standard recommended hand washing (Fig 1) uses different detergents for hand washing three times. Para-chloro-meta-xylenol (PCMX) is used as a disinfectant in the hand soap used in the first and third hand washing. A formulation (isodine) having a main ingredient of povidone iodine for disinfection of fingers and the skin of the palms of hands was used in the second hand washing.

After examining the effect of detergents, hand soap which uses the disinfectant triclosan reportedly having a more potent antimicrobial activity than PCMX was employed

3. ATP Luminescence Measurement Method

The ATP swab test method (ATP Luminescence Measurement Method) was used to quantify the results of swab tests. E. L. Larson et.al.(2003) negatively assess the ATP method as a quick method widely used in the food service industry, which can be a rough indicator for the amount of microbial contamination, but has no correlation with risks from food poisoning-causing microbes. However, the ATP method is used extensively and is proven to be effective as an indicator capable of quickly assessing microbial contamination in hospital environments and the like, where infection would be problematic (H. SHIBATA (2014), N.Kajigaya(2015)) .

Before and after hand and finger washing, Kikkoman Biochemifa Company's Lumitester PD-30 and a dedicated measurement reagent "Luci Pac Pen" were used in swab tests on hands and fingers, where the resulting RLU (Relative Luminessence Unit) indicates the level of contamination of the hands and fingers. For the principle of Lumitester, Suzuki et. al. and Sakakibara et. al. (1999) have examined the relationship between RLUs obtained from the ATP swab test method using a luciferase reaction and microbial test results.

4. Examination of Time of Hand Washing

Each employee practices the standard recommended hand washing according to the "Hand Washing Manual" shown in Fig. 1 prior to starting their shift. Specifically, employees first wash their hands upon reporting to work by using hand soap (first hand washing), and then wash their hands using isodine (second hand washing). After changing into their uniforms, the employees further wash their hands by using hand soap prior to entering the workplace such as the kitchen (third hand washing hand washing including hand disinfection).

Figure 1: Standard Recommended Hand Washing

Several examinations were conducted on the effect of hand washing due to the frequency of hand washing and time of one hand washing etc.

1) Washing the palm and back of the hand for the frequency stipulated in the manual and rinsing at half the frequency of washing required 30 seconds or more. Employees who washed their hands by this method are considered to be part of the standard recommended hand washing group.

2) Washing and rinsing the palm and back of the hand 5 times each could be completed in 30 seconds or less. Employees who have washed their hands by this method are considered to be part of the simplified hand washing group.

The effect of hand washing was compared between the two aforementioned groups, while the baseline of acceptable hand washing was RLU of 1,500 or less in accordance with value recommended by Kikkoman[17].

5. Statistical Analysis

Statistical estimation and test were based on the significance level of $\alpha = 0.05$. The 95% confidence interval was calculated by assuming a normal distribution. Although data is partially derived from the same employees, such data was limited. The difference in two ratios was thus tested by using $\chi 2$ -**test** (Yates' correction, F. Yates (1934)) in a 2x2 contingency table while assuming independence

Result and Discussion

Restaurant characteristics subjected to experiment

The Corporate Motto is “Delicious Sushi Fills the Belly, Delicious Sushi Also Fills the Heart”. The mission is to provide "delicious sushi! of course, and to also satisfy the customer's heart” including maintaining a relaxing store environment, and our employees recite this corporate motto every day.

The company's biggest characteristic is “product appeal”, for which they take great pride. In particularly they have a strong preference for “in-store food preparation”. For example, the sushi rice is cooked each day for that day's portion and is never rolled over to the next day. Maguro tuna uses the same method as the maguro tuna specialty shops in Tsukiji (where is Japanese famous fish market in Tokyo), i.e., thawing two times each day, and slicing by the employees. For yellowtail sea bream, or other fresh fish, as well, the company has set a rule to provide, as much as possible, “fresh slice” in “amounts just enough to supply the next 30 minutes”. They also prepare side menus one at a time. The chawan-

mushi (a steamed egg custard), red miso soup, noodles, and other soup stock are also made in-house.

Organization and Main Operations of the Quality Control Office

Since the company so much prefer in-store food preparation, at the store they perform quite a large number of "manual operations". As a result, they have a strong awareness that, within the store, they "must firmly engage in strict sanitation control at the same level as food product plants".

To perform sanitation control thoroughly, both "establishment of a framework and rules" and "efforts to observe the framework and rules" are extremely important. As a result! at their company, they established the Quality Control Office as shown in Fig. 2 that is engaged in (1) store controls (store audits and employee education, etc.) and (2) plant controls (plant audits, food materials [bacteria count, quality] controls). Below is an introduction to the main efforts in which they have engaged.

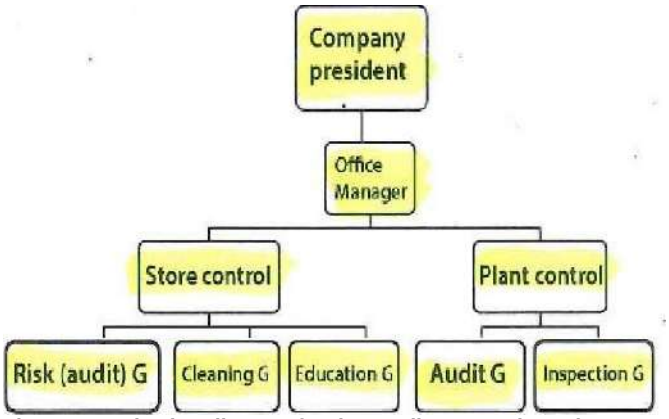

Fig. 2: Organization diagram for the Quality Control-Section

Store controls/Store audit by QC Office

They target all 362 stores, performing audits once every two months. The people in charge of audits number nine persons, the audit requires about 3-4 hours for each store (including the time required for the audit, plus the time for feedback of the audit results to the store manager), and about 130 audit items are set. The persons in charge of audits perform audits at pace of two stores per day.

The audit results are shown on cards which is divided into three colors: black, red, and yellow (in order of audit result from bad to good), and stores with excellent sanitation control are granted card with an "Excellent" mark on it. The black and red are classified in-house as "risk stores", and the section manager or area manager who have jurisdiction over the store, are handed an improvement order, after which they perform improvement actions.

Employee educations

Employee education can be broadly divided into (1) group training, (2) publication of "Sanitation Control Diary" (each month), and (3) mobiles test (once every 3 months).

l) About "group education"

Group education is divided broadly into training that targets new employees (for 3 hours on the day following company entry ceremony), training received when promoted to store manager or assistant store manager (2 hours), and training for "noro-virus countermeasures" targeting the store manager before noro-virus spreads (30 minutes to 1 hour every year in October).

2) About the "Sanitation Control Diary"

Every month, a series titled the "Sanitation Control Diary" is published. Here, what is difficult is that although some store managers actively make use of the "Sanitation Control Diary" in employee education, there are others who do not. However, unless the store manager firmly understands the content written in the 'Sanitation Control Diary" (basic knowledge related to sanitation management) and conveys that to the store staff it could lead to the occurrence of food poisoning.

Accordingly, the company need to exert some degree of "coercion", in getting the store manager to express the content of the "Sanitation Control Diary" to staff members. For examples QC audit could, in the interviews during an audit, ask, "have you conveyed the "Sanitation Control Diary" to staff members?" or place a telephone call to the store immediately after the "Sanitation Control Diary" has been issued and say, "Please tell us the content of today's Morning Assembly; did you convey the content of the "Sanitation Control Diary" ?", thereby confirming its use. The fact is that if the QC audit members do not do this, employee education will be difficult.

3) Regarding the "Mobile phone test"

To confirm that employee education is even reaching staff members without terminals, QC office perform a test utilizing mobile phones. This involves using a mobile phone to read the QR code, whereupon a URL is displayed, and clicking on the URL displays the question.

As many as 50 questions regarding basic problems have been prepared, of which 10 questions are set out. Staff members can answer the questions whenever they have a free moment during work hours. In the past, a paper-based test was used, but with the increase in employee numbers, then QC office switched to this style of using mobile phones. At present, for the test results QC office can use "point scores for all stores", "average for all stores", "average by area", "average by social level" and various other classifications to perceive the situation. In addition, for each store, since QC office can learn "who is not understanding the rules?", the store manager can also provide individual guidance to staff members with bad scores. In addition, the QC office can perform individual guidance for stores with bad scores.

Plant audits

The company does not manage their own company plants. For this reason, in QC audits of outside plants to which manufacturing is consigned have an extremely important position.

These audits are performed by three auditors (audit time per plant is about 6 hours). The frequency of plant audits is determined by the "food materials risk" (for example, "is raw food being processed?", "has the plant obtained HACCP certification?" etc.). The number of items audited is 185.

Microbial Contamination Routes and Its Preventive measures

In order to determine major process for hygiene control in Sushi restaurant chain, analysis was made on microbiological testing results from all stores of the chain to extract issues to threaten hygiene control.

According to the data analysis, it turned out that there are two routes of microbiological contamination. One is a parallel contamination through wiping cloth, and the other is vertical contamination from floors to working tables, for instance, by putting cookware on the floor and then onto the working table.

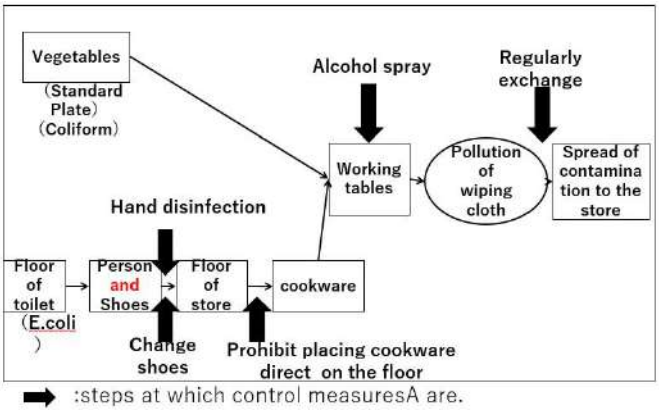

Figure 3: Estimation of pollution root and prevention measures introduced

Considering the results, it is shown that cutting off of these two routes is critical. Taking account into consideration above, the authors set 4 control measures: 1) introduction of disposable wiping cloth, 2) prevention of cookware direct contact, 3) routine spraying of alcohol, and 4) education of "hand washing" to employees.

Examination of Value for Determining Acceptability with RLU

When Kikkoman Biochemifa Company's Lumitester PD-30 and dedicated measurement reagent "Luci Pac Pen" are used to determine the effect of hand washing, Kikkoman suggestion RLU of 1,500 or less as an acceptable level and any value greater as a non-acceptable level. Table 1 shows the distribution of RLU values when hands were washed by the method in Fig 1 (standard recommended method). The values prior to hand washing were most frequently 5,000-10,000. 5 out of 67 employees had a value of 1,500 or less (7.46%: 95% confidence interval 1.17-13.8%), where the values were close to the threshold 1,500. Two employees had the most contaminated hands, exhibiting a maximum value of 50,001 or greater.

The distribution shifted materially toward smaller values from washing hands once with hand soap, such that the number of employees with an acceptable value of 1,500 or less was 52 out of 66 (78.8%: 68.9-88.7%), and the maximum RLU value of employees with non-acceptable levels was 10,000 or less. After washing hands three times with hand soap, isodine, and hand soap, the number of employees with an acceptable value of 1,500 or less was 64 out of 65 (98.5%: 95.5-100%), and the RLU value of employee with a non-acceptable level was 2,000 or less, such that most employees had a value close to an acceptable level.

Judging from such distributions, it is concluded that it is reasonable to determine RLU of 1,500 or less for acceptable employees' hand washing value. The effect of hand washing was examined hereinafter in accordance with this judgment.

Examination of Hand Washing Method

Effectiveness of hand washing was examined. Table 1 shows results, such as the number of employees exhibiting an acceptable RLU value of 1,500 or less after washing hands three times by standard recommended hand washing (Fig. 1), which is 30 seconds or more of hand washing using hand soap. The number of employees with an acceptable value prior to hand washing (0 times) was 5, which is 7.46% (95% confidence interval 1.17-13.8%) (Table 1-(1)). Meanwhile, the number of employees with an acceptable value after washing hands once with hand soap was 52, which is 78.8% (68.9-88.7%) (Table 1-(2)). It can thus be understood that the number of employees with an acceptable value is significantly higher after washing hands once.

Table 1-(3) shows results after washing hands three times, in order, with hand soap, isodine, and hand soap. The number of employees with an acceptable value after washing hands 3 times was 64, which is 98.5% (95.5-100%). The number of employees with an acceptable value after washing hands 3 times naturally is significantly higher than the number prior to washing. The results were able to show the effectiveness of hand washing. However, although the number of employees with an acceptable value after washing hands once was 52 which is 78.8%, the number of employees with an acceptable value after washing hands 3 times was 64 which is 98.5%. When washing hands once is compared to washing hands 3 times, the number of employees with an acceptable value is higher for washing hands 3 times, but a significant difference was not found there between. It was also found that the ratio of having an acceptable value was nearly 100% after washing hands 3 times.

Table 1: Effects of time and frequency on hand washing

		Frequency of hand washing	x	n	p	P-	P+
(1)	Conventional detergent/Standard recommended hand washing	0 times	5	67	0.07	0.01	0.14
(2)		1 time	52	66	0.79	0.69	0.89
(3)		3 times	64	65	0.98	0.95	1.01
(4)	New detergent/Simplified hand washing	0 times	1	64	0.02	-0.01	0.05
(5)		1 time	33	62	0.53	0.41	0.66
(6)		3 times	55	63	0.87	0.79	0.96
(7)	New detergent/Simplified hand washing (after one month)	0 times	1	63	0.02	-0.01	0.05
(8)		1 time	33	63	0.52	0.40	0.65
(9)		3 times	58	63	0.92	0.85	0.99

The effectiveness of hand washing was similarly examined with simplified hand washing for less than 30 seconds. The results are shown in Table 1-(4) to (6). The number of employees with an acceptable value prior to hand washing (0 times) was 1, which is 1.56% (0-4.6%). Meanwhile, the number of employees with an acceptable value after washing hands once was 33, which is 53.2% (40.8-65.6%). The number of employees with an acceptable value becomes significantly higher after 1 simplified hand washing, thus establishing that the hand washing method shown in Figure 4 is also effective.

Figure 4: Simplified Hand Washing

Standard recommended hand washing was compared with simplified hand washing. The number of employees with an acceptable value after simplified hand washing was 33, which is 53.2%, while the number of employees with an acceptable value after standard recommended hand washing was 52, which is 78.8%. It can be understood from the test results of both values that the number of employees with an acceptable value is significantly higher after standard recommended hand washing.

The same comparison was made after washing hands three times. The number of employees with an acceptable value after simplified hand washing was 55, which is 87.3% (79.1-95.5%), and the number of employees with an acceptable value after standard recommended hand washing was 64, which is 98.5%. It can be understood from the test results that the number of employees with an acceptable value is significantly higher after standard recommended hand washing. In view of the results, standard recommended hand washing, which takes 30 seconds or more, can be considered more effective.

Hand washing shown in Fig. 3 requires 30 seconds or more. However, employees expressed a desire to reduce the frequency of rubbing the palm and back of the hand to 5 times instead of 10 times such that hand washing can be completed in 30 seconds or less in order to shorten the time of hand washing. Thus, the effect of hand washing was examined when the conventional detergent that has been in use was replaced with an effective new detergent with a main ingredient of triclosan, which is considered to have a more potent antimicrobial activity, and rubbing and washing of the palm and back of the hand was reduced to 5 times to employ the simplified hand washing, which allows employees to wash their hands in 30 seconds or less.

The time and frequency of hand washing were examined. Table 1-(2) and (8) show results of comparing new detergent/simplified hand washing with conventional detergent/standard recommended hand washing after washing hands once. The number of employees with an acceptable value using the new detergent/simplified hand washing was 33, which is 53.2% (40.8-65.6%), while the number of employees with an acceptable value using the conventional detergent/standard recommended hand washing was 52, which is 78.8% (68.9-88.7%). It can be understood that the number is significantly higher for hand washing for 30 seconds or more, even with the conventional detergent, than hand washing for less than 30 seconds with a new detergent. Subsequently, new detergent/simplified hand washing and conventional detergent/standard recommended hand washing after washing hands three times were compared (Table 1-(3) and (9)). The number of employees with an acceptable value using the new detergent/simplified hand washing was 58, which is 87.3% (79.1-95.5%), while the number of employees with an acceptable value using the conventional detergent/standard recommended hand washing was 64, which is 78.8% (68.9-88.7%). A significant difference was not found there between.

In view of the above, standard recommended hand washing by the method of Fig. 4 can increase the effect of hand washing to an acceptable level while being barely affected by external factors such as detergent/ frequency/ period of washing. It was also found that the new detergent/ simplified hand washing can attain, after washing hands three times, almost the same hand washing effect as standard recommended hand washing of 30 seconds or more, such that requests of the employees involved in the operation of the restaurant could be answered.

However, the resulting data, when analyzed in detail after classification into groups for each restaurant, showed that the ratio of acceptable hand washing results, although not statistically significant, is a value that is less than 90% in 1 (restaurant A) out of 4 restaurants for new detergent/ simplified hand washing (Table 2). Since this value was problematic, it was decided to examine a method of standard recommended hand washing using a new detergent.

Table 2-(6) shows RLU values when hands and fingers were washed by using the new detergent according to the manual. The effect is prominent relative to the conventional detergent.

Table 2: Restaurant A: New detergent /Standard recommended hand washing

		Frequency of Hand Washing	x	n	p	P−	P+
(1)	Conventional detergent/Standard recommended hand washing	0 times	2	18	0.11	-0.03	0.26
(2)		1 time	15	18	0.83	0.66	1.01
(3)		3 times	18	18	1.00	1.00	1.00
(4)	New detergent/ Simplified hand washing	0 times	1	16	0.06	-0.06	0.18
(5)		1 time	5	16	0.31	0.09	0.54
(6)		3 times	13	16	0.81	0.62	1.00
(7)	New detergent/ Simplified hand washing (after one month)	0 times	0	16	0.00	0.00	0.00
(8)		1 time	4	16	0.25	0.04	0.46
(9)		3 times	15	16	0.94	0.82	1.06

Analysis of hand-washing results between some stratified employee groups

Hereinafter, focus is placed on the first hand washing. Table 3-(1) to (6) shows the number of employees with an acceptable value after washing once by standard recommended hand washing with hand soap for each number of years of employment

Table 3: Number of employees with passing value by years of employment/age group/shift time

			x	n	p	P-	P+
(1)	Years of employment	Less than 1 year	10	10	1	1	1
(2)		1 year to less than 5 years	24	33	0.73	0.58	0.88
(3)		5 years to less than 10 years	9	12	0.75	0.51	1.00
(4)		10 years to less than 15 years	6	6	1.00	1.00	1.00
(5)		15 years to less than 20 years	1	3	0.33	-0.20	0.87
(6)		20 years or more	2	2	1.00	1.00	1.00
(7)	Age group	10	3	8	0.38	0.04	0.71
(8)		20s	9	10	0.90	0.71	1.09
(9)		30s	7	7	1.00	1.00	1.00
(10)		40s	12	16	0.75	0.54	0.96
(11)		50s	6	7	0.86	0.60	1.12
(12)		60s	3	6	0.50	0.10	0.90
(13)	Shift time	AM shift	32	37	0.86	0.75	0.98
(14)		PM shift	21	30	0.70	0.54	0.86

The ratio of employees with an acceptable value was 100% for the groups with less than 1 year, 10-15 years, and 20 years or more of employment.

The number of employees with 1 to less than 5 years of employment was 33, accounting for 50% of the total. The number of employees with a non-acceptable value in this group was 9, which is 64.3% of all employees with a non-acceptable value and 13.6% of the total employees of 66. The group with 1 to less than 5 years of employment is thus

considered worthy of attention. Table 5 shows the ratio of employees with a non-acceptable value in this group. The ratio of employees with a non-acceptable value, 40%, was the highest after 1 to less than 2 years after joining the firm. It is conceivable that employees have relaxed after gaining some experience after joining the firm. For this reason, re-education regarding hand washing is considered necessary 1 year after joining the firm, in addition to the education provided immediately after joining the firm.

Similar several analysis was done, and decided education plan on hygiene to employees. For thoroughness of hand and finger washing by employees in conveyor belt sushi chain restaurant, authors examined the appropriate timing of education of employees and the like by utilizing ATP method, which can quickly assess results of hand washing and is used extensively in Japanese food industry. The authors were able to identify the timing of education to be 1 year after joining the firm and identify the target employee group as teenage employees and employees in their 60s.

CONCLUSION

For restaurants in the foodservice industry, not only management/processing of ingredients, but also management of the environment, employees, etc. are important in order to provide delectable food and to have customers enjoy safely prepared meals. Authors firstly focused on environmental management in the kitchen space to examine the transmission route of contaminating microorganisms in a restaurant and examine a method of stopping the transmission, and secondly examined hand and finger washing, which is the cornerstone of sanitation education/sanitation management, while targeting employees in a restaurant.

The ATP method was used to reevaluate the effect of hand washing by the hand washing method taught in conventional in-house training systems, and the data acquired was analyzed for each group with respect to several factors, resulting in some information on hand washing method itself as well as an education method therefor and the like.

In order to re-examine the education plan, the quality of hand washing was investigated by using the ATP method. It was decided, as a result of data analysis, to change the conventionally-used hand soap formulation. The disinfectant contained in the new detergent is triclosan, the effect of which has been already assessed and reported in many articles.

When employees in each restaurant were analyzed in groups such as age, years of employment etc., a characteristic RLU values after hand washing was found in the young teenage student part-time employee group and the group of employees in their 60s with increased wrinkles from aging. These groups especially need careful education.

This concept was applied to a chain restaurant, where most employees are hired when the restaurant opens. It was thus found that the closest path to effectively stabilize the sanitary state of a restaurant is re-education in terms of hand washing at the restaurant, prior to its grand opening and 1 year after its grand opening. As a result, it was found that it is necessary to provide education upon joining the firm and to provide re-education to teenagers and employees in their 60s after 1 year from joining the firm.

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SEASONAL VARIATIONS OF FORMALDEHYDE AND RISK ASSESSMENT OF MARKETED FISH CONTAMINATED WITH FORMALDEHYDE: FISH AND FOOD SAFETY ISSUE

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Abstract

The study was undertaken to determine the formaldehyde (FA) content in marketed fish and analyze risk assessment of consumers. Five species of fish (*Stromateus chinensis, Ompok pabda, Labeo rohita, Tenualosa ilisha* and *Amblypharyngodon mola*) from Kawran Bazaar of Dhaka city were analyzed under different conditions: fresh, dipped in plain water, dipped in 5% brine, fried in oil, boiled in water and cooked as curry. Dipping treatments in all cases were done for 1 hour. Boiling water where the fish is cooked and the gravy in the fish curry were also tested for FA. The FA contents, determined by spectrophotometric method, were found to be 9.42±2.68 to 19.23±4.32 mg kg^{-1} in fresh fish (October to March), 13.67±6.59 to 24.53±7.17 mg kg^{-1} in fresh fish (April to September), 5.26±1.41 to 12.67±4.06 mg kg^{-1} in fish dipped in water, 3.76±0.98 to 10.1±2.20 mg kg^{-1} in 5% brine water, 0.87±0.25 to 2.59±0.61 mg kg^{-1} in fried fish, 0.67±0.27 to 2.29±0.18 mg kg^{-1} in cooked fish, 0.57±0.10 to 1.53±0.22 mg kg^{-1} in boiled fish, 0.61±0.14 to 1.66±0.10 mg kg^{-1} in boiled fish water and 0.77±0.14 to 2.47±0.12 mg kg^{-1} in the gravy from fish curry. High priced commercial fishes (*Stromateus chinensis*) contained more FA than low price small fishes (*Labeo rohita*). FA contents in all fishes and conditions were still very low compared to oral reference value (0.2 mg kg^{-1}), as assigned by United States Environmental Protection Agency. The effects of dipping in water and 5% brine, or frying, boiling or cooking showed a significant ($p<0.05$) reduction of FA contents in all the samples. Study indicates that collected fish species obtained from wet market were still found to be safe for human consumption.

Keywords*:* Marketed fish, Formaldehyde, Seasonal variation and Risk assessment

INTRODUCTION

Fishes are one of the major sources of food and protein to human being. About 60% of the total animal protein intake is coming from fish (Department of Fisheries, 2015). In Bangladesh, different food items are reported to be adulterated by unsafe chemicals at different marketing steps from farms to consumers. Recently, there have been complaints from the consumers that many food items including fresh fish, fruits, vegetables and even milk available in the domestic markets are contaminated with formaldehyde; which might have been used intentionally to improve their appearance and extend shelf life (Reza et al., 2009, Yeasmin et al., 2010). Consumable fishes are being contaminated by formaldehyde by some evil traders. The fresh fish are sprayed with or dipped into formaldehyde by the fish traders while transporting through domestic market chain (Nowsad, 2010).

Formaldehyde is a natural component of a variety of foodstuffs (International Agency for Research on Cancer, 1995). Recently, International Agency for Research on Cancer (IARC) has classified formaldehyde as a Group 1 carcinogenic to humans (World Health Organization, 2002; International Agency for Research on Cancer, 2012). Formaldehyde develops postmortem in marine fish and crustaceans, from the enzymatic reduction of trimethylamine oxide to formaldehyde and dimethylamine (Sotelo et al., 1995; Badii & Howell, 2002). The highest concentrations of formaldehyde naturally occurring in foods (i.e., up to 60 mg kg^{-1}) are in some fruits (Tsuchiya et al., 1975) and marine fish (Tsuda et al., 1988). Formaldehyde concentrations in fish show higher extreme values: 220–290 mg kg^{-1}; however, averages are between 2 and 50 mg kg^{-1} (Bianchi et al., 2007; Weng et al., 2009). However, formaldehyde accumulates during the frozen storage of some fish species, including cod, pollack, and haddock (Sotelo et al., 1995).

According to the United State Environmental Protection Agency (EPA), maximum daily oral reference dose (R_fD_0) for formaldehyde is 0.2 mg\kg body weight\day (Wang et al., 2007) and 0.15 mg kg^{-1} body $weight^{-1}$ day^{-1} (Xuang et al., 2009). In 1985, Italian Ministry of Health has proposed formaldehyde values of 60 mg kg^{-1} and 10 mg kg^{-1} for *Gadidae* and crustaceans, respectively (Bianchi et al., 2007). Taking into account that fish and fish products fulfill an important role in human nutrition, the aim of our study was to determine the formaldehyde content of different wet marketed fish and risk assessment of consumers.

2. Materials and Methods

2.1 Quantitative estimation of formaldehyde

2.1.1 Sample size

Five types of commercial fish were selected as target sample. Fish species were analyzed under different circumstances; dipped in water for 1 h, dipped in 5% brine for 1 h, fresh, fried, boiled, cooked, water used in boil and fish gravy.

2.1.2 Determination of formaldehyde

The fish samples were cut into small pieces and 30 g of the sample was homogenized with 60 ml of 6% w/w TCA. The mixture was filtered and the pH of the filtrate was adjusted to 7.0 with 30% w/w KOH and stored in ice for 1 h. The test was performed by mixing 5 ml of the standard solution, TCA, fish extract; 2 ml of Nash's Reagent and then heated in the water bath at 60˚C for 30 min. The absorbance at 415 nm was measured immediately by UV/vis spectrophotometer (Shimadzu, UV-1800). Triplicate of the absorbance was prepared for each sample and recorded for further calculation. The sample reading was placed on the standard curve for the calculation of formaldehyde content of the sample.

2.2 Risk assessment

2.2.1 Exposure analysis

Available national data of average fish consumption (g day^{-1} $person^{-1}$) and primary data of residual formaldehyde concentration in fish (mg kg^{-1}) was used for exposure analysis. In case of body weight based on national data of different age group subpopulation such as children, adolescent and adult, were used for the distribution of body weight. Exposure was calculated as per following equation.

Exposure or Daily Intake Rate (DIR) = Concentration of contaminant (mg kg^{-1}) x Consumption (kg day^{-1}) / Body weight (kg)

2.2.2 Hazardous quotient (HQ)

Hazardous quotient (HQ) for the consumers through the consumption of formaldehyde treated fish was assessed by the ratio of daily intake rate (DIR) to the oral reference dose (R_fD_0) for each fish (US-EPA 2013). If the value of HQ is less than 1, then the exposed population is said to be safe, if HQ is equal to or higher than 1, is considered as not safe for human health, therefore potential health risk occurred, and related interventions and protective measurements should be taken (US-EPA, 2013). An estimate of risk to human health (HQ) through consumption of formaldehyde treated fish was calculated by the following equation:

$$\mathbf{HQ = DIR / R_fD_0}$$

R_fD_0 is an estimate of a daily oral exposure for the human population, which does not cause deleterious effects during a lifetime (US-EPA, 2009).

2.3 Data analysis

Probable risk was assessed based on the available fish consumption and formaldehyde concentration data. Initially available consumption and concentration data was fit into descriptive analyses using Microsoft Office Excel 2007 and XL-stat version 16 for DMRT to understand the differences of the variables. Estimated formaldehyde intake (maximum, minimum, median, mean, and SD) was determined per different scenarios separately.

3. Results and Discussion

3.1 Seasonal variation of formaldehyde

Seasonal variation of formaldehyde content in selected fish species in Kawran bazaar was found significantly difference ($p<0.05$) in table 1. The ideal ice to fish ratio should be at least 2:1 in summer and 1:1 in winter (Clucas and Ward, 1996). This indicates that in summer fish are more susceptible to spoilage than winter. This is because of most of wholesalers, retailers and vendors use formaldehyde in high amount on marketed fish in summer than winter. So, formaldehyde content was low in fish in October to March than April to September.

Table 1: Seasonal variation of formaldehyde in fish

Type of fish	Formaldehyde content (mg kg^{-1})	
	October to March	April to September
Rup Chanda (*Stromateus chinensis*)	19.23(±4.32)^{e}	24.53(±7.17)^{d}
Pabda (*Ompok pabda*)	12.17(±5.11)^{d}	17.39(±4.55)^{a}
Rui (*Labeo rohita*)	10.18(±3.06)^{c}	13.67(±2.91)^{b}
Hilsa (*Tenualosa ilisha*)	9.42(±2.68)^{a}	20.91(±8.19)^{c}
Mola (*Amblypharyngodon mola*)	10.21(±3.71)^{b}	21.17(±6.59)^{e}

*****The values in the same column having similar superscripts do not differ significantly ($p<0.05$)

3.2 Water dipped, 5% brine water dipped and different cooking practiced to decreased the formaldehyde concentration

Formaldehyde concentration gradually decreased by dipping treatment in water and 5% brine (Table 2, 3). In case of 1 h dipping water 49-57% formaldehyde decreased from fish body. On the other hand, dipping 5% brine reduced 59-66% from fish body. It was found that formaldehyde in the fish body was removed within 1 h by washing with tap water or when kept in melting ice and the time were directly proportional to the concentration of formaldehyde used (Yeasmin et al., 2013). Formalin degraded more quickly at high temperature than at low temperature in water. It took 72 hr for formalin to degrade completely at 30 and 35^{0}C. No effect of salinity on formalin degradation was found in water. After 72 hours' formalin was almost completely degraded in water with different salinities at 25^{0}C (XU et al., 1995). On the other hand, in the present study selected fish were dipped for 1 hr in plain water and 5% brine. However, formaldehyde concentration decreased more by dipping 5% brine than dipping in plain water.

Different cooking methods were used to reduce the formaldehyde concentration from fish muscle. The formaldehyde content generally decreased in all of the fish species analyzed after cooking. Formaldehyde content was in the range of 10.40 to 27.80 mg kg^{-1}in fresh fish, 2.15 to 5.75 mg kg^{-1} in fried fish, 1.98 to 5.30 mg kg^{-1} in fish gravy, 1.94 to 5.19 mg kg^{-1} in cooked fish and 1.36 to 3.64 mg kg^{-1} in boiled fish water and 1.25 to 3.33 mg kg^{-1} in boiled fish. The decrease in levels occurred when the samples were cooked in open pots, thus allowing the evaporation of formaldehyde during the cooking process since the formaldehyde is soluble in water at 20^{0}C. In addition, the boiling point for formaldehyde is 101^{0}C, so the evaporation of formaldehyde maybe occurred due to its low volatility (Leslie, 1994). Thus, the present results were in agreement that exposure to formaldehyde would decrease if the fish was cooked (Benchman, 1996). Decrement of formaldehyde concentration was also observed for the shrimps and cuttle-fish by using canning, cooking, boiling and roasting methods (Bianchi et al., 2007).

Table 2: Formaldehyde content in fish dipped in plain water

Type of fish	Formaldehyde content (mg kg^{-1})						
	Fresh fish	Dipped fish	Fried fish	Boiled fish	Boiled fish water	Cooked fish	Fish gravy
Mola	28.46(±11.42)^{e}	12.67(±4.06)^{e}	2.59(±.61)^{e}	1.53(±.22)^{e}	1.66(±.10)^{e}	2.29(±.18)^{e}	2.47(±.12)^{e}
Rup chanda	23.73(±4.47)^{d}	10.43(±5.11)^{d}	2.11(±.37)^{d}	1.27(±.36)^{d}	1.36(±.31)^{d}	1.91(±.41)^{d}	1.98(±.22)^{d}
Hilsa	19.98(±7.81)^{c}	8.96(±1.54)^{c}	1.87(±.13)^{c}	1.08(±.21)^{c}	1.18(±.13)^{c}	1.69(±.11)^{c}	1.73(±.34)^{c}
Rui	11.74(±3.85)^{b}	5.50(±3.22)^{b}	1.14(±.21)^{b}	0.66(±.17)^{b}	0.72(±.26)^{b}	1.03(±.21)^{b}	1.05(±.29)^{b}
Pabda	11.66(±2.93)^{a}	5.26(±1.41)^{a}	1.11(±.16)^{a}	0.64(±.11)^{a}	0.70(±.12)^{a}	0.97(±.16)^{a}	1.01(±.05)^{a}

*****The values in the same column having similar superscripts do not differ significantly ($p<0.05$)

Table 3: Formaldehyde content in fish dipped in 5% brine water

Type of fish	Formaldehyde content (mg kg^{-1})						
	Fresh fish	5% brine dipped fish	Fried fish	Boiled fish	Boiled fish water	Cooked fish	Fish gravy
Mola	32.57(±13.13)^{e}	10.19(±2.20)^{e}	2.36(±.73)^{e}	1.37(±.17)^{e}	1.49(±.39)^{e}	2.12(±.12)^{e}	2.16(±.28)^{e}
Rup chanda	23.54(±11.09)^{d}	8.70(±3.72)^{d}	1.93(±.44)^{d}	1.07(±.62)^{d}	1.21(±.31)^{d}	1.67(±.33)^{d}	1.71(±.39)^{d}
Hilsa	21.31(±9.64)^{c}	8.31(±2.17)^{c}	1.71(±.37)^{c}	0.98(±.11)^{c}	1.09(±.16)^{c}	1.54(±.58)^{c}	1.61(±.04)^{c}
Rui	12.44(±3.18)^{b}	4.84(±1.01)^{b}	1.13(±.71)^{b}	0.61(±.29)^{b}	0.68(±.22)^{b}	0.91(±.63)^{b}	0.93(±.47)^{b}
Pabda	10.37(±4.16)^{a}	3.76(±.98)^{a}	0.87(±.25)^{a}	0.57(±.10)^{a}	0.61(±.14)^{a}	0.67(±.27)^{a}	0.77(±.14)^{a}

*****The values in the same column having similar superscripts do not differ significantly ($p<0.05$)

3.3 Formaldehyde content in marketed fish

Formaldehyde contents in marketed fish in October to March and April to September were found 9.42 to 19.23 mg kg^{-1} and 13.67 to 24.53 mg kg^{-1} respectively (Table 4). Formaldehyde increased from 0.50 to 1.4 mg kg^{-1} during frozen storage of rake-gill mackerel at -20^{0}C; whilst in lizard fish it increased from 16.8 to 42.5 mg kg^{-1} after six months (Tunham et al., 2010). Formaldehyde content in haddock and mullet were 1.47-4.87 mg kg^{-1} and average values in cod was around 100 mg kg^{-1} (Bianchi et al., 2007). Formaldehyde naturally formed from the post-mortem of enzymatic reduction of TMAO to equimolar amounts of formaldehyde and dimethylamine (DMA) (Bianchi et al., 2005). Formaldehyde content was in the range of 0.38 to 15.75 mg kg^{-1} in fresh fish (Noordiana et al., 2011) and 3.95 to 13.40 mg kg^{-1} in fish markets (Hossain et al., 2008).

Table 4: Formaldehyde content in marketed fish

	Formaldehyde content (mg kg^{-1})	
	October to March	April to September
Max.	19.23	24.53
Min.	9.42	13.67
Median	13.68	19.42
Mean	12.54	18.16
Stdev.	3.78	5.88

3.4 Risk assessment from consuming marketed fish

The average daily intake rate in October to March and April to September were used for human health risk assessment (Table 5). The daily intake of formaldehyde from food consumption is dependent on the fish and amount of fish consumed. Assessment of human health risk from ingestion of fish treated with formaldehyde required information on the quantities of fish consumed. National data showed that, on an average, population consumed 58.65 g day^{-1} of fish. Protein sources in the diet were mainly obtained from fish. The risks were computed as an exposure analysis and hazard quotient (HQ) in case of children; adolescent and adult people were found to be less than 0.2 mg kg^{-1} and 1 respectively (Fig. 1). In case of the value of HQ is less than 1; the concentration of formaldehyde in fish is unlikely to cause adverse effects on human when the fish are consumed. In fact, the average daily intake rate (0.2 mg kg^{-1} day^{-1}) is sufficient to guarantee the consumer safety (Xuang et al., 2009). It was assumed that oral exposure to formaldehyde in humans from dietary sources would not exceed 100 mg formaldehyde per day corresponding to 1.7 and 1.4 mg kg^{-1} body weight per day for 60 kg and 70 kg respectively (European Food Safety Authority, 2014). However, the study showed that the consumption of commercial fish could influence the risk of formaldehyde.

Table 5: The daily intake rate and hazard quotient for individual's formaldehyde via dietary intake of marketed fish

Individuals		Exposure rate (mg kg^{-1})		Hazard quotient (HQ)	
		October to March	April to September	October to March	April to September
Max.	Adult	0.0211	0.0305	0.1054	0.1526
	Adolescent	0.0232	0.0335	0.1158	0.1677
	Children	0.1048	0.1517	0.5238	0.7585
Min.	Adult	0.0136	0.0196	0.0678	0.0982
	Adolescent	0.0166	0.0239	0.0829	0.1199
	Children	0.0222	0.0322	0.1110	0.1607
Median	Adult	0.0166	0.0241	0.0831	0.1203
	Adolescent	0.0192	0.0278	0.0961	0.1392
	Children	0.0359	0.0521	0.1798	0.2605
Mean	Adult	0.0168	0.0244	0.0841	0.1219
	Adolescent	0.0196	0.0283	0.0979	0.1417
	Children	0.0436	0.0632	0.2181	0.3158
Stdev.	Adult	0.0023	0.0033	0.0113	0.0164
	Adolescent	0.0021	0.0031	0.0107	0.0155
	Children	0.0214	0.0309	0.1070	0.1549

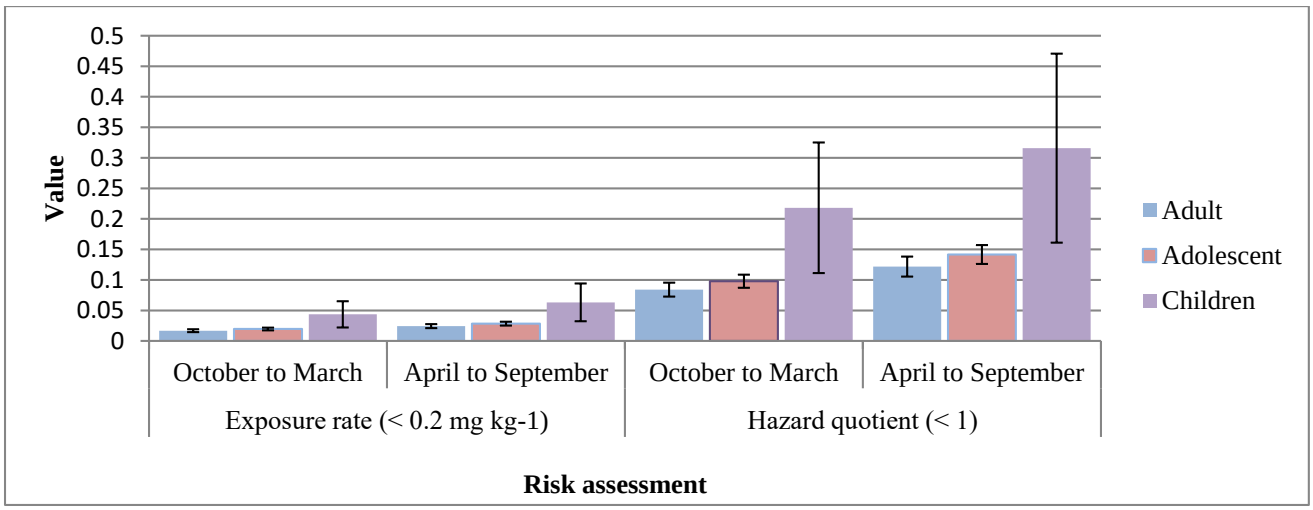

Fig. 1. Exposure rate and hazard quotient of consuming marketed fish

4. CONCLUSION

There were no adverse health effects on human due to the formaldehyde contaminated fish consumption in the whole year based on the risk assessment. Thus, the fish from wet market can be taken for consumption with caution that formaldehyde content might be kept in control. However, all fishes taken from the market should be washed thoroughly by dipping at least 1 h before cooking and cooking must be done under sufficient heat treatment to remove residual formaldehyde in fish.

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EFFECT OF SWEETENED CONDENSED MILK, GLUCOSE SYRUP AND WHEAT FLOUR ON THE STRUCTURE AND SENSORY ASPECTS OF MILK CANDY

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Abstract

The objective of this research is to invest the impact of main ingredients on structure and sweetness of local milk candy in Dong Nai Province, Vietnam. Samples are prepared with 70 - 80% sweetened condensed milk, 2 – 6% glucose syrup and 16 - 26% wheat flour (w/w). Texture profile analysis was performed using Instron universal testing machine to evaluate hardness of the candy samples. Influence of these ingredients on sensory evaluation has been also studied. Mixture design was used to optimize ingredient composition of the candy. The results showed that sweetened condensed milk and wheat flour are the two main factors which impact structure of product. Candy hardness was significantly increased with increasing amounts of sweetened condensed milk and wheat flour. Moreover, there were also significant interactions between these two components with glucose syrup on structural properties of the samples. Optimum composition of the candy was finally found at 70.62% sweetened condensed milk, 4.62% glucose syrup and 19.76% wheat flour.

Keywords: confectionery, caramel, sweetened condensed milk, glucose syrup, milk candy

1. INTRODUCTION

Milk candy is a complex blend of gel system of protein – protein, protein – other ingredients such as sugar, water, and water dispersed in fat globules. Sweetened condensed milk, glucose syrup and wheat flour are the factors which impact sweetness of product.

Inclusion of milk solids is the essential feature of a milk candy recipe and it is the milk solids that cause the product to be difference in its properties from other types of confectioneries (Pyrz ,1976; Guelfi, 1988). The presence of milk solids has an effect on texture, flavor and colour of product (Heathcock, 1985; Gilmore, 1988). The higher the level of milk solids contained in candy, the harder will be the candy (Lees và Jackson, 1975; Gilmore, 1988). Milk solid are normally added as full cream as either skimmed sweetened condensed milk or dried whey powders are sometimes used (Gilmore, 1988). The milk proteins in sweetened condensed milks contribute significantly to the emulsification of fats and give body, texture and mouthfeel to final product (Jackson, 1990). Whey protein interaction with casein, this mixture was associated mainly with the fat globular layer. Candy hardness was significantly increased with increasing amounts of protein. However, this is governed by type, concentration and the level of denaturation of milk protein (Chandrani, 1997).

Glucose syrup, also known as confectioner's glucose, is made from the hydrolysis of starch. Glucose syrup is important major in candy processing. Glucose syrup containing over 90% glucose is used in industrial fermentation. However syrups used in confectionery contain varying amounts of glucose, maltose and oligosaccharides with typically 10% to 43% of glucose depending on quality grade. Glucose syrup is used in foods in order to sweeten, soften texture and increase volume of the products. By converting some of the glucose in corn syrup into fructose by using an enzymatic process, a sweeter product of high fructose corn syrup can be produced (Jackson, 1990). Corn syrup can retard the sucrose crystal growth. According to (Hartel and Shastry 1991) potential mechanisms of sucrose growth inhibition by corn syrup fractions are: (1) Corn syrup molecules impede diffusional motion of sucrose molecules to the crystal surface and thereby, slow down crystal growth. (2) Corn syrup molecules adsorb to the crystal surface and inhibit incorporation of sucrose molecules into the crystal lattice. In such case, sucrose molecules would have to displace the oligosaccharide molecules in order for growth to occur. (3) Corn syrup molecules are incorporated into the sucrose crystal lattice. The type and amount of corn syrup used can affect sweetness, texture, color development and moisture uptake (hygroscopicity) of the final product. The most important properties include the DE (dextrose equivalent) value, specific saccharide composition and viscosity. DE is a measure of reducing power of a product calculated as glucose and expressed as percent of total dry substance. 42 DE corn syrup is the most frequently used in confections (Kitt, 1993; Hofberger, 2009). Lower DE corn syrups lead to tougher finished caramels (Steiner et al., 2003; Hofberger, 2009) due to increased viscosity caused by higher amounts of large molecular weight molecules (Steiner et al., 2003; Cakebread, 1970; McMaster et al., 1987). The lower the DE of a corn

syrup, the more viscous it is. Excessively high levels of corn syrup lead to the defect 'cold flow' (Cakebread, 1971, 1972), defined as the tendency of a material to deform under its own weight, over time (Warnecke, 1995). Higher levels of corn syrup solids decrease the amount of sucrose that crystallizes out and increase the amount of dissolved solids in the continuous phase (Hartel, 2001).

In confectionery, starches are used to create structure of product so that the viscosity is one of the important properties of the starches. It affects the quality and texture of many food product (James, 2000). Wheat flour is a relatively cheap ingredient and acts both as a bulking and as a gelling agent; it has the property of changing from a thick semifuid cold slurry to a viscous semisolid when gelatinized by heat, and on cooling sets to a firm gel the basis of the manufacture of candy.

A typical wheat flour that can be used satisfactorily in the manufacture of candy include 14% moisture (by mass), max 1.5% fat, 8-10% protein 67-75% carbohydrate max 1.0% ash and 0.5-1.0% fiber.

2. Materials and Methods

2.1. Materials

Sweetened condensed milk was supplied by local milk candy company in Dong Nai Province, Vietnam. Milk content is 74% min and sugar content varies from 30-32%.

Glucose syrup was supplied by Minh Tung company, Vietnam. Glucose syrup has dry matter concentration from 74-82%, transparent, comparable, colorless or slightly yellow, sweet taste.

Flour (Grade 8) was supplied by VIKYBOMI, Viet Nam.

2.2. Research Methodology

2.2.1. Process for the production of candy

Sweetened condensed milk was mixed with glucose syrup and eggs. Then they were co oked at a temperature of about 90-100°C. Adding flour and additive Texim B2 is obtained at nearly the end of the process. A typical composition of 100 g of ingredients contains 6 g glucose syrup, 9 g flour, 4 g egg, 1 g Texim and 75 g sweetened condensed milk (SCM). At the end of the cooking, water content of candy is reduced to 6%. Rolling, cooling, cutting and packaging candy are the next steps of the manufacturing process.

Products were preserved during a week to ensure stability of the structure. Texture profile analysis was performed using Instron universal testing machine to evaluate hardness of the candy samples. Influence of these ingredients on sensory evaluation and hardess of milk product has been also studied.

Sensory analysis was performed using 9-point hedonic scale (Ha Duyen Tu, 2006). Sensory panel include 10 specialists from the local milk candy company in Dong Nai Province.

2.2.2. Optimize candy composition with sweetened condensed milk, glucose syrup and wheat flour

A mixture design was used to optimize ingredient composition of the candy. Ingredient composition levels and coded values for the factors used in developing the experimental data are presented in Table 1. The selected response variable was the hardness (N) of the candy.

Table 1. Treatment levels and coded values of the independent factors

Factor	Coded level		
	–1	0	+1
Sweetened condensed milk (%)	70	75	80
Glucose syrup (%)	2	4	6
Wheat flour(%)	16	21	26

2.2.3. Data Processing

The experimental were evaluated by using JMP 9.0.2 software (SAS Institute Inc., 2011, USA) to analysis of variance ANOVA, comparing average by Tukey's HSD method, Minitab 16.

3. Results and Discussion

3.1. Effect of sweetened condensed milk on structure and sweetness of candy

The overall trend indicated that the higher the content of sweetened condensed milk, the higher the hardness of a candy (Figure 1). Tukey's HSD post hoc test showed that all of the hardness means were statistically different ($p < 0.05$) except for the candy made with 60% and 65% sweetened condensed milk, which were not statistically different from each other ($p > 0.05$). However, at 75% SCM, the hardness (347.65 N) is closest to that of reference sample (348.77 N). These results agree with the literature where higher SCM content yields a firmer product (Chandrani, 1997)

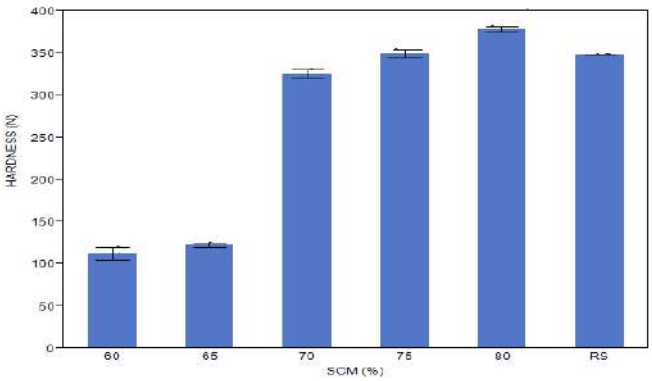

Fig 1. Candy hardness at different content of sweetened condensed milk

This result can be explained by three main reasons: the first is the ability to network and interact with other components of the protein in milk, the second is due to the change of the state of sugar molecules in the condensed milk materials and the third is the moisture movement.

In the next steps, the products were assessed the hardness and sweetness by asking assessors from Dong Nai candy company to sort the samples from the less preferred to the most preferred.

Using Friedman test, sample different preferred levels are presented in Table 2. Candy with 60% SCM and candy with 65% SCM had the preferred level of sweetness and structure was the lowest. It was not statistically different from each other ($p > 0.05$). Candy with 70% SCM, 75% SCM

and 80% SCM had higher preferred levels of sweetness and structure compared to those of 60% SCM and 65% SCM.

There was not statistically different from 3 higher SCM samples ($p > 0.05$). However, they had difference in structure preferred level ($p < 0.05$).

Therefore, the content of 75% SCM was selected as fixed factor to conduct the next experiments. The range of SCM content from 70 to 80% was used to optimize the content of the ingredients in the recipe by Mixture design.

Table 2: Sensory panel score for sweetness and structure of candy sample

	Candy with 60% SCM	Candy with 65% SCM	Candy with 70% SCM	Candy with 75% SCM	Candy with 80% SCM
Sweetness	14^a	16^a	41^b	45^b	34^b
Structure	13^a	17^a	37^b	49^c	34^d

3.2. Effect of glucose syrup on structure and sweetness of candy

The overall trend indicated that the higher the content of glucose syrup, the lower the hardness of a candy (Figure 2). The candy with content of 4% glucose syrup had hardness (350,99 N) close to the most reference sample (348.77 N) and there were statistically different from each other ($p < 0.05$).

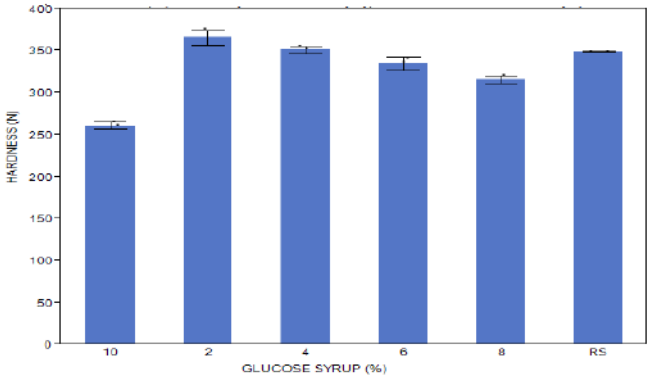

Fig 2. Candy hardness at different content of glucose syrup

When the content of glucose syrup increased, the content of maltodextrin also increased while protein and sucrose in product decreased. These made hardness of the candy decrease. On the other hand, glucose syrup contains oligosaccharide which creates plasticity and high hygroscopic to make a softer product.

Influence of this ingredient on sensory evaluation has been also studied. Table 3 shows that candy with 8% glucose syrup or 10% of glucose syrup had lower sweetness and heterogeneous structure due to they are less preferred. Two samples were not statistically different ($p > 0.05$) in sweetness, but there were statistically different in structure ($p < 0.05$). Candy with glucose syrup of 2%, 4% and 6% had higher preferred level than those of 8% and 10%. For structure, candy with 2% glucose syrup had the highest preferred level.

These results showed that 4% glucose syrup was selected as fixed factor to conduct the next experiments. The range of glucose syrup content from 2 to 6% was used to optimize the content of the ingredients in the recipe by Mixture design.

Table 3. Sensory panel score for sweetness of candy sample at different glucose syrup content

	Candy with 2% syrup	Candy with 4% syrup	Candy with 6% syrup	Candy with 8% syrup	Candy with 10% syrup
Sweetness	35^a	42^a	41^a	19^b	13^b
Structure	35^a	47^b	38^a	20^c	10^d

3.3. Effect of wheat flour on structure and sweetness of candy

Figure 3 showed that the higher the content of wheat flour, the higher the hardness of a candy. At the content of 21% wheat flour (349.67 N) candy had hardness close to the most reference samples (348.77 N) and there was statistically different from other sample ($p > 0.05$).

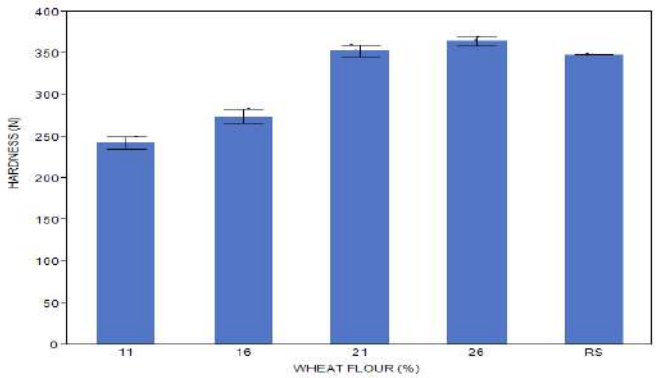

Fig 3. Candy hardness at different content of wheat flour

With a low level of starch and suitable amount of water in the mixture, the starch will have gelatinized making candy complex and becomes flexible and softer in the cooking process. When the content of starch in the mixture increases at a constant amount of water, the starch granules will not be completely swollen, candy complex become drier and harder.

On the other hand, there is a competition between wheat flour molecular and sugar molecular in structural stability stages. Due to hygroscopic property of sugar molecules is higher than flour molecular, water molecules will move from flour to sugar. This made the hardness of the product increase during the storage after a week.

Table 4. Sensory panel score for sweetness of candy sample at different flour content

	Candy with 11 % flour	Candy with 16 % flour	Candy with 21 % flour	Candy with 26% flour
Sweetness	12^{c}	18^{c}	40^{a}	30^{b}
Structure	11^{c}	38^{a}	26^{b}	25^{b}

The results of Table 4 showed that sample 3 (21% wheat flour) had the highest preferred level of sweetness. Tukey's HSD showed that the sweetness of samples was statistically different ($p > 0.05$). But sample 2 (16% wheat flour) had the highest preferred level of structure. There were statistically different ($p < 0.05$) among samples. Therefore, the range of wheat flour content from 16 % to 26 % was used to optimize the content of the ingredients in the recipe by Mixture design.

3.4. Optimization of ingredient composition of the candy

Mixture design of JMP 9.0.2 was used to optimize ingredient composition of the candy. The content of ingredient composition is 70 – 80% sweetened condensed milk, 2-6% glucose syrup, 16-26% wheat flour. The result was showed in Table 5.

Table 5. Hardess of candy at different ingredient composition

Run	The ratio of SCM	The ratio of glucose syrup	The ratio of wheat flour	Hardness (N)
1	0.7	0.055	0.245	337.36
2	0.73	0.02	0.25	352.33
3	0.8	0.06	0.14	333.58
4	0.7	0.05	0.25	337.36
5	0.715	0.035	0.25	352.21
6	0.8	0.02	0.18	349.99
7	0.75	0.06	0.19	330.05
8	0.8	0.04	0.16	347.29
9	0.765	0.02	0.215	355.73
10	0.7	0.06	0.24	320.37

Experimental values and predicted values of hardness had a high degree of compatibility, the value shown in the $R^2 = 0.97$ (Figure 4). This means experimental values distributed near diagonal on the chart and the majority value in the range of 3% margin of error, 97% empirical values are compatible with the value calculated according to a mathematical model to analyze the stiffness. In summary it can be concluded by the mathematical model can be used to represent and explain the relationship between material composition and structure of the finished candy.

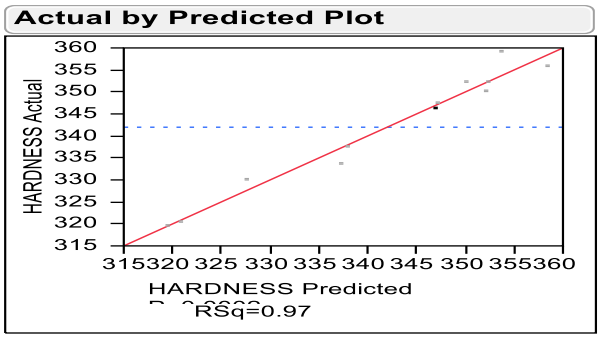

Fig 4. Actual by predicted plot

Influence of the ingredients to the hardness of the candy is illustrated in Figure 5. Sweetened condensed milk and flour were the two factors which had significantly affect the structure or the hardness of the product. In Figure 3.5, value of glucose syrup is negative (-1.36) which means that the higher the content of glucose syrup, the lower the hardness of a candy. But with low content of glucose syrup, it has significantly impact on the structure of the candy. Coordinating pairs as shown in Figure 3.5 are an interaction between the milk – glucose syrup, glucose syrup - flour, milk - flour can significantly affect the structure of the product.

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
(SCM-0.7)/0.12	343.359	5.618007	61.12	<.0001 *
(WHEAT FLOUR-0.16)/0.12	340.3365	5.618007	60.58	<.0001 *
GLUCOSE SYRUP*WHEAT FLOUR	585.81273	126.5031	4.63	0.0036 *
SCM*GLUCOSE SYRUP	570.54676	126.5031	4.51	0.0041 *
SCM*WHEAT FLOUR	66.310298	26.08211	2.54	0.0439 *
(GLUCOSE SYRUP-0.02)/0.12	-108.7473	80.15628	-1.36	0.2237

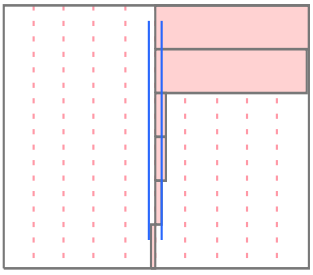

Fig 5. Interaction of ingredient composition

The results showed that sweetened condensed milk and wheat flour are the two main factors which impact structure of product.

Under the support of JMP 9.0.2 software it is showed that the ingredient composition of candy is 74.34% SCM, 4.86% glucose syrup, 20.80% wheat flour. It is equivalent to experiment formula of candy with 70.62% SCM, 4.62% glucose syrup, 19.76% wheat flour, 4% egg and 1.0% Texim. The hardness of candy was 349,47 (N) which was not statistically different from the predicted value by the model (348.98 N).

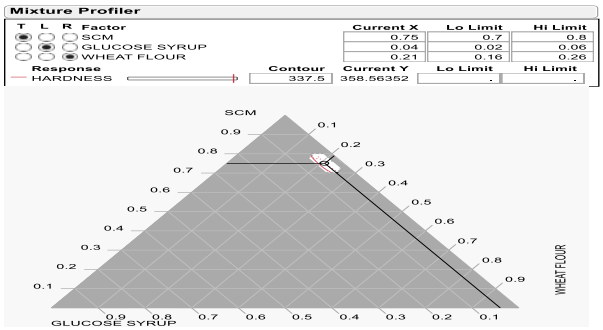

Fig 6. *Mixing formula proposed by software*

Through optimization of ingredient composition of the candy with the support of JMP 9.0.2 software, the optimal formula for the structure has been determined and the hardness of the new developed candy is similar to commercial reference products available in the candy market.

4. CONCLUSION

There were also significant interactions between sweetened condensed milk and wheat flour with glucose syrup on structural properties of the candy. Sweetened condensed milk and wheat flour are the two main factors which impact the hardness of candy product. Mixture design was successfully used to optimize ingredient composition of the candy. Optimum composition of the candy was finally found at 70.62% sweetened condensed milk, 4.62% glucose syrup, 19.76% wheat flour 4% egg and 1.0% Texim.

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EFFECTS OF DIFFERENT PACKING MATERIALS AND STORAGE CONDITIONS ON QUALITY OF BELL PEPPER

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Abstract

Extending the shelf-life of Bell pepper (*Capsicum* annuum) with no compromise of their final quality is an important aspect of food security. This study determined effect of different packing materials and storage conditions on the weight loss, firmness, elasticity, Total Soluble Sugar (TSS) and decay development. Bell peppers were collected from the Department of Postharvest Science of Fresh Produce. X-tend® and Polyethylene were used to pack the peppers. The bagged and unbagged (control) peppers were put in storage at 1 and 7 ºC for 14 days. The peppers were then analyzed for weight loss, firmness, elasticity, decay development, chilling injury, gas composition and TSS. Data obtained were subjected to descriptive statistic and correlation. X-tend® had the least weight lost, best firmness of 7.3 to 7.2 mm, elasticity: 3.8 to 3.8 mm after storage at 7 ºC. There was significant ($p < 0.05$) positive correlation between firmness and elasticity. There was significant ($p < 0.05$) negative correlation between carbon (iv) oxide and oxygen. X-tend® had lower reduction in TSS compared to Polyethylene after storage at 7 ºC for 14 days. Water condensation, decay and CO_2 injury were observed in the bell peppers of Polyethylene. Less or no water condensation, minimal decay and physiological disorders, were observed in X-tend. At 1 °C, highest decay of 80 % was observed in Polyethylene while 70 % shriveling was observed in control. About 100 % decay and 60 % CO_2 injury were observed in Polyethylene while 50 % shriveling was observed in control at 7 °C. At 17 ºC, 100 % shriveling and 40 % decay were observed in control while 40 % shriveling was observed in the Polyethylene. However, X-tend had the least percentage of decay at 1 (10 %), 7 (20 %) and 17 (30 %) ºC.

Keywords: Packages, Storage conditions, Pepper, Quality

1. INTRODUCTION

Bell peppers are excellent source of natural, micronutrient antioxidants (vitamins C, E and carotenoids) which play important role in preventing or reducing chronic and age-related diseases (Palevitch & Craker, 1996). Peppers are considerably more susceptible to water loss, sun scald, and heat damage after they have been harvested than before. These problems are likely to occur if the bulk bins are allowed to sit for more than an hour in direct sunlight. Harvested peppers should be placed in the shade immediately after harvest and cooled as soon as possible.

Proper storage system reduces wastes, adds value and makes the product qualitatively and quantitatively acceptable. Respiration rate and gas exchange through the package material are the processes involved in creating a modified atmosphere inside a package that will extend shelf life of fresh bell peppers (Wills, Lee, Graham, McGlasson, & Hall, 1981; Susana et al., 2002). Manolopoulou, Xanthopoulos, Douros, & Lambrinos (2010) stated that ascorbic acid did not change significantly in peppers packed in the two Poly Ethylene films throughout the cold storage period. Peppers packed in Low Density Poly Ethylene film, had insignificant higher ascorbic acid values compared to Medium Density Poly Ethylene film at 10 ºC storage. In a study conducted by Opal et al. (2005) found in a comparative study between diffusion channel (4, 7 and 10 cm tube length) and silicon membrane (50.29, 98.56 and 158.43 cm^2 silicon membrane window area) system that all diffusion channel treatments were observed to have lower values of total soluble solids than membrane treatments and ripe controls.

Bell peppers are highly perishable, deteriorate rapidly and have short shelf-life. Therefore, is important to extend their shelf-life through different packing materials and storage conditions. The objectives of this study were to determine effect of different packing materials (XF and PE) on the shelf life of bell peppers; evaluate effect of different storage temperatures on peppers quality; and determine firmness and springiness due to chilling, high CO_2 percentage and fungal decay under different storage conditions.

2.0 Materials and Methods

2.1 Raw material

Bell peppers (*Capsicum* annuum) were collected from the Department of Postharvest Science of Fresh Produce, Institute of Postharvest and Food Sciences, Agricultural Research Organization, Volcani Center, Israel.

2.2 Pre- packing analysis of the peppers

Fresh weight, firmness, elasticity and Total Soluble Solid (TSS) were determined using weighing balance, pressure meter and Manual Refractometer respectively.

2.3 Packing and storage conditions

Packing was done using X-tend® film (XF) and Polyethylene (PE) bag. After packing, gas composition was determined using Oxybaby™. The bagged and unbagged peppers were put in storage at 1 and 7 ºC for 11 days. After 11th day, they were removed from the cooling and subjected to 20 ºC for 3 days while those (bagged and unbagged) designated for 17 ºC were put in the storage for 14 days.

2.4 Experimental treatments

1. Unbagged (Control) 7 ºC
2. XF-7 ºC
3. PE-7 ºC
4. Unbagged (Control) 1 ºC
5. XF-1 ºC
6. PE-1 ºC
7. Unbagged (Control) 17 ºC
8. XF-17 ºC
9. PE-17 ºC

2.5 Post packing and storage analysis

After removing the peppers from the storage, they were analyzed for weight loss, firmness, elasticity, decay development, chilling injury, gas composition and TSS.

2.6 Statistical analysis

Data obtained were subjected to descriptive statistics and Pearson correlation coefficient.

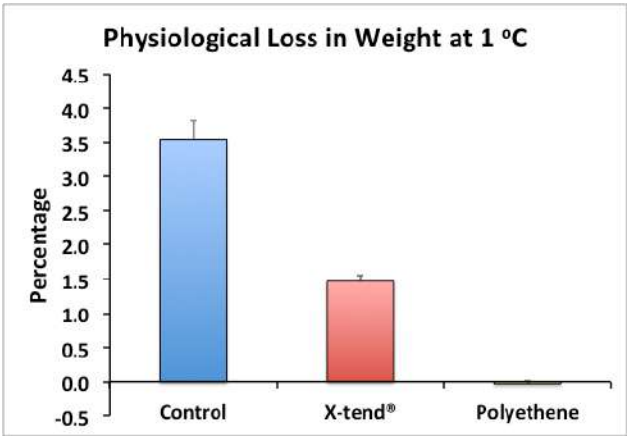
3.0 RESULTS AND DISCUSSION

3.1 Effects of different packing materials and temperatures on weight loss of bell pepper

At 1°C, unbagged (control) and X-tend bag peppers decreased in weight by 3.54% and 1.47% respectively while peppers in the Polyethylene bag increased in weight by 0.03 % [Figure 1(a)]. Decrease in weight of peppers by 3.5% and 2.13% were observed in the control and X-tend at 7 °C while peppers in Polyethylene increased in weight by 0.01 % [Figure 1(b)]. Decreases in weight of peppers were observed in unbagged (13.02 %), X-tend (4.82 %) and Polyethylene (0.17 %) at 17 °C [Figure 1(c)]. Unbagged peppers decreased in weight more than bagged peppers; this might be due to packing materials used. Packing generally maintained the various quality attributes better than unpackaged condition (Watada *et al*., 1987).

3.2 Effects of different packing materials and temperatures on the firmness of bell pepper

Firmness of unbagged bell peppers before and after storage increased from 5.9 – 7.9 mm while the firmness of the peppers in X-tend and Polyethylene decreased from 7.3 – 5.9 mm and 6.6 –



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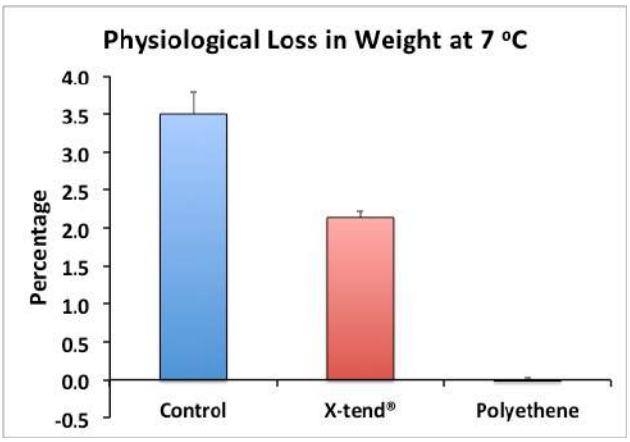

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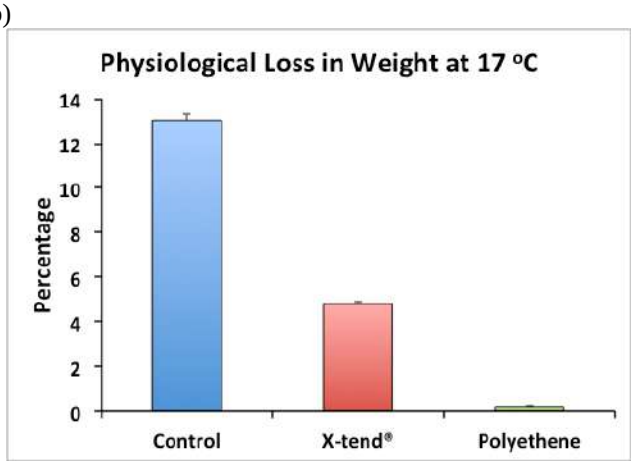


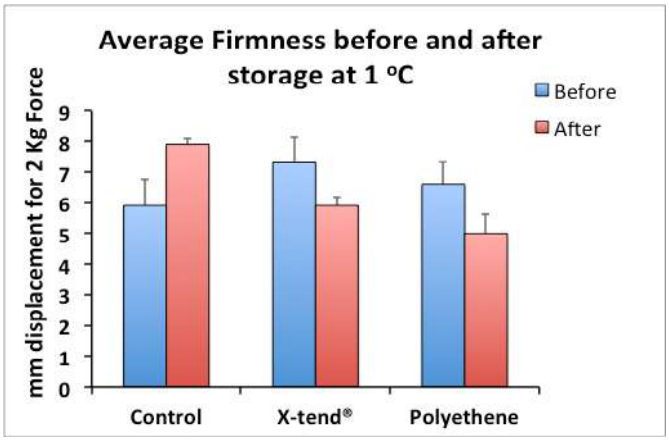
(c)

Figure 1: Effect of packing materials on the weight of bell pepper at 1°C (a), 7 °C (b) and 17 °C (c)

5.0 mm respectively at 1°C [Figure 2(a)]. At 7 °C, firmness of the peppers reduced from 7.3 – 7.2 mm in X-tend, 6.6 – 5.8 mm in Polyethylene and increased from 5.9 – 7.1 mm in the unbagged [Figure 2(b)]. However, firmness of the peppers increased in the unbagged (5.9 – 13.8 mm) and X-tend (7.3 – 8.5 mm) while it decreased in Polyethylene (6.6 – 5.0 mm) at 17 °C [Figure 2(c)]. This is in agreement with several other authors (Ben-yehoshua, Shapiro, Chen, & Lurie, 1983; Watada, Kim, Kim, & Harris., 1987) who found that the main benefit of film packaging of peppers was a reduction in fruit water loss and firmness.

3.3 Effects of different packing materials and temperatures on the elasticity of bell peppers

Elasticity of bell peppers in X-tend and Polyethylene decreased from 3.8 – 2.9 mm and 3.35 – 2.5 mm respectively and increased in the unbagged from 2.9 – 4.4 mm before and after storage at 1°C [Figure 3(a)]. There was no change in the elasticity of bell peppers in the X-tend bag while the elasticity of bell peppers increased by 0.9 mm in the unbagged and decreased by 0.55 mm in the Polyethylene bag at 7 °C [Figure (3b)]. Elasticity of peppers decreased by 0.95 mm in Polyethylene; and increased by 7.7 and 1.1 mm in the unbagged and X-tend bag respectively at 17°C [Figure (3c)]. Film packaging reduces water loss and firmness (Ben-Yehoshua *et al*., 1983; Watada *et al*., 1987).



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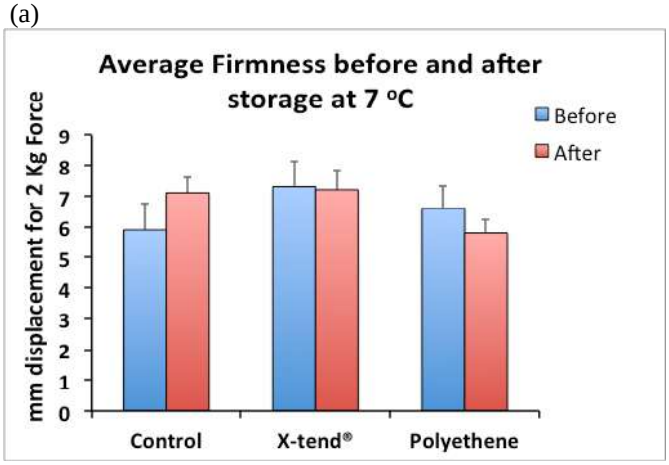

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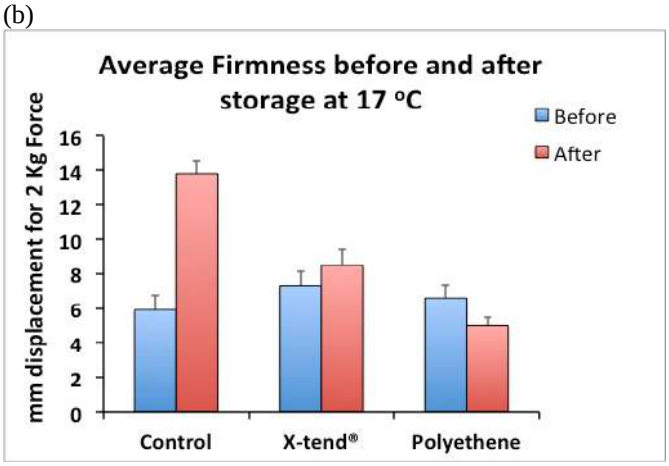

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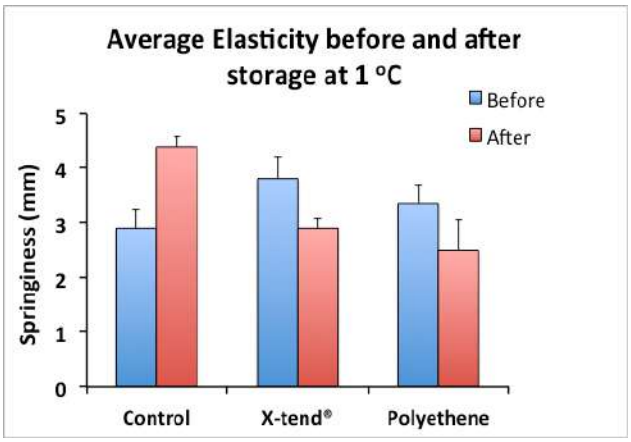
Figure 2: Effect of different packing materials on the firmness of bell pepper at 1°C (a), 7 °C (b) and 17 °C (c)



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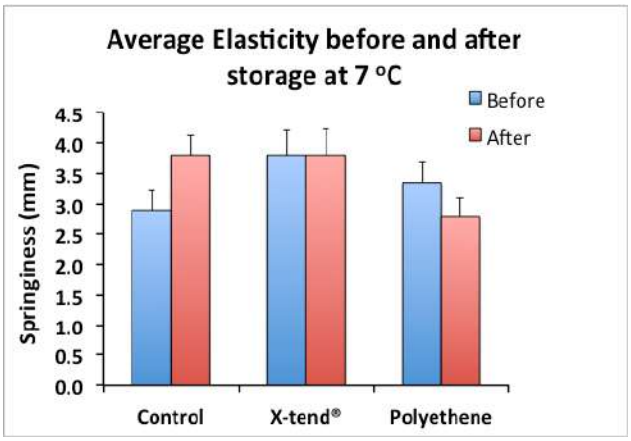

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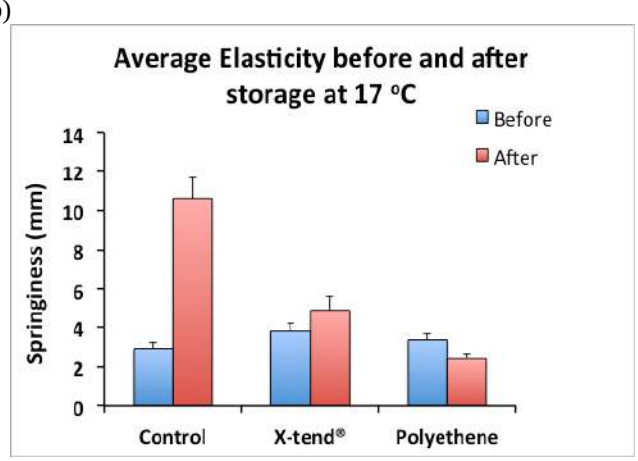


(c)

Figure 3: Effect of packing materials on the elasticity of bell pepper at 1°C (a), 7 °C (b) and 17 °C (c)

3.4 Correlation coefficient between firmness and elasticity of bell peppers

There was significant ($p < 0.05$) positive correlation between firmness and elasticity of bell peppers (Figure 4).

3.5 Effects of different packing materials and temperatures on the Total Soluble Solid of bell peppers

Total Soluble Solid (TSS) of bell peppers decreased by 0.28, 0.24 and 0.72 Brix in the control, X-tend and Polyethylene bag respectively at 1°C [Figure 5(a)] while the TSS decreased by 0.38, 0.46 and 0.48 Brix in the control, X-tend and Polyethylene bag respectively at 7 °C [Figure 5(b)]. However, 0.32, 0.22 and 0.66 Brix decrease was observed in the control, X-tend and Polyethylene bag at 17 °C [Figure 5(c)].

3.6 Effects of packing materials and temperatures on the Carbon (iv) oxide of bell peppers

At 1°C, Carbon (iv) oxide content remain the same before and after storage in the control and increased by 0.7% and 2.5% in X-tend and Polyethylene respectively [Figure 6(a)]. Carbon (iv) oxide increased by 0.5% and 2.1% in X-tend and Polyethylene respectively while there was no variation in the CO_2 content of the control before and after storage at 7 °C [Figure 6(b)]. Similarly, there was no difference between CO_2 content of the control while 0.5% and 1.4% increase were observed in X-tend and Polyethylene at 17 °C [Figure 6(c)].

3.7 Correlation coefficient between Carbon (iv) oxide and oxygen contents of bell peppers

Significant ($p < 0.05$) negative correlation ($r = -0.99$) was observed between carbon (iv) oxide and oxygen contents of bell peppers (Figure 7).

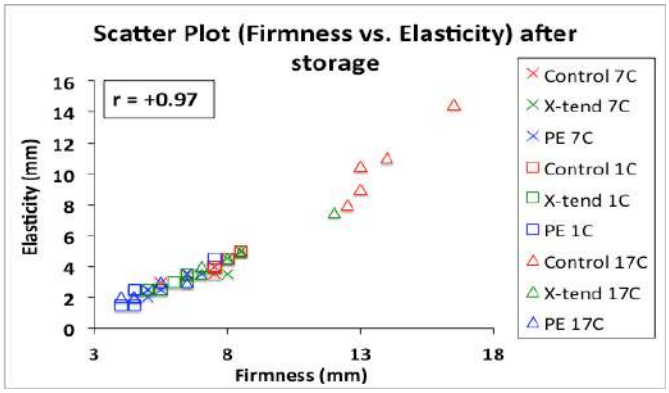
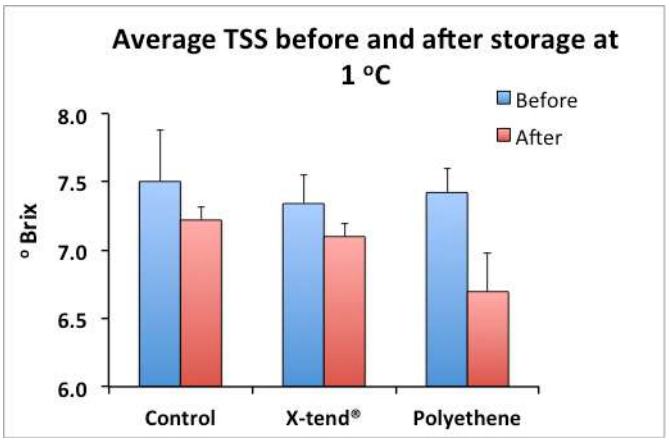


Figure 4: Scatter Plot (Firmness vs. Elasticity) after storage (r = + 0.97)



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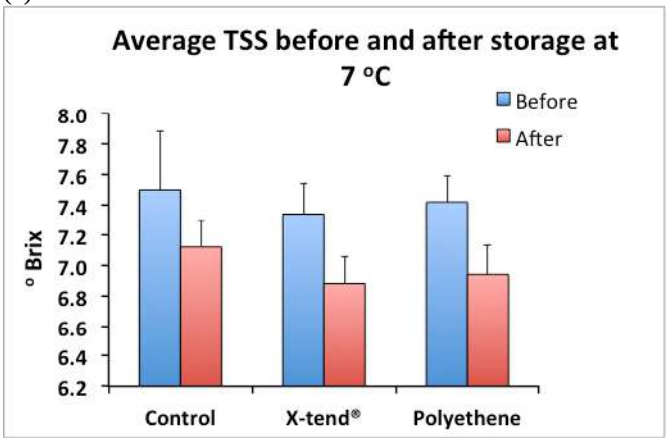

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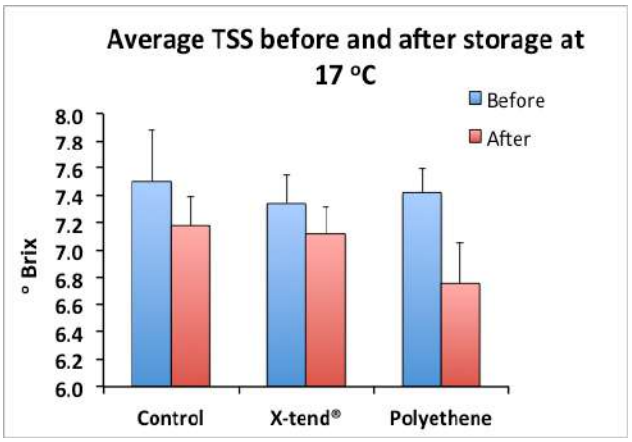

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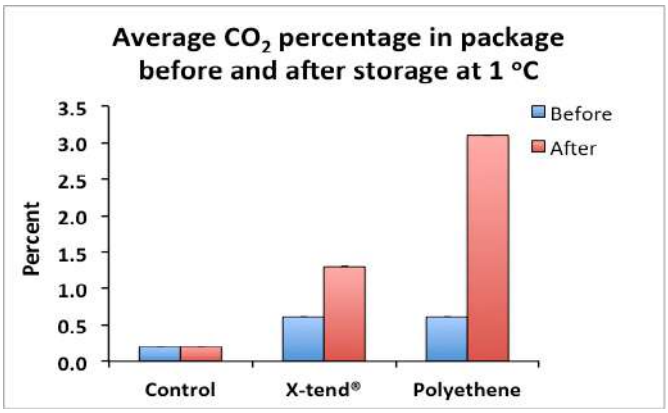
Figure 5: Effect of packing materials on the Total Soluble Solid of bell peppers at 1°C (a), 7 °C (b) and 17 °C (c)



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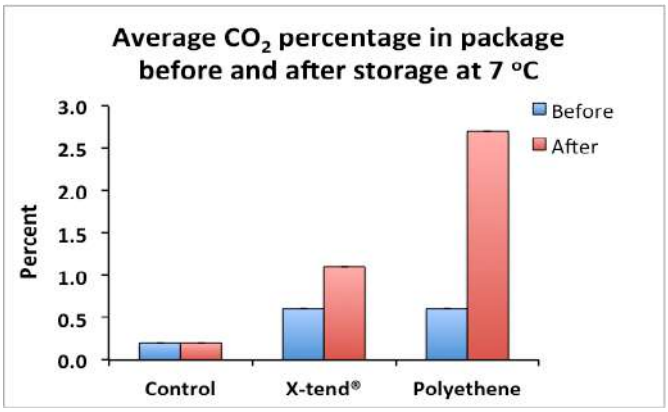

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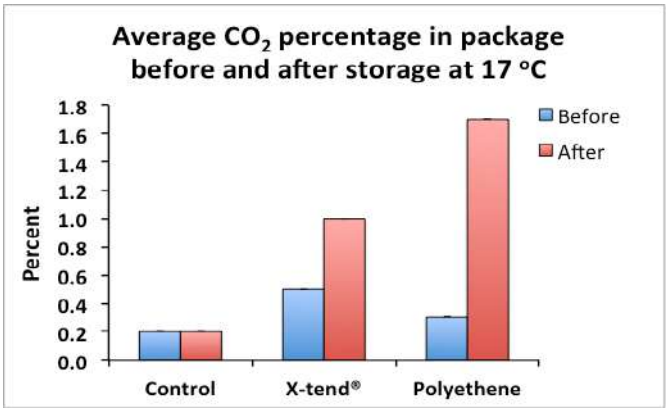

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Figure 6: Effects of packing materials and temperatures on Carbon (iv) oxide of bell peppers at 1°C (a), 7 °C (b) and 17°C (c)

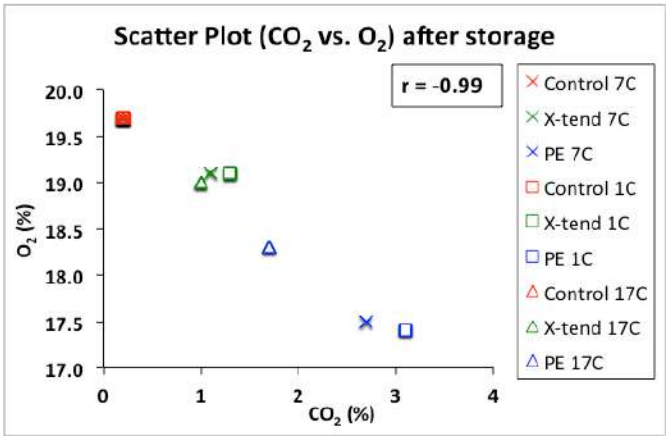

Figure 7: Scatter Plot (CO_2 vs. O_2) after storage (r = -0.99)

Plate 1: Bell peppers in Polyethylene

2: Bell peppers in X-tend

3.8 Effects of packing materials and temperatures on the physiological injury of bell peppers

Water condensation (Plate 1), decay and CO_2 injury (Plate 3) were observed in the bell peppers of Polyethylene. Less or no water condensation (Plate 2); minimal decay and physiological disorders (Plate 3) were observed in X-tend. At 1 °C, highest decay of 80% was observed in Polyethylene while 70% shriveling was observed in the control. About 100% decay and 60% CO_2 injury were observed in Polyethylene while 50% shriveling was observed in control at 7 °C. At 17 ºC, 100% shriveling and 40 % decay were observed in control while 40% shriveling was observed in the Polyethylene. However, X-tend had the least percentage of decay at 1 ºC (10 %), 7 ºC (20 %) and 17 ºC (30 %) as shown in Table 1.

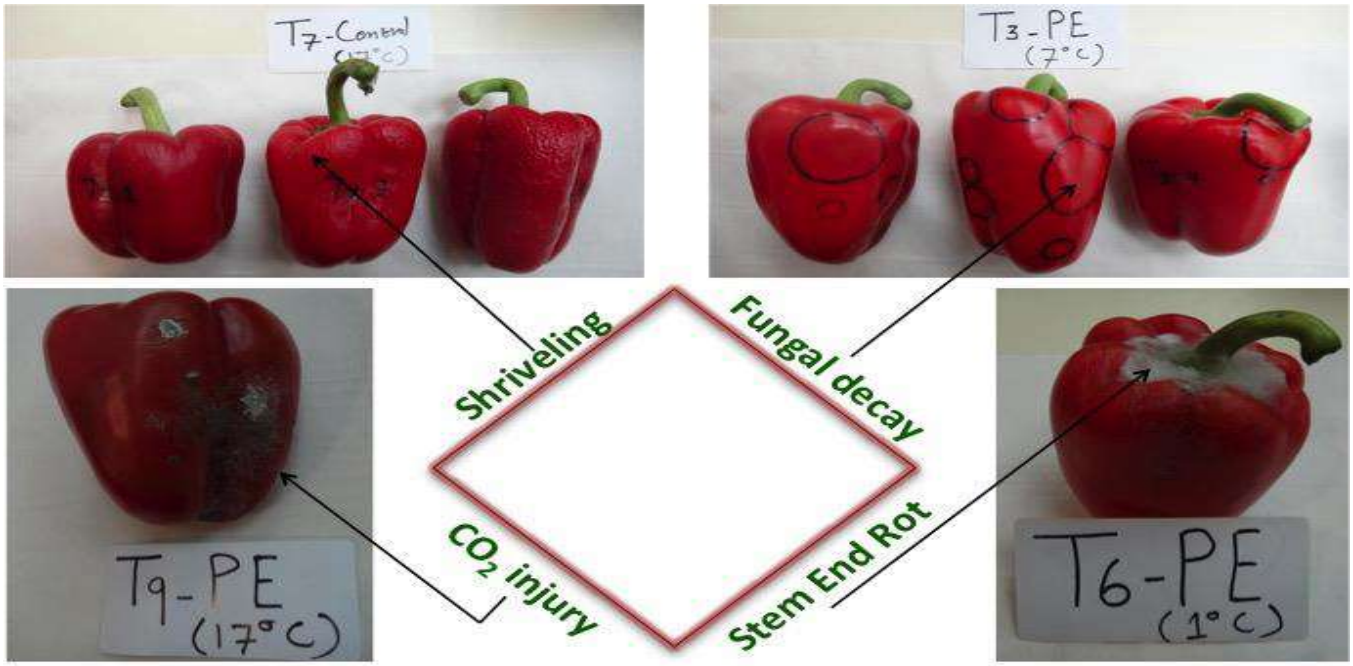

Plate 3: Physiological injuries observed after storage

Table 1: Effects of packing materials and temperatures on physiological injury of bell peppers

Treatments		CO_2 injury	Shriveling
Control	1 °C	-	70
X-tend®		-	30
Polyethene		-	10
Control	7 °C	-	50
X-tend®		-	30
Polyethene		60	-
Control	17 °C	-	100
X-tend®		-	20
Polyethene		10	40

4. CONCLUSION

Best firmness, elasticity and total soluble solid reduction were obtained with X-tend at 7 ºC. X-tend had no water condensation, minimal decay and physiological disorders. Storage temperature of 7 ºC with X-tend packing is suitable to increase the shelf-life of bell pepper.

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CORBICULLA JAPONICA SHELL POWDER ARAGONITE IMPROVED LIVER FUNCTIONS

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Abstract

Aragonite type of shell powder created by firing bivalve *Corbicula japonica* shell at 200℃ for 40min was provided for the bio-activity studies using the alcohol-fed C57BL/6 mice model. Solubility of the aragonite shell powder in 1/10 N acetic acid was 85%, and that of non-fired shell powder being also aragonite was 70%. On the tests used by the alcohol-fed C57BL/6 mice model, the aragonite type of fired shell powder effectively worked to lower AST and ALT values in sera to compare with the values of the saline-fed or just alcohol-fed control mice group. Aragonite shell powder-fed mice additionally lowered level of neutral lipid and cholesterol against control groups in the alcohol-fed mice sera. The aragonite type of shell powder also demonstrated functional roles to restore alcohol-caused liver deterioration, accompanying with lipid decreasing activity.

Keywords: *Corbicula japonica*, aragonite type of fired shell powder, restoration of liver function, lowering lipid value in serum, alcohol-fed C57BL/6 mice model

INTRODUCTION

Aomori prefecture in Japan is a well-known place to produce the high quality bivalve *Corbicula japonica,* and the meat-extract is one of the hot-selling products as a liver protective drink. On one side the meat-removed shells were discarded as a waste without any concrete recycling manner.

Villagers around the lake have been using the fired shell powder of *Corbicula japonica* for a long period as a folk medicine to improve liver function. Lately we could confirm the anti-hepatitis efficacy of the calcite type of shell powder fired at 400-500℃ by the LEC rat hepatitis model [1)]. LEC rat is a special animal used for liver function study due to development of disease around at age 4-6 months, and shifts to cirrhosis stage, followed by development of liver cancer at one and half years age[2)].

Calcite type of shell powder has been already commercialized and selling as a liver function improvement supplement, and the efficiencies based on clinical data are reporting from the clients. However, the aragonite type of shell powder was a different story due to lack of studies on the bio-function.

Present study was then carried out to know whether the aragonite type of *Corbicula japonica* shell powder possessed any biological function, which will eventually lead to development of the health enhancement agent. Aragonite shell powder showed a liver function restorable potency like the calcite against the alcohol-induced liver deterioration, accompanying with improvement of the hyper neutral-lipemia and cholesterolemia in the alcohol-fed mice model.

1. Material and Methods

1-1 Nature of Bivalve Corbicula japonica

Photo 1 Bivalve *Corbicula japonica*
(http://beachmollu.exblog.jp/15282918/)

Bivalve *Corbicula japonica* is a widespread shell fish living in brackish water lakes and tidal flats of river from South of Japan to South of Sakhalin[3)]. For the experiment shell of *Corbicula japonica* was extensively washed in clean water to remove debris of meat and dried completely for the next step experiment of shell firing.

1-2 Crystal structure analysis of the fired shell powder

Three types of shell powders were prepared by changing firing condition from at 200, 360 and 500^{0}C and provided for analyses by both X-ray diffraction apparatus (XD-610 Shimazu Ltd., Japan) and by scanning electron microscopic observation (JEOL Ltd. Japan).

Table 1 Firing condition and structural relation of shell powder

1.	Non-fired naïve shell powder	Aragonite type (100%)
2.	Fired at 200℃, 40 min	Aragonite type (100%)
3.	Fired at 360℃, 120 min	Aragonite + Calcite type (mixture)
4.	Fired at 500℃, 120 min	Calcite type (100%)

1. Non-processed naive shell powder (aragonite type): Non-fired shell powder was 100 % aragonite in crystal structure.
2. Fired at 200℃ for 40 min (aragonite type): Extensively washed shell in water was fired at 200℃ for 40 min with the electronic firing equipment (AT-E 58, Isuzu Ltd., Japan) and cooled down to the room temperature, followed by milling to create fine powder (granule) at 10 ㎛in diameter. This powder was 100% aragonit**e** in crystal structure.
3. Fired at 360℃ for 120 min (mixture of aragonite and calcite): Powder (10㎛ granule in diameter) was a mixture of aragonite and calcite.
4. Fired at 500℃ for 120 min (calcite type): Complete calcite type of powder was obtained.

Sample 1 (non-fired shell powder) and 2 (fired at 200℃ for 40 min) were provided for the bio-functional tests. Sample 3 (fired at 360℃ for 120 min) and 4 (fired at 500℃ for 120 min) were used as reference for crystal structure analysis to compare with sample 1 and 2 (Fig. 1).

1-3 Solubility of the fired shell powder in 1/10 N acetic acid

Above samples listed were subjected to solubility test in 1/10 N acetic acid. Briefly each test sample at 0.5 gr. was stirred in 40 mL acetic acid for 30 min, and insoluble residues were weighted to evaluate solubility of test sample (% w/w).

1-4 Amino acid analysis of Conchiolin in the fired shell powder

Amino acid was measured by the Amino Acid Analyzer (JLC-500V2 JEOL Japan) to know whether or not there remained the heat-resistant *Conchiolin* in shell powder even after fired at 200℃ for 40 min.

1-5 Liver function improvement potency and lipid lowering efficacy of the fired shell powder in the alcohol-fed C57BL/6 mice model

Two-month-old female mice (18-22 gr.) were obtained from the Animal Laboratory Center, Shanghai China to examine the bioactive potency of the fired *Corbicula japonica* aragonite, and formed four experimental groups (5 mice per each group) for the tests;

Table 2 Mice grouping for tests of the aragonite shell powder

G1; (**Physiological saline**/1.0mL, *ip group) (Control)
G2; (**10% ethanol/1.0 mL**, ip group) (Control)
G3; (**10% ethanol/1.0 mL ip + Non-fired shell powder**, oral administration group)
G4; (**10% ethanol/1.0 mL ip + Shell powder fired at 200**℃ **for 40 min**, oral administration group)

*ip: intra-peritoneal injection

For evaluation of bio-activity of aragonite, test sample of the shell powder was serially administered for 18 days into mouse by manner described below.

Alcohol (1.0 ml at 10% alcohol/mouse/day) or distilled physiological saline (1.0 ml/mouse/day) was respectively injected into peritoneal cavity of mouse in the morning, and the non-fired or fired shell powder was orally administered at 50 mg/0.5 ml in distilled water using catheter in the afternoon.

Animal experiments were conducted with the Approvals of the Animal Ethics Committee of University, stating to avoid an excess distress burden to the animals through the experiments.

1-6 Statistical analysis

Student's t-test (SPSS for Windows 16.0) was employed for the evaluation of the statistical significance.

RESULTS

2-1 Solubility of the fired shell powder in 1/10 N acetic acid

Solubility of *Corbicula japonica* shell powder in acetic acid increased in proportion to rising of firing treatment temperature (Table 1). Shell powder fired at 360℃, being a mixture of aragonite and calcite, showed the highest solubility among the samples tested.

Table 3 Solubility of the fired shell powder in 1/10 N acetic acid (% w/w)

Non-fired shell powder (aragonite type)	70 %
Fired powder at 200^{0}C for 40 min(aragonite type)	85
Fired powder at 360^{0}C for 120 min (aragonite + calcite type)	95

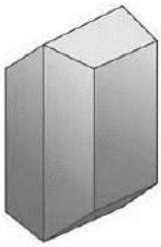
2-2 Scanning electron microscopy of the fired shell powder

High temperature treated shell powder formed fine granule in size (Photo. 2) correlating with high solubility in acetic acid (Table 1).

Photo. 2 Scanning electron micrographs of the fired shell powder
(From left; non-fired (aragonite), 200℃ (aragonite) and 360℃ (aragonite + calcite) respectively)

Structural and chemical feature of aragonite and calcite was listed below to help bio-activity difference between two forms.

Calcite	Aragonite
Chemical Formula: CaCO3	Chemical Formula: CaCO3
Molecular Weight: 100.09g	Molecular Weight: 100.09g
Calcium: 40.04% Ca	Calcium: 40.04% Ca
Carbon: 12.00% C	Carbon: 12.00% C
Oxygen: 47.96% O	Oxygen: 47.96% O
Calcite	Aragonite



(www.carefreeconditioners.com)

2-3 X ray diffraction analysis of the fired shell powder

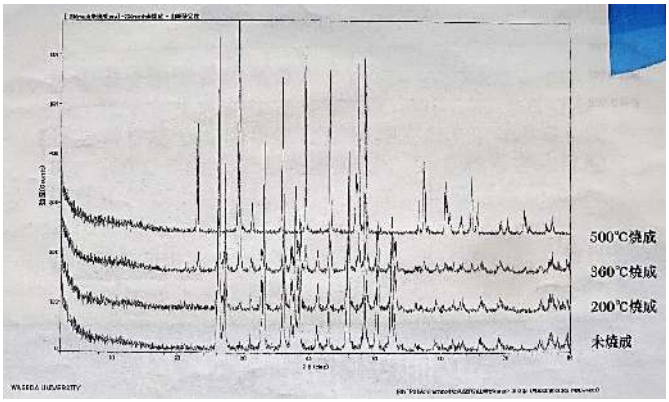


Fig. 1 X ray diffraction feature of the fired shell powder
(From upper; 500, 360, 200^{0}C treatment and non-fired-control) X ray diffraction pattern of the fired shell powder was demonstrated in Fig. 1. Each of four test samples showed the specific profile in detail.

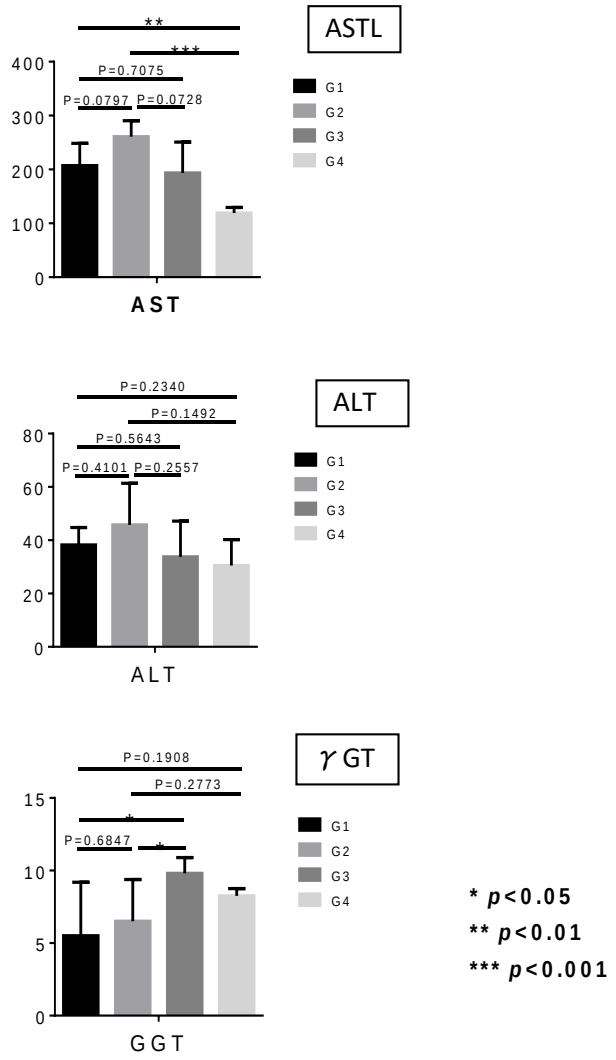
2-4 Amino acid analysis of the 200℃ for 40 min treated shell powder

Amino acids were detectable at 0.85 g/100 g in the 200℃ treated shell powder by analysis, and *Conchiolin*[4] was not burnt out by treatment at 200℃ for 40 min treatment. Amino acids detected were aspartic acid 16.5% (w/w), glycine 36.5%, tyrosine 8.2%, phenyl-alanine 5.9%, glutamic acid 4.7% and proline 4.7%. No amino acid was detected in the sample fired at 500℃ for 120 min.

Conchiolin was a part of matrix of organic macromolecules, mainly consisted of proteins and polysaccharides to hold and bind the crystals of aragonite to give shells stiffness. Its role was known to facilitate calcification during shell formation[5], but bio-logical function remains unclear up to now.

2-5 Improvement of liver dis-function by the aragonite type of shell powder fired at 200℃ for 40 min in the alcohol-fed mice model

Shell powder fired at 200℃ for 40 min (G4 group; aragonite group) effectively worked to lower AST (aspartate aminotransferase) and ALT (alanine aminotransferase) values in the alcohol-fed mice, however it was less effective against γ-GT (γ-glutamyl transferase) value. Non-fired Aragonite type of shell powder slightly worked to keep AST and ALT low in level (Fig. 2).



G1; Control (Physiological saline, ip)
G2; Control (Alcohol, ip)
G3; 10% ethanol/1.0 mL ip + Non-fired shell powder, oral administration
G4; 10% ethanol/1.0 mL ip + Aragonite shell powder fired at 200℃ for 40 min, oral administration

*ip: intra-peritoneal injection

Fig. 2 Liver damage protective potency of the aragonite type of shell powder in the alcohol–fed mice model.

2-6 Lipid lowering potency of the aragonite type of shell powder fired at 200℃ for 40 min in the alcohol-fed mice model

Aragonite type of shell powder was orally given to the alcohol-fed mice for 18 serial days to assess lipid lowering efficacy, and the outcomes were summarized in Table 2. Aragonite powder-fed mice lowered the serum lipid level of neutral lipid and total cholesterol compared with those of saline-fed, alcohol-fed, and non-fired shell powder-fed mice.

Table 4 Lipid lowering potency of the aragonite type of shell powder in the alcohol-fed C57BL/6 mice model

Treatment	Neutral lipid (mil mol. /L)	Total cholesterol (mil mol. /L)
Physiological saline	Median; 1.2 (1.0〜1.3)	2.4 (2.0〜2.8)
Alcohol alone	Median; 1.7 (1.5〜1.9)	4.1 (3.5〜4.8)
Alcohol + Non fired Sample	Median; 1.7 (1.6〜1.8)	3.8 (3.0〜4.5)
Alcohol + Fired A Sample	Median; 0.5 (0.3〜0.6)	2.6 (2.3〜2.9)

Discussion

The calcite type of shell powder prepared from the bivalve *Corbicula japonica* by firing at 500℃ was effective to improve mal-function of liver in the LEC rat hepatitis mode[1)]. Our concern is this time toward to know how the aragonite type of shell powder works, and whether it has any biological potency, because the aragonite type of shell powder was discarded as useless waste.

In present studies it became obvious that the aragonite type of shell powder, regardless of fired or non-fired, had similar bio-active potency such as lowering AST (aspartate aminotransferase) and ALT (alanine aminotransferase) value in alcohol-fed mice (Fig. 2), even though its activity of the non-fired (naïve) was somewhat weak in anti-deterioration activity.

Prominent bio-logical effect of the aragonite of shell powder was that neutral lipid and total cholesterol values in the aragonite-fed mice were powerfully affected by reducing their values from 1.7→0.46 mil mol (neutral lipid) and 4.1→2.6 mil mol (cholesterol) in the alcohol-fed control mice (Table 4).

Persuasive explanation to this phenomenon was not at the moment in our hand that why the aragonite or calcite type of shell powder worked to lower the hepatocellular enzyme and serum lipid level in the alcohol-fed animal model, but the shell powder solubility might somewhat affect to bio-activity elevation *in vivo* (Table 3, Fig. 2).

On the cell culture analysis of human adipocytes[6)], increasing calcitriol (a form of vitamin D that is used to treat and prevent low levels of calcium in the blood) can increase lipogenesis, decrease lipolysis, and increase messenger RNA expression of a number of fat-metabolism-related genes by stimulating calcium influx. However, it is not enough data to accept that way of thinking and more experimental information should be accumulated for well understanding of mechanisms.

The biggest issue on this work still remains unsolved why the aragonite or calcite shell powder works, and why the calcite is more effective in bio-functional activity than those of the aragonite. Both agents have same chemical structure, and only difference between them is the physical structure as seen in the text. It is a fascinating subject to do research in future.

CONCLUSION

The aragonite type of shell powder prepared from *Corbicula japonica* worked likely to be the *Corbicula japonica* calcite to restore the alcohol-damaged liver cells. It also demonstrated lipid lowering activity in serum in the alcohol-fed mice model. The aragonite or calcite type of shell powder prepared by firing of *Corbicula japonica* shell is a fascinating calcium supplemental material armed with the important bio-functions, even though several unsolved problems still remained.

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A STUDY ON USE OF LOCAL FOOD STUFFS AND TRADITIONAL KNOWLEDGE FOR IMPROVING PUBLIC HEALTH NUTRITION IN MAYURBHANJ DISTRICT, ODISHA

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Abstract

Major health problems of the 21st century include nutritional deficiencies and dietary changes in both rural and tribal settings. Nutritional analysis, combined with an understanding of traditional systems and resources, can help to identify the biological and socio-cultural components in solution of dietary and health problems associated with dietary change and adaptive strategies for the future. Addressing nutritional needs offers a primary rational for the preservation of traditional knowledge and life-style, the conservation of wild and cultivated resources and the sustainable use of the environment in which they are located. The objectives of the present study were to discuss about the practice of traditional knowledge and culture in public health nutrition among tribal population of Mayurbhanj district of Odisha, which forms one of the mega biodiversity zones of the country rich in flora and fauna. One hundred households were selected by random purposive sampling method for collection of data and information on traditional food and health practices was collected by interview cum questionnaire method. The results of the study revealed that they used different types of locally available foods for their health benefits such as Palta medicines for son and Babul leaves for fair baby during pregnancy, Kalibahu andGai chira(one type of root) for recovery after delivery, Bottlle gourd and sago dana kheer for better lactation, Handia for better health and getting relief from tiredness .For getting relief from diseased conditions they used various types of things such as Wild ant chutney for cough, Gangasiuli leaves juice for Malaria fever, Amar poi leaves for Diarrhea, Powder of Black berry seeds for Diabetes, Bhalia for Eczema, Neem leaves and oil for scabies, Handia rasi, Pedipedica leaves and Mehendi roots for Jaundice, Bug with banana for Piles, etc. All types of medical facilities are available in that locality but the people were not utilizing it because of their misconception and lack of knowledge. Thus emphasis should be given on nutrition and health education of the local people and further in depth scientific research is required in this direction to adopt new strategies for future generation.

Keywords: Food habits, Babul leaves, Pedipedica leaves, Traditional knowledge

INTRODUCTION:

India has a concentration of 104.3 million tribal people and considered to be the second largest in the world next to Africa. These Tribal people constitute 8.6 percent of total Indian population (census of India, 2011) of which 93.8% resides in rural areas. The country today is placed in a piquant position having succeeded in solving some problems while new ones are emerging. Current development in various dimensions has not been able to offer succor of the poor especially the tribal population. Indeed, the difference between the poor tribal people and other population groups is widening. Various International and National Organization provides many schemes for them but the tribal people are downtrodden and vulnerable often suffering from various socio-economic, demographic, physical, nutritional and health problems. Most of the developmental indicators like level of poverty, nutrition, per capita income, basic education. IMR, MMR, employment etc. appear to be very severe in backward states of India.

Among 30 States, Odisha, being socio-economically backward and culturally sound, occupies a unique place in the tribal map of the country having largest number of tribal communities with a population of 9.59 million constituting 22.86% of states population and 9.17% of the total tribal population of the country (census of India, 2011). Despite remarkable worldwide progress in the field of diagnostics and curative and preventive health, still the tribal people of Odisha are living in isolated area, far away from civilization with their traditional values, customs, beliefs and myth intact. They manage their livelihood through agriculture and maintained an indigenous life with their own knowledge system. They used to maintain the long standing traditions from their ancestors and spread the knowledge in different spheres of their life which is popularly called local knowledge or indigenous knowledge. The concept of indigenous knowledge gained its worldwide recognition through the United Nation conference on Environment and Education in 1992, World Conservation strategy of International Union and Conservations of Natural Resources in 1980, Brundtland Commission and World Commission on Environment and Development, 1987. With modernization of the present society, the needs of those disadvantaged populations were brought to the limelight and their knowledges system was given the importance and treated as real knowledge for survival. This paper is intended to unfurl the practice of traditional knowledge and culture in improving the public health nutrition of the tribal people of Mayurbhanj district of Odisha.

The objective of the study was

1) To study the socio-economic conditions of the respondents.
2) To know the food habits and consumption of traditional foods in their daily diet.
3) To study the use of indigenous foods in treating some diseases.
4) To analyze practice of indigenous knowledge for betterment of reproductive health.
5) To study different myths in treating some health problems.

Materials and Methodology

The study was carried out in Jashipur block of Mayurbhanj districts of Odisha. One hundred households were selected for the study by random purposive sampling method. The data was collected by questionnaire cum interview method with the help of pretested and modified questions. The head of the family was interviewed for the collection of data. 24 hours' recall method was used to know their food habits. Information on indigenous knowledge for treating different health problems was collected by interviewing the participants. The collected data was tabulated and analyzed with the help of statistical tools and techniques and are discussed below.

Results and Discussion-

The results of the study were compiled and discussed below.

a) Socio-Economic indicator of the respondents-

The socio-economic conditions of the respondents provide relevant information regarding their family background. It was observed that majority of the respondents belong to the age group 25 to 60 yrs i.e. All of them were Hindu by religion and were scheduled tribe. 95% of them were literate. Nuclear family system was found to be prevalent in that area. All of them like to be in joint family but they preferred to be in nuclear family system to avail government facilities such as ration card, BPL Card, Indira Abas Yojana etc. Primary occupation of the respondents was found to be agriculture (83%) and all (100%) of them belonged to low income group.

Table -1: Socio-Economic Indicators

Sl	Socio- economic Indicators	Characteristics	Percentage
1	Age	25 - 60 years	72
2	Religion	Hindu	100
3	Education	Literate	95
4	Marital status	Married	100
5	Types of family	Nuclear	92
6	Occupation	Agriculture	83
7	Types of house	Kucha	82
8	Farm animal	Cow, Goat, Hen, Cock, Pig. etc.	100
9	Income	(Rs. 4000-10000) Month	100

Most of them were staying in their kutcha houses and kept poultry, goat, sheep, cow, pig in their houses for the purpose of meat.

b) Food habits and food consumption pattern

It was observed that all respondents were non vegetarian and took three meals per day. They took a heavy breakfast i.e. Pakhal (Flooded rice) / Rice flake / Puffed rice with vegetables or dried fish, chili, onion etc. and walked out for their work place with packed lunch. Generally, they cooked their food only in the night and kept it for the next day. They prepared non-vegetarian food on every market day i.e. at least twice per week either broiler or fish or chicken. They consumed mutton occasionally during festivals.

Rice was their staple food. They cultivated and consumed different types of high yielding varieties of rice such as babailaccha, mossori, swarna, lalat etc. Different types of locally grown and seasonal pulses, vegetables, leafy vegetable mushrooms, fruits and meat fish and eggs were included in their daily diet. The availability of different local food stuffs is shown in Table No.2. Various uncommon food stuffs consumed by them were dal eg. khesaridal, horsegram dal, vegetables such as pindra, dimri, kaunara etc., leafy vegetables such as bathua, hirmichia, khapra, keendali, pitagama, kaunra, kachu etc., mushrooms such as rutka chhattu, bali chhattu, kukuda chgattu, dashra chhattu, kadhan chhattu etc., Fruits such as charkoli, cusum koli, kanta koli etc. They consumed different types of meat such a bat meat, pigeon meat, snail meat, kurkuti meat in their diet according to availability.

Handia, Handiarasi and Mahuli were the common beverages consumed daily by the respondents Handia is prepared by fermenteding soft cooked rice with Ranu Powder in a covered mud pot or silver pot for three days. Ranu powder is prepared out of bark of Kankada tree and rice powder. After three days of fermentation Handia will be prepared. The top water is discarded carefully which is known as Rasi. The remaining fermented rice is sieved with the help of bamboo net mixing little bit water which is known as Handia. Mahuli is prepared out of Mahula one type of fruit.

Process of food preparation and food preservation was found to be hygienic. They dried all excess stuff in the sun without mixing salt or turmeric and kept it in bamboo basket. Due to exposure to air and moisture the foods may be spoiled by contamination with fungus, bacteria or worm etc. But sometimes they consumed those without looking into its freshness and suffer from health problems such a cholera, diarrhea and dysentery etc. Loss of life was also reported in some cases due to consumption of poisonous mushroom and other un hygienic foods.

Table - 2: Traditional foods consumed in the locality

Sl No.	Food Group	Types of foods	Frequency of consumption
1	Cereal	Different kind of rice	Daily
2	Pulses	Khesari (*lathyrus sativus)*, barbatti (*vigma catjang)*, Horsegram / black gram, Red gram (*cajanus cajan)*, lentil (*cicer aientinum)*, green gram	Weekly but according to seasonal availability
3	Vegetables	Pindra (*morinda citrifolia)*, dimiri *(ficushispida*), cabbage(*brassica oleracea)*, kunduri, kaunra, radish, brinjal, tomato, bitter gurd, bottle gurd,pumpkin etc,bamboo	Seasonally / according to availability
4	Leafy Vegetables	Bathua (*cheno podium album)*, bhaji (*amaranthus viridis)*, hirmichiya (*enhydra tluctuans)*, kalam (*ipomea reptans)*, drumstick (*moringa oleifera)*, spinach, radish leave, khapra (*trianthema monogyna)*, kundali (*ipomea batata)*, pita ghima, tentuli leave (*tamarindus indica)*, kaunra (*abelmoschus manihot)*, colecossia (*colocasia esculenta)*, kachu	Seasonally / according to availability
5	Mushrooms	Rutka, bali, kukuda, dashra, kadhan,	Specially in rainy or winter season.
6	Meat, fish, egg	Mutton ,chicken, pigeon,bat, crab, snail, kurkuti (*oecophylla smaragdina)*, and different kind of fish, egg of hen and duck	At least twice per week and also according to availability
7	Fruits	Kendu *(dios pyros melanoxylon)*, black berry (*syzygium cumini)*,charkoli (*buchanania lanzan)*,mango, jack fruits, kusuma (*schleichera oleosa)*, ambada (*spondias pinnata)* koli (*ziziphus mauritiana)*	Seasonal
8	Beverage	Handia, rashi, mahuli	Daily / Weekly
9	Preserved food	Vegetables, Meat, Fish, Mushrooms	Occasionally

c) Health culture & health practice of the respondents:

Health culture and health practices of the tribal vary according to their communities and geographical location. However, education, media and Govt. involvement has some impact on their health practices. Information regarding different types of treatment adopted, use of traditional knowledge for improving public health & nutrition were collected and discussed below.

(i) Types of treatment adopted to improve health condition:

It was observed that 100% of the respondents believe in magic treatment and herbal treatment but only 46% of them practice magic treatment, especially for fever, diarrhea, cholera, colic pain etc. They also believe in medical treatment and went to nearby to hospital with ANM, Asha Karmi or Anganwadi worker for their health problem. But in case of severity of the disease, they went to both local gunia as well as consult doctor and follow their treatment.

Table No. 3: Health problem:

Sl. No	Health problem	Frequency & %	Treatment adopted
1.	Malaria/Cold/ fever/ cough	88 12	Medical & Herbal/ Magic treatment
2.	Joint pain/Arthritis/ Rheumatism	85	Herbal medicine
3.	Eczema/ Skin problem	72	local treatment/Indigenous medicine
4.	Problems related to reproduction	12	Doctor
		88	local treatment/Indigenous medicine
5.	Diabetes/Heart Problem	14	Doctor & Indigenous medicine
6.	Diarrhea / Dysentery	100%	Indigenous medicine

(ii) Foods taken during disease condition

It was interesting to note that they consumed different types of foods to get recovery from illness which is shown in table no. 4

Generally, they used the above food materials & leaves for getting relief from diseases at their household level & in case of emergency they consult doctor. Coconut ladoo was given to relieve back pain and to provide strength. Verma (2002) reported in her studies that buttermilk is beneficial for diarrhea. Sachan et.al, (2012) found in their study that tribal of Similipal biosphere reserve use different types of mushrooms as a source of food as well as for treating malnutrition, weakness and other nutritional disorders.

Table No. 4: Food taken during disease condition to get recovery

Sl No	Disease	Remedies	Frequency	Percentage
1	Cough	Basanga (*Adhatoda zeylanica)* leaf, wild ant (kurkuti-*Oecophylla smaragdinaS*) chutney.	89	89
2	Cold	Durmstic leaves with masur dal and torani (Soaked water of cooked rice)	73	73
3	Indigestion	Black pepper with bael leaves	54	54
4	Diarrhea	Amarpoei (*Kalanchoe pinnata)* leaves and guava leave	83	83
5	Dysentery	Burn skin and ear of goat	59	59
6	Piles	Bug with banana	64	64
7	Malaria	Gangasiuli(*Nyctanthes arbortristis)* leaves juice with black peper, ginger and honey	100	100
8	Jaundice	Handia rasi, mehendi root, Redgram (*Cajanus cajan)* leave, pedi pedika (*Abutilon indica)* leaves.	100	100
9	Mouth disease	Warm discarded water of cooked rice. Pig oil, green chilly	94	94
10	Ear in infection	Putting kunduru leave juice in ear	56	56
11	Pimples	Smearing Pigeons stool , massor dal paste on pimples	73	73
12	Scabies	Neem leaves & neem oil	67	67
13	Diabetes	Powder of blackberry seeds	14	14
14	Weakness, Tiredness	Handia	99	99
15	Better health	Mushroom	59	59

iii) Health practices related to reproductive health

Table No. 5 Foods / Remedies related to reproduction

Sl No	Causes	Remedies	Percentage
1	For son child	Palta medicine made by local kabiraj,	88
		small raw gadisa fish with ripe banana	10
2	For fair baby	Powder of of Babul (*Acacia nilotica)* leaves	10
3	Quick recovery after child birth	Sutika goli made by local kabiraj	99
4	For abortion	Runja (*Abrus precatorius)* seeds	05
5	Mensuration delay	Through Mustard seeds under the bed	80
6	For better milk secretion	Bottle gourd sabjee & sago dana kheer	79

The information on food or remedies adopted by the people of studied area was found to be interesting. 88% of respondents used palta medicine for son where as 10% of the respondents took small gadish fish inside a ripe banana for getting son during pregnancy. Palta medicine is prepared by the local Kabiraj with combination of dung of black female goat having only male calve and blackgram (dung + black gram). For fair child they took powder of babul leaves from third month of pregnancy up to nine months. They took one spoon of it in the morning in empty stomach with water for first fifteen days of third month to 9th month. 99% of the respondents found to take sutika goli prepared with Kalibahu and gaichira roots for getting quick recovery after delivery. For better milk secretion, they took sago dana kheer & bottle gourd sabjee during lactation. Telesara (2000) found out Gond Ladoo was given to pregnant & lactating mothers to increase milk output and prevent excessive bleeding.

iv) Myth in some health problem

Table No. - 6: Practice of myths in some health problems

Sl No	Disease	Remedies	Frequency & Percentage
1	Eczema	Putting warm molten Bhalia on affected area	70
2	Migrain	Burn with hot iron rod (Nia chenka)	40
3	Colic pain	Burn with hot iron (Nia chenka) on 21st day of birth and on Makar Sankranti	95
4	Pimples	Smearing Pigeon Stool, Lentil paste on pimple	73
5	Eye allergy	Smearing dung of black buffalo on head	43

There are many instances of death of children in Odisha due to burn with hot iron (Nia chenka) in stomach but still the practice persists among tribal in 95% cases. Putting warm Molten Bhalia on affected area of eczema was also observed. Some of them used pigeon stool on pimples and smeared black buffalo's dung on the head to get relive from eye allergy. Those practices were sometimes virulent and create serious health problems in some cases. Similar findings were also observed by Dash (2014) and Pedi et.al (2013).

Elizabeth et.al. (2015) found in their discussion that common beliefs, customs, practices related to health and disease of the people are influencing their health seeking behaviour, low productivity and poverty.

Conclusion:

Tribal preserve, enrich and enliven the cultural diversity of India besides making up a substantial portion of total population of the country Odisha has a large number of tribal communities who love to live in nature and maintained their livelihood with their own indigenous / traditional knowledge system specially the disadvantaged ones who are deprived of economic, social and political benefits. Further, abundance of tribal people's access to forest product and indigenous health care system contributes positively to the tribal health. They have their own system of diagnosis and cure. They prepare their own medicine usually using herbs and other items collected from the nature and processed locally. These natural resources and skills are disappearing. Moreover, traditional system cannot treat most of the present new emerging diseases that modern medicine can do. Health and sanitation are often worse in regions where tribal peoples live.

Thus herculean efforts should be taken in all direction to address the health and nutritional problems of the tribal people by educating them on food safety and security, creating awareness about available health care services at their door step. Emphasis should be given on preserving indigenous knowledge of tribal regarding health benefits & further scientific research should be carried out in this direction to adopt new strategies for future generation.

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THE EFFECT OF HYDRAULIC RETENTION TIME AND LOADING RATE ON THE REMOVAL OF POLLUTANTS FROM FISH PROCESSING WASTEWATER BY ANAEROBIC PROCESS

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Abstract

Anaerobic treatment model treats fish processing wastewater to be necessary for a small and medium factory that is very popular in Vietnam and other countries. Several techniques have been proposed. However, they are quite expensive and hardly operation, especially in remote areas. In this study, the hydraulic retention times (HRT) including 3, 5, and 7 hours with a various organic loading rate of 1.5 to 6.5 kg COD/m3/day were investigated. Biomass concentration as mix-liquor volatile suspended solid (MLVSS) in the model is at 6,000 to 9,000 mg/L. On the basis of the result the optimal HRT with a 4.0 kg COD/m3/day organic loading rate was 8 hours which BOD5, COD removal efficiency were 92.18, 87.36 percent respectively. By the end of the optimal hydraulic retention times, the total methane gas volume as a by-product was collected with 2.6 liters.

Keywords*:* Wastewater treatment, anaerobic process, organic loading rate.

1. INTRODUCTION

Global consumption of fish has doubled since 1973, and the developing world has been responsible for nearly all of this growth. The total per capita consumption of food fish in the developing world has increased from 7.3 kg/capita/year to 14.0 kg/capita/year from 1973 to 1997 while it has come down from 22.6 kg/capita/ year to 21.7 kg/capita/year from 1973 to 1997 in the case of the developed world. The projected per capita consumption of food fish in the year 2020 are estimated to be 16.2 kg/capita/year and 21.5 kg/capita/year for the developing world and developed world, respectively (Chowdhury, Viraraghavan et al. 2010).

Water consumption in a fish-processing industry and high-strength wastewater from such an industry are of great concern world-wide. Liquid effluent regulations are becoming more stringent day by day. The anaerobic treatment process can be considered as the core method of a resource preservation and environmental protection (EPRP) technology, and it, therefore, represents - combined with other proper methods - the advanced sustainable technology society urgently needs. Despite the persisting reluctant attitude of the established wastewater pollution control world, anaerobic treatment is assured of increased usage in the future, the more so because the potentials of the method are far bigger than expected a few years ago(G. Lettinga 1997). The big benefits of the anaerobic wastewater treatment concept compared to conventional aerobic methods should be known and will not be discussed here again. Accepting that the anaerobic treatment principle is a pre-treatment method, at the present state of knowledge, little if any serious drawbacks can be brought up against it any more. Previously mentioned drawbacks have vanished almost completely, like its presumed low stability.

The anaerobic treatment of wastewaters from the seafood-processing industry was studied in a 15 m3 industrial pilot-plant. These effluents have a high organic content (10–60 g COD/liter), with protein percentages between 25 and 70%, and a salinity similar to sea water: sodium (5–12 g/l), chloride (8–19 g/l) and sulphate (0·6–2·7 g/l). This high concentration of salts, together with the production of sulphide and ammonia due to sulphate reduction and protein breakdown, respectively, produces important inhibitory/toxic effects on non-adapted biomass. After an initial start-up procedure, where the acclimation of the biomass was the objective, 70–90% organic matter removal was achieved (Omil, Méndez et al. 1995).

The performance of one-step UASB reactors treating fish processing wastewater of different lipid levels was determined using artificially generated influent simulating that of the canning of sardines and tuna. The organic loading rates (OLR) and the hydraulic retention times (HRT) were 5–8 g COD.L−1. d−1 and 11–12 hours, respectively. In treating a wastewater that contains 3–4 g.L−1 total COD of which 5–9% was lipids, the COD removal and conversion to methane were ca.78% and 61%, respectively (A. Palenzuela-Rollon 2002).

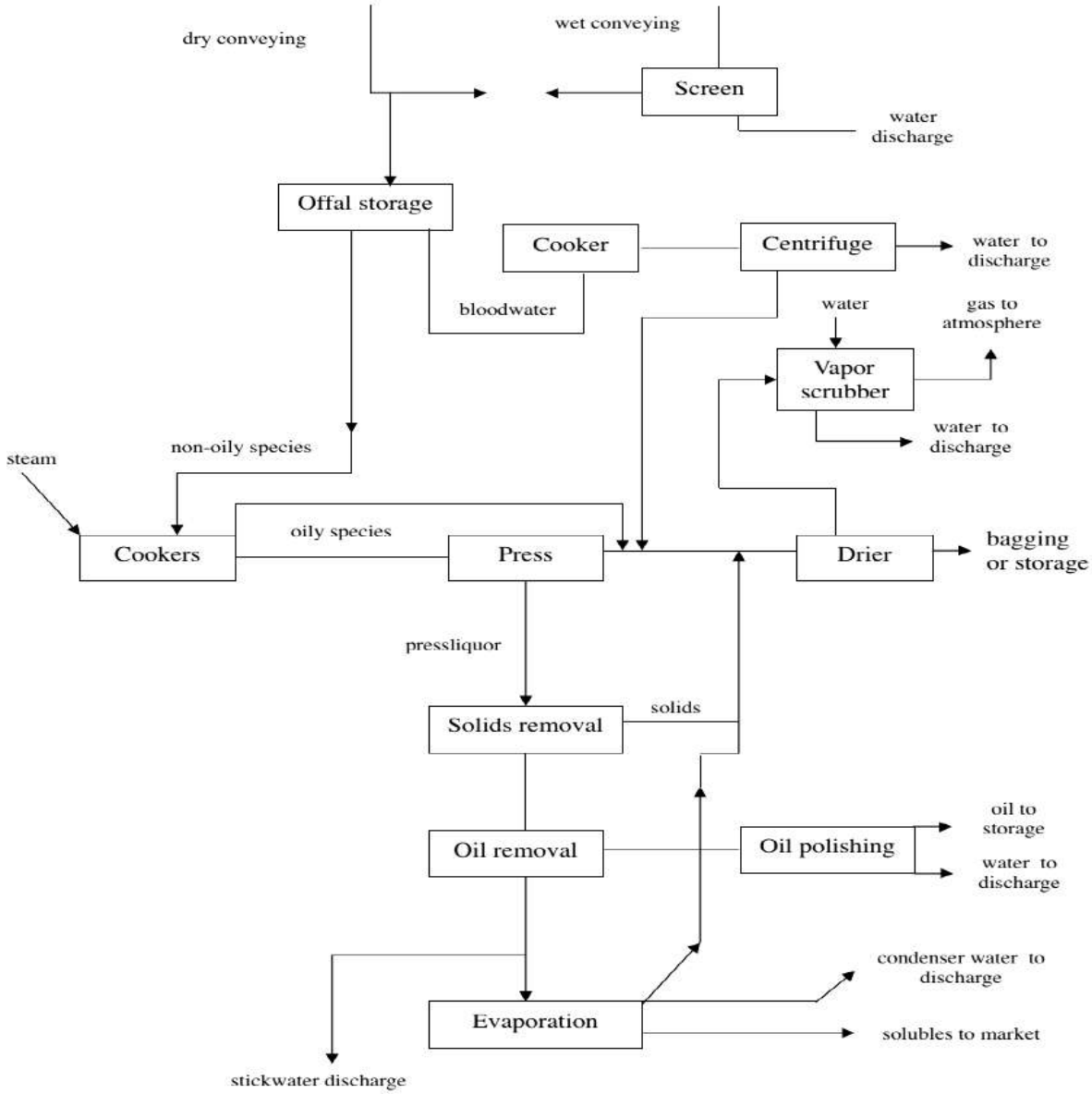

Fig. 1. Flow diagram for fish meal production (adapted from (Riddle 1973))

2. Experiments

2.1 Lab-scale model setup

The system was designed with a 7-litter anaerobic tank used in this study was made with a column of 120mm inner diameter, a total volume of 7 liters including reaction section of 6.5 liters and a gas zone of 0.5 liters. The wastewater was introduced at the bottom of the reactor through a tube of about 2mm. Sludge concentration in the reactor was 12.5gSS/L (11.3gVSS/L).

The methane gas was collected to a column of 5 liters' tank that was filled with sodium hydroxide solution (5 percent) to absorpt carbon dioxide (CO2) and hydrosulfite (H2S) gases. Influent and effluent samples were analyzed for pH, alkalinity, COD, BOD5, suspended solid (SS).

The system was started up in 30 days at 1.0 kg COD/m^3/d before analyzing all parameters.

2.2 Methods of analysis

COD, BOD5, SS, VSS, TN, TP was measured depending on Standard Method (1993), respectively. SS in effluent and VSS in sludge samples were measured by Total suspended solids dried at 103 – 1050C and volatile solids ignited at 5500C based on Standard Method (1993), respectively.

Table 1. The parameters of raw wastewater

Time (Days)	OLR (kgCOD/m³/d)	HRT (h)	COD_{in} (mg/l)	BOD_{in} (mg/l)	pH_{in}
1	1.5	7	1231	-	7.1±0.3
3			1050	-	
5			1035	-	
7			1127	-	
8			1341	-	
9			1110	-	
12			1020	-	
13			1224	832	
15			1080	-	
19			1350	-	
21			1180	-	
22			1425	926	
24			1025	-	
26			1025	-	
29	3.5	5	1100	-	7.2±0.5
31			1099	-	
33			1054	-	
35			1032	-	
37			1157	-	
41			1389	931	
42			1279	-	
44			1076	-	
47			1055	-	
51			1077	-	
53			1179	-	
55			1290	-	
57			1390	917	
60	6.5	3	1378	-	6.9±0.2
62			1364	-	
63			979	-	
65			1188	-	
66			1056	-	
69			1349	-	
71			1382	903	
73			1017	-	
74			1051	-	
77			1093	-	
80			1067	-	
81			1178	-	
83			1193	776	
85			966	-	
7			1328	-	
90			1084	-	

3. Results and Discussions

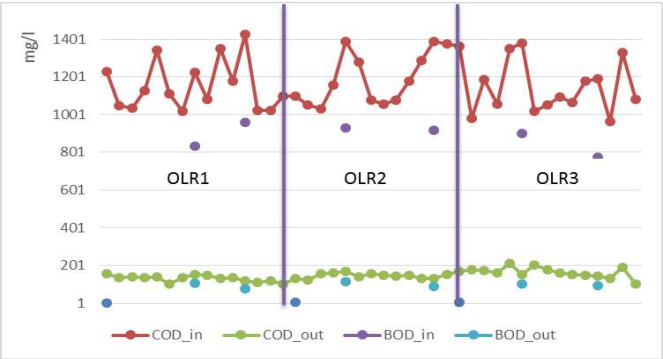

Fig .2. The changes in COD, BOD inlet, and outlet through 3 OLRs

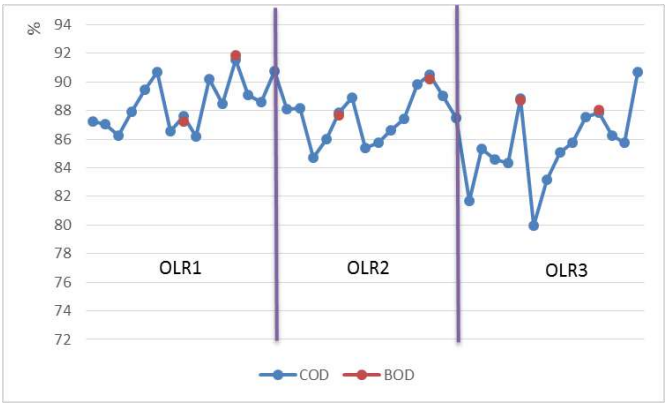

Fig .3. The changes in COD, BOD efficiency through 3 OLRs

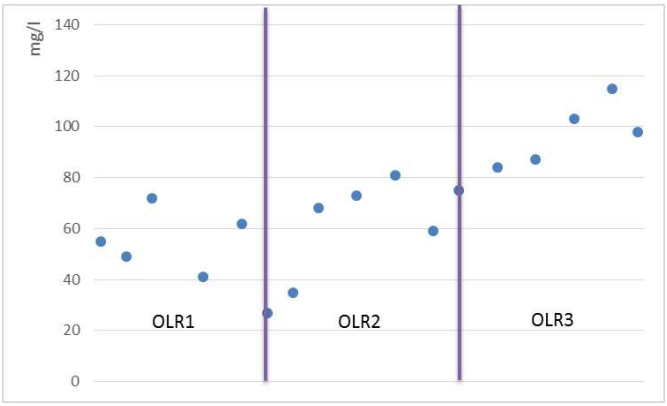

Fig .4. The changes in SS outlet through 3 OLRs

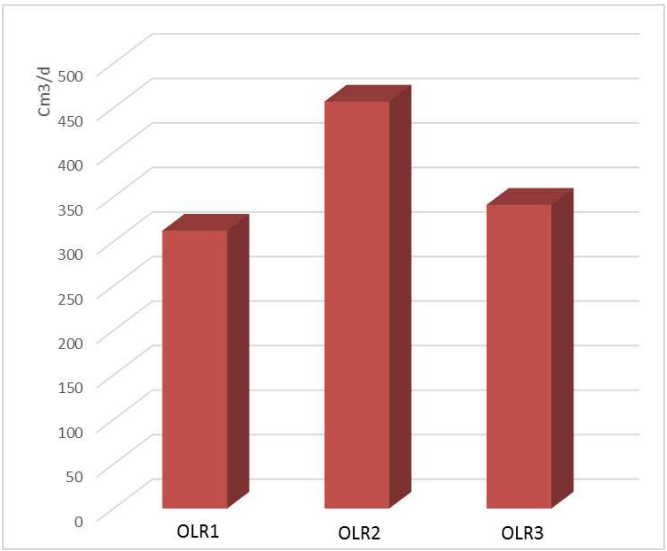

Fig .5. The changes in methane gas producing as by-product through 3 OLRs

The model was operated continuously for 120 days (including 30 days for startup), one loading rate each month, from 1.5 to 6.5 kg COD/m3/d.

Figure 2, 3 show that there were little changes in BOD and COD of raw wastewater, the average of COD inlet was 1164 (mg/l) during all operating time while was 887 (mg/l) with BOD. At first OLR1 (1.5 COD/m3/d), the average outlet of COD was 131 (±16) (mg/l) and the efficiency removal remained stable at around over 88%, that was significantly high. This figure of BOD was nearly 90%. Regarding OLR2 (3.5 COD/m3/d), there was clear that the outlet of both COD and BOD was slightly higher than OLR1 with 146 (±16) (mg/l) and 103 (±10) (mg/l), respectively. It could be seen that the efficiency removal reached 87 (±2) % and 89 (±2) %, respectively.

Figure 4 unveils that the model could remove SS efficiently. The concentration of inlet SS often maintained at around over 500 (mg/l) while the outlet was stable at 80 (mg/l) and tended to increase through OLRs.

Methane gas is a by-product that appears in any anaerobic process using bacteria and people often utilize this kind of gas for producing electricity, lighting, burning, etc. During the operating period, this system produced about 300 cm3/d at the first OLR and rose dramatically to 450 cm3/d before went down again to around 310 cm3/d.

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4. CONCLUSIONS

The results achieved in this research revealed that the anaerobic process could be applied as an effective solution for fish processing wastewater in tropical regions such as Vietnam, India where fishery resource play an important role in the economy. Based on the results, here the following conclusions could be drawn:

The optimum HRT and OLR for treating fish processing wastewater through the anaerobic process were 3.5 kg COD/m3/d and 5 hours. As these conditions, the average removal efficiency for COD and BOD5 was 87 and 89 percent, respectively. The SS concentration effluent often below 120 mg/L. The methane volume produced of 450 cm3/d.

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COMPARISON OF EMULSIONS STABILISED BY ANIONIC AND CATIONIC BIOPOLYMERS

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Abstract

Emulsions are used in the formulation of processed foods making it necessary to be stable over an extended period under varying stress conditions. In this study, oil-in-water emulsions were prepared by dispersing the oil into a water phase by using energy from ultrasonication. All the emulsions were prepared by sonicating for 5 minutes with a duty cycle of 0.5 at 20 kHz at using a sonotrode probe of 12 mm tip diameter. Two types of emulsions, a cationic and an anionic emulsion were formed by using two different food-grade emulsifiers and oil at 1% (w/v). The emulsifiers used for the study are sodium caseinate and gum Arabic that forms cationic and anionic oil droplets respectively, at pH 3. The emulsion quality was assessed by measuring the surface charge, particle size distribution and creaming stability. Gum Arabic stabilised emulsions had negative zeta-potential of -5.39 ± 0.779 mV while, sodium caseinate stabilised emulsions had a positive zeta-potential of +16.13 ± 0.929 mV at an emulsifier concentration of 12 mg/ml. The particle size of cationic emulsions varied from 641.7 ± 39.47 to 402.93 ± 18.31 nm for sodium caseinate concentration ranging from 2 to 12 mg/ml. The stability of emulsions to various environmental stresses like the changes in pH (3 to 9), temperature (30 to 90 ºC) and salt concentration (50, 100, 500 mM NaCl) was determined. Gum Arabic stabilised emulsions were free from flocculation on the addition of sodium chloride up to 500 mM while cationic sodium caseinate emulsions destabilised

Keywords: ζ-potential, creaming stability, freeze-thaw cycling, ultra-sonication

1. INTRODUCTION

Manufactured foods exist in structures such as emulsions and foams. The control of stability of such structures is an essential skill for food technologist. A wide range of materials for food use with emulsifying and stabilising ability are available. This includes surfactants (Tweens, lecithins, sorbitan esters, monoglycerides), proteins (whey protein isolate, β-lactoglobulin, gelatin, sodium caseinate) and polysaccharides (gum arabic, pectin, chitosan). Most of these species behave as surface-active agents that act by lowering the surface tension at air-water (foam) or oil-water (emulsion) interfaces. The ability of biopolymers to prevent recoalescence of the separated phases of the dispersion is attributed to the viscoelastic membrane that forms structural barriers (Dickinson, 2015). Many processed food systems are structured in the form of an emulsion, for example, milk, beverages and sauces. A thorough understanding of the interactions between the components of the emulsion system is necessary to ensure long term stability. Conventional oil-in-water emulsion is the most basic and widely found emulsion. (McClements, 2015).

Emulsions are stabilised by a combination of thermodynamic and kinetics factors. Oil-in-water emulsions have an innate nature to separate into cream and serum phases due to thermodynamic factors. Though thermodynamically unstable, food emulsions could be kinetically stable for years (McClements, 2015). Several approaches are employed to bring stability in an emulsion system such as increasing the concentration of droplets, reducing the size of droplets, shrinking the density difference between the oil and aqueous phases and rheological modification of interface, continuous phase or the dispersed phase (Perrechil and Cunha, 2010).

Several food biopolymers such as gums, proteins, starches and phospholipids are known to have emulsifying capacity. Emulsifiers are substances that align themselves on the oil/water interface to reduce the surface tension, thus ensuring stability of emulsion. The potency of a hydrocolloid is dictated by several factors including: (1) molecular properties like concentration, branching, molar mass, charge, conformation, hydrophobicity and interactions (2) bulk physicochemical characteristics like gelling, thickening and light scattering (Chung et al., 2013).

Cationic emulsion droplets can be formed by sodium caseinate (NaCas) at pH below its isoelectric point. Sodium caseinate is a dairy ingredient prepared from skim milk using numerous unit operations including acid precipitation and resuspension of caseins, neutralization using sodium hydroxide and drying. The commercial product has 86% protein and 5% ash (Zhang and Zhong, 2013). NaCas is a soluble mixture (α_{s1}, α_{s2}, β and κ) of disordered lipophilic protein with a high tendency to align as casein submicelles that co-exist with free casein under equilibrium (Dickinson et al., 1997; Dickinson et al., 2003). On the other hand, gum Arabic forms anionic droplets throughout the pH employed for food system. Gum Arabic is obtained by natural exudation from *Acacia senegal* trees. It is a potent emulsifier due to its

amphiphilic nature, water solubility and ability to create solutions of low viscosity at high concentration in relation to similar hydrocolloid gum. Gum Arabic comprises of highly branched organization of simple sugars that are structurally bound to a small amount of protein (~ 2% w/w). Though small, the protein fraction confers the hydrocolloid its emulsion stabilising activity (McNamee et al., 1998).

Formation of emulsion with desired property depends upon a variety of interrelated factors including viscosity and composition of continuous and dispersed phases, the choice of surface-active material and emulsifying technique. Emulsification is achieved mostly by using energy intensive mechanical shearing operations such as colloid milling, high-speed mixing, microfluidization, high pressure homogenization and ultrasonication. The choice of emulsifying technique dictates the droplet size and its distribution (Purwanti et al., 2016). Ultrasonication is a convenient method to produce emulsions with small droplet size and lower polydispersity in view of the fact that it consumes less energy and surfactant (Hashtjin and Abbasi, 2015). On the contrary, the technique suffers certain disadvantages such as probable titanium contamination, complication in scaling up and acceleration of chemical degradative reactions (Quintanar-Guerrero et al., 1998).

In this study, two biopolymers of which one is a protein and the other is a polysaccharide were studied for their physical stability. The protein used here was sodium caseinate that formed positively charged droplets and the polysaccharide was gum Arabic that formed negatively charged droplets at pH 3. The response of the two emulsions to stress factors affecting the emulsions system such as variation in pH, temperature, salt concentration and freeze-thaw cycle are discussed.

2. Materials and methods

2.1. Materials

Gum Arabic (GA) powder, analytical grade sodium acetate anhydrous (99%), sodium chloride (99.9%), sodium hydroxide (NaOH) and acetic acid glacial (99.6%) were procured from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Sodium caseinate (NaCas) was purchased from Sigma-Aldrich. Oil was obtained from Kamani Oil Industries Pvt. Ltd., Khopoli, India and used as such without further purification. All the solutions were prepared using Milli-Q water acquired from Millipore water purification system.

2.2. Solution preparation

Buffer solution was obtained by first preparing a 50 mM sodium acetate solution in Milli-Q water and consequently adjusting pH using acetic acid or 1 M NaOH. NaCas stock solution was prepared by dissolving 1.2% (w/v) sodium caseinate in acetate buffer (50mM, pH 3) for not less than 3 hours at room temperature using magnetic stirrer (Perrechil and Cunha, 2010). Gum Arabic stock solution was prepared by mixing 1.2% GA powder in buffer for not less than 3 hours to ensure complete hydration. Lower concentrations of emulsifier solutions were prepared by diluting with required quantity of buffer at the same pH and ionic strength.

2.3. Emulsion preparation

Primary emulsions of NaCas and GA were formed by homogenizing 1% (w/v) vegetable oil in buffer containing emulsifier of concentrations 0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2 %. Homogenization was achieved by first blending the oil and aqueous phases and subsequently subjecting to ultrasonication (QSonica 700) at an amplitude of 50% for a duration of 5 minutes with a duty cycle of 0.5 (Noshad et al., 2016). The tip diameter of the probe was 12 mm. The pH of the emulsion formed was not adjusted after sonication.

2.4. Particle size measurement

The particle size of the emulsion samples is given by Z-Average size. It is the intensity weighted mean hydrodynamic size of a conglomeration of oil particles dispersed in aqueous phase. The emulsions were analyzed for size by Dynamic Light Scattering (DLS) using Zetasizer Nano, Malvern Instruments. The device uses the Stokes–Einstein Equation to correlate the size to Brownian movement of particles. Brownian movement of particles are measured and size is determined under the assumption that the larger particles have slower movement (Leong et al., 2009). The emulsions were diluted 100 times prior to particle size measurement.

2.5. ζ-potential

The velocity and direction of movement of droplets in a well-defined electric field determines the basis of its charge indicated by ζ-potential (Aoki et al., 2005). Particle electrophoresis instrument, Zetasizer Nano ZS, Malvern Instrument, UK was used to measure ζ-potential of diluted emulsion samples. The prepared emulsions were diluted 100 fold with buffer of the same pH and ionic strength in order to prevent multiple scattering effect (Noshad et al., 2016).

2.6. Emulsion stability measurement

Creaming index was used to study emulsion stability over time. 8 mL of the emulsion samples were stored in uniformly sized centrifuge tubes for a period of one week and the height of the separated serum layer from the bottom and the total emulsion height were recorded regularly. Creaming index was defined as the ratio of serum height to the emulsion height, expressed in percentage. Creaming index could be evaluated from the following formula, where HE represents total height of emulsions, HC represents the thickness of cream separated on top (Nikbakht Nasrabadi et al., 2016).

$$\mathrm{CI}\% = \frac{(\mathrm{HE} - \mathrm{HC})}{\mathrm{HE}} \times 100$$

2.7. Emulsion environmental stresses

The effect of environmental stresses (pH, temperature, NaCl concentration and freeze thaw) on Z-Average size and ζ-potential of 1% vegetable oil-in-water primary emulsions of sodium caseinate and gum Arabic were compared.

2.7.1. pH:

Emulsions using NaCas and gum Arabic emulsifiers of varying pH (3, 5, 7 and 9) were prepared and their Z-Average size and ζ-potential were recorded and compared. The pH variations were brought about by preparing sodium acetate buffer of required pH and emulsifier and oil were added and homogenized subsequently.

2.7.2. Heat treatment:

5mL of prepared emulsion sample was drawn in a glass test tube of dimensions-outer diameter 15 mm and height 150 mm. These were heated in a water bath for temperatures 30, 50, 70 and 90 °C, over a time of 30 minutes. The emulsions, after heating, were brought to 30 °C (Ogawa et al., 2003) and sampled for analysis.

2.7.3. Freeze-thaw cycling:

To assess the freeze thaw stability of NaCas and GA emulsions, 10 g of the sample was taken in capped plastic tubes of internal diameter 10 mm and frozen at -20 ± 2 °C for 22 hours. The frozen sample was thawed for not less than 2 hours at 25 ±2 °C (room temperature), thus completing a freeze-thaw cycle. The cycle was run twice and its effect on emulsion properties were analysed before and after each cycle (Ariyaprakai and Tananuwong, 2015).

2.7.4. Sodium chloride (NaCl) addition:

Food processing operations demand situations when ionic strength of medium elevates. Therefore, it is beneficial to form emulsion system that does not change the droplet size on adding salt (Li et al., 2016). Sodium caseinate and gum Arabic primary emulsions with varying salt concentration (0 to 500mM) were prepared by adding and dissolving powdered sodium chloride into prepared emulsions (Ogawa et al., 2003). The emulsions with added salt were sampled for ζ-potential and Z-Average size analysis.

3. Results and Discussion

3.1. Effect of emulsifier concentration on sodium caseinate and gum Arabic emulsion properties

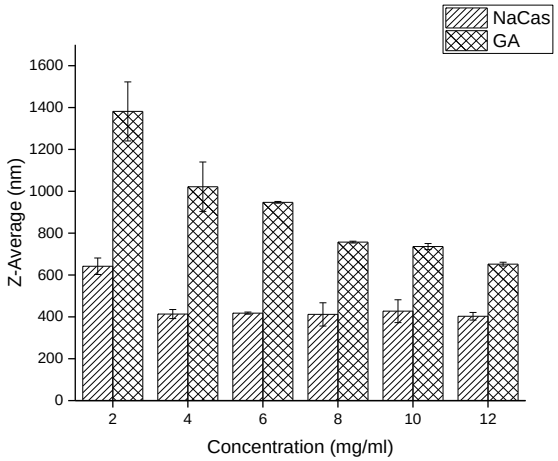


Figure 1: Z-Average size (nm) of sodium caseinate and gum Arabic stabilised oil-in-water emulsions, with the emulsifier concentration varied.

The quantity of emulsifier that is required to form a reasonably stable emulsion with desired properties is to be known from a practical viewpoint (van Aken, 2006). Sodium caseinate stabilised 10 mg/ml oil-in-water emulsions had a consistent Z-Average size around 400 nm for emulsifier concentrations 4, 6, 8, 10 and 12 mg/ml as observed from Figure 1. This observation is consistent with the results of experiments conducted by Sánchez and Patino (2005) at pH 7 and Srinivasan et al. (1999). However, at 2 mg/ml NaCas concentration, the emulsion size hiked up to 641.7 nm (polydispersity index, PDI<0.4) may be due to insufficient emulsifier to fully coat the oil surface that led the droplets to increase the size in order to reduce the surface area that requires protein coverage. Therefore, it can be inferred that a critical concentration of emulsifier is required to coat the oil droplets that gives minimum Z-average size. Further increase in concentration of NaCas emulsifier does not yield smaller droplets under the prevailing pH, ionic strength and emulsion forming condition but may form multilayers on the surface or remain in solution (Sánchez and Patino, 2005).

From Figure 1, it could be observed that as the concentration of gum Arabic is increased from 2 to 12 mg/mL, the droplet size gradually falls, indicating that the higher the concentration of gum Arabic that is available, the smaller the size of droplets. However, this observation does not hold true for indefinite increase in emulsifier concentration. Nakauma et al. (2008) noted that after a certain level, increase in gum Arabic concentration does not reduce the droplet size and present study has obtained similar results. When the comparison between NaCas (protein) and GA (polysaccharide) emulsions are made, we observe that more quantity of carbohydrate is required to cover the oil surface in order to impart stability against creaming and coagulation. Higher amount of gum Arabic is required as less hydrophobic residues are available in the polysaccharide than in protein (NaCas) molecules.

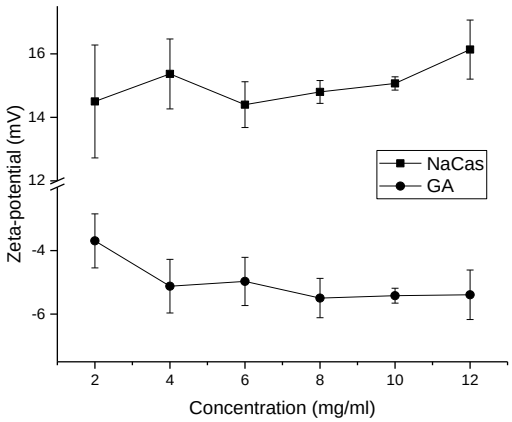
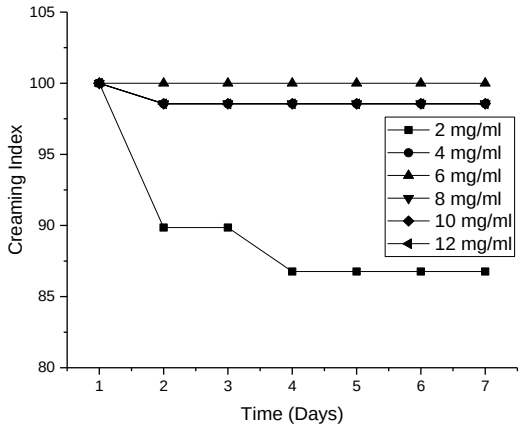


Figure 2: Zeta-potential of sodium caseinate and gum Arabic stabilised oil-in-water emulsions, with the emulsifier concentration varied at pH 3.

The stability of emulsion structure depends on the charge possessed by the oil droplets in the aqueous phase. When the electrostatic repulsion between the droplets are high, the emulsified oil tends to be dispersed in the aqueous phase more effectively over longer period of time. From Figure 2, it can be observed that NaCas at pH 3 forms cationic emulsion droplets while gum Arabic at the same pH produces anionic droplet. Irrespective of the nature of charge, the magnitude of charge plays a significant role in providing stability to emulsions. The greater the magnitude of positive or negative charge on the droplets, the better repulsion between them and subsequently have fair amount of stability. Sodium caseinate emulsions have ζ-potential +14.4 to +16.13 mV for emulsifier concentration of 6 mg/mL and 12 mg/mL respectively. This positive charge could give stability to emulsions by electrostatic stabilisation (Dickinson et al., 2003). In gum Arabic emulsion, charge varies in the order of -5 mV. In spite of having a low negative charge, the stability of gum Arabic emulsions are comparable to sodium caseinate emulsions due to steric stabilisation offered by the polysaccharide on the interface (Nakauma et al., 2008). In both the types of emulsions, no considerable variation in ζ-potential is observed in response to variation in concentration of emulsifier within the evaluated range (2-12 mg/mL).

(3a)



(3b)

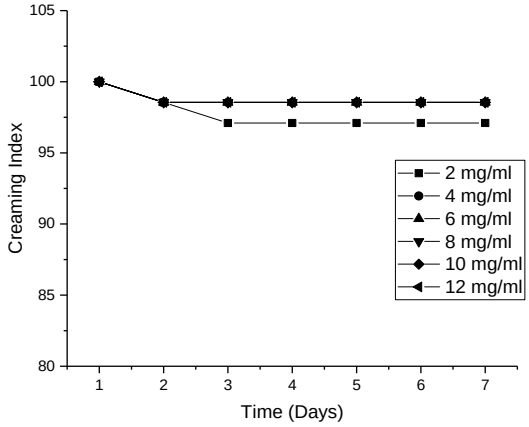


Figure 3: (a): Creaming index of oil-in-water emulsions stabilised by sodium caseinate. (b): Creaming index of oil-in-water emulsions stabilised by gum Arabic as emulsifier.

Sodium caseinate and gum arabic stabilised emulsions exhibited good stability to creaming over the period studied. Only the sodium caseinate emulsions with least concentration of emulsifier (2 mg/mL) destabilised by creaming as obseverd in Figure 3(a). This may be due to insufficient protein concentration. Meanwhile, we can observe that gum arabic coated emulsions at the same concentration are more stable than sodium caseinate emulsions. This observation contracts the theory that more amount of carbohydrate is required to stabilise oil droplets than protein. Nevertheless, no other concentration variation of emulsifier studied had creaming index variation of significance.

Sodium caseinate and gum Arabic stabilised oil-in-water emulsions at an oil concentration of 10 mg/ml and emulsifier concentration of 10 mg/ml were used for studying environmental stress factors like change in pH, NaCl concentration, freeze-thaw cycling and temperature. Therefore, all further references to sodium caseinate stabilised emulsions and gum Arabic emulsions in this article refer to an emulsion prepared with 10 mg/ml oil and 10 mg/ml emulsifier.

3.2. Effect of pH on sodium caseinate and gum Arabic emulsion properties

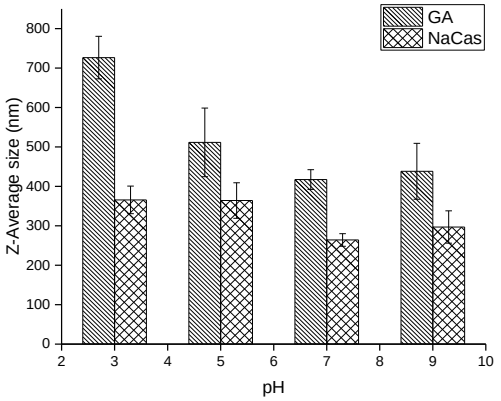


Figure 4: Effect of variation of pH on gum Arabic and sodium caseinate stabilised oil-in-water emulsions on Z-Average size of droplets.

Emulsions prepared using protein and then subjected to pH variations coagulated as the pH values had to go through the isoelectric point (pI) of sodium caseinate (Surh et al., 2006) which is around 4.6 (Belyakova et al., 2003). Therefore, buffers of various pH (3, 5, 7 and 9) were prepared and emulsions were formed separately using sodium caseinate emulsifier and oil. The same protocol was followed to evaluate the effect of variation of pH of gum Arabic stabilised emulsions in an attempt to maintain uniform conditions between the emulsions compared.

The protein and polysaccharide emulsions with 10 mg/ml oil concentration and 10 mg/ml emulsifier show a varied droplet size due to pH change. From Figure 4, it is seen that the protein stabilised oil droplets could form smaller size than the carbohydrate stabilised droplets. Proteins in general are more effective at coating the lipid droplet surface due to their amphiphilic nature. Gum Arabic emulsions exhibit sizes of 0.4 - 0.5 µm at all studied pH except 3. This may be due to insufficient charge to repel the surrounding oil droplets, leading to large sized oil droplets. Sodium caseinate stabilised emulsions exhibited slightly higher droplet size (Z-Average size) at pH 3 and 5 than the sizes recorded at pH 7 and 9. The close proximity of pH 5 and 3 to isoelectric point of the emulsifier is held responsible for probable coalescence and slightly higher particle size (Surh et al., 2006).

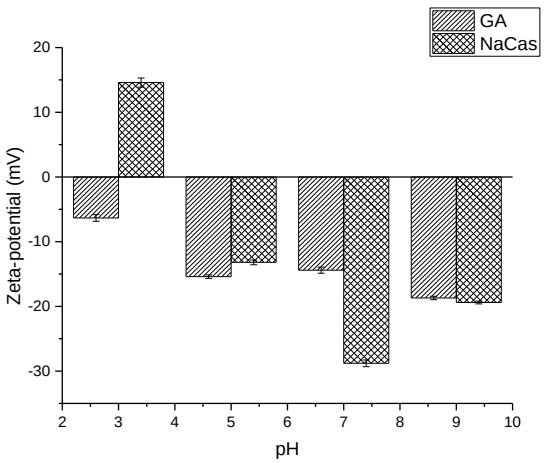


Figure 5: Effect of variation of pH on gum arabic and sodium caseinate stabilised oil-in-water emulsions on zeta-potential of droplets.

The effect of pH on the ζ-potential of NaCas and GA stabilised emulsions vary significantly as one could view NaCas and GA as two different types of biopolymers- one is a protein and the other is a polysaccharide. Polysaccharides exhibit the same sign charge throughout the pH range, though the magnitude of the charge of the emulsions they stabilise varies. From Figure 5, it is evident that gum Arabic stabilised primary emulsions exhibited negative charge throughout the tested pH range (3-9). The overall negative charge of GA emulsions may be attributed to the presence of $–COO^-$ group on the acidic polysaccharide. Also the magnitude of the charge on gum Arabic stabilised emulsion remains fairly constant at pH 5, 7 and 9. Only at a low pH of 3, the ζ-potential takes considerably reduced value of -6.33 mV. The minor fraction of proteins present in gum Arabic, at significantly low pH could exhibit cationic behavior and may have contributed to reduced ζ-potential at pH 3 (Chanamai and McClements, 2002). As widely accepted, gum Arabic is a natural conjugate between protein and polysaccharide, with polysaccharide portion making up the major fraction and protein fraction a meager 1.7% (Matsumura et al., 2003). However, this study has been conducted well above the isoelectric point of gum Arabic which is predicted as 1.8 (Jayme et al., 1999).

Stable emulsions of sodium caseinate were formed at all evaluated pH- 3, 5, 7 and 9. From Figure 5, it could be seen that at pH 3, sodium caseinate emulsions have a positive ζ-potential of +14.6 mV. NaCas emulsions at other pH values of 5, 7 and 9 show negative ζ-potential values. Proteins are zwitterionic biomolecules and their overall charge can be decided by the ions present in the surrounding solution. Therefore, at pH 3 positive charge exists on protein coated emulsions. This charge is neutralized at isoelectric pH (pI ≈ 4.6) and protein in solution precipitate. When emulsion pH is increased from pH 3 to pH 5, precipitation occurs as pH of aqueous phase nears pI. At pH fairly above pI, proteins solubilize again with a reversed charge. Therefore, we observe negative ζ-potential for emulsions at pH 5 and above. Except around pI of protein, the emulsified droplets have sufficient repulsive interaction among them to keep them in dispersed state. This repulsive interaction collapse only around isoelectric point, resulting in droplet aggregation (Perrechil and Cunha, 2010).

3.3. Effect of salt addition on sodium caseinate and gum Arabic emulsion properties

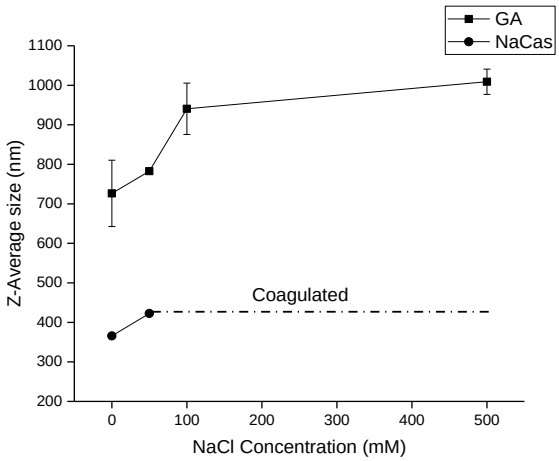


Figure 6: Effect of added sodium chloride on gum Arabic and sodium caseinate stabilised oil-in-water emulsions on Z-Average size of droplets.

The effect of salt concentration on the stability of emulsions is a crucial factor in order to use the emulsions for practical purposes. It is evident from figure 6 that the gum Arabic emulsions could withstand variations in salt concentration and exist in emulsified state when salt up to a concentration of 500mM is added. Nakamura et al. (2004) indicated that gum Arabic emulsions are stabilised predominantly by steric stabilisation and electrical effects imparted by minerals present in the aqueous phase have little effect on overall emulsion structure. This is true due to the fact that emulsions are stable to flocculation. However, we can observe that gum Arabic the droplet size has significantly increased with rise in NaCl concentration through 50, 100 and 500 mM, indicating that electrostatic forces imparted by small protein fraction also play their roles.

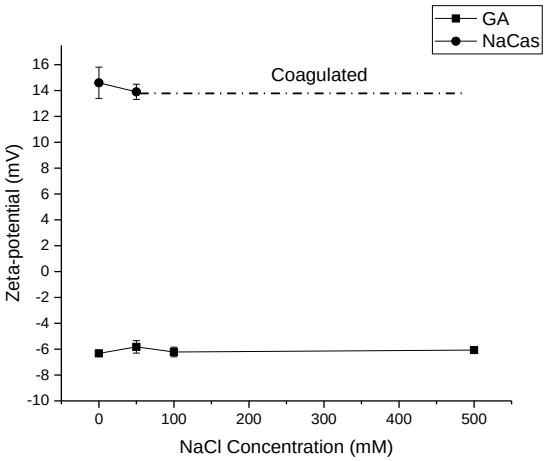


Figure 7: Effect of added sodium chloride on gum Arabic and sodium caseinate stabilised oil-in-water emulsions on zeta-potential of droplets.

The increase in salt concentration of 1 % gum Arabic stabilised oil-in-water emulsions show no considerable change in ζ-potential when the salt concentration is varied (Figure 7). The ζ-potential dropped only from -6.33 to -6.07 on varying the salt amount from 0 mM to 500 mM. Addition of salt would have reduced surface charge due to compression of the electrical double layer (Jayme et al., 1999). However, the reduction in ζ-potential is not prominent due to the highly acidic pH used in the study.

3.4. Effect of freeze-thaw cycling on sodium caseinate and gum Arabic emulsion properties

Table 1: *Z-Average size and zeta-potential of emulsions stabilised by sodium caseinate and gum Arabic subjected to two freeze-thaw cycles.*

Emulsion type	**Sodium caseinate emulsions**		**Gum Arabic emulsions**	
Measured property	**Z-Average size**	**ζ-potential**	**Z-Average size**	**ζ-potential**
Fresh emulsion	427.27 ± 54	15.07 ± 0.21	735.9 ± 14	-5.42 ± 0.23
Cycle 1	961.2 ± 89	13.4 ± 0.57	868.6 ± 59	-6.17 ± 0.26
Cycle 2	Coagulated	Coagulated	1119 ± 67	-6.09 ± 1.3

The emulsions prepared from protein and polysaccharide as emulsifiers in our study showed varied responses to freeze-thaw treatment. Gum Arabic stabilised emulsions showed significant increase in Z-Average size as the number of freeze-thaw cycle increased (Table 1). In addition, the polysaccharide emulsions maintained their physical integrity and did not coagulate on subjecting to two freeze-thaw cycles. On the other hand, sodium caseinate stabilised emulsions had a tremendous size increase in the first cycle and coagulated after the second freeze-thaw cycle. The emulsion destabilisation may have occurred because of the following physicochemical changes during freezing: (1) the formation of ice crystals in the aqueous phase, bringing the oil droplets close together, (2) the formation of ice crystals may have led to a situation where the emulsifier molecules are devoid of water and are not sufficiently hydrated, (3) the concentration of ions in the available aqueous phase after water cystallisation would have been considerably high, thus screening the electrostatic repulsive interactions between droplets, (4) the ice crystal could rupture the oil globule membrane, thus collapsing the structure of emulsion, (5) the fat crystals could also penetrate the oil globule membrane leading to partial coalescence on freezing and complete coalescence on thawing (McClements, 2004; O'Regan and Mulvihill, 2010)

3.5. Effect of heating on sodium caseinate and gum Arabic emulsion properties

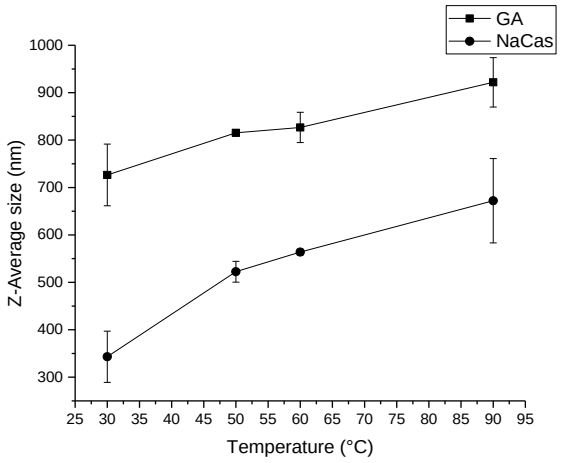


Figure 8: Effect of temperature on gum Arabic and sodium caseinate stabilised oil-in-water emulsions on Z-Average size of droplets.

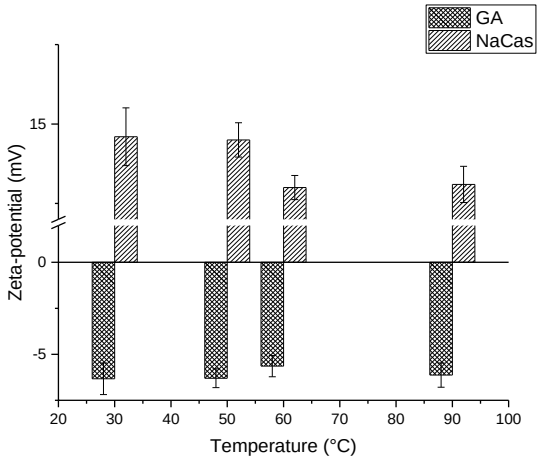


Figure 9: Effect of temperature on gum Arabic and sodium caseinate stabilised oil-in-water emulsions on zeta-potential of droplets.

On subjecting sodium caseinate and gum Arabic emulsions to varying temperatures of 30, 50, 60 and 90 °C, the Z-Average size of the droplets showed a slightly increasing trend (Figure 8). The droplets size of sodium caseinate emulsions at 30 °C was 343.1 ± 54 nm which increased to 672.1 ± 89 nm on heating to 90 °C. Our result shows that heating of sodium caseinate emulsions increases their droplet size. This result is consistent with the studies of Srinivasan et al. (2002) which indicated that larger diameters of NaCas stabilised oil droplets were obtained on heating than unheated control emulsions at a given protein concentration. A review on heat treatment of milk protein stabilised emulsions by Raikos (2010) suggested that non-adsorbed proteins present in the serum interacted with the adsorbed proteins of interface causing fat globule aggregation resulting in increased particle size.

The increase in Z-Average size of gum Arabic stabilised oil-in-water emulsions on heating is less prominent than sodium caseinate stabilised emulsions. On increasing the temperature of gum Arabic emulsions from 30 °C to 90 °C the droplet size varied from 726.5 ± 65 nm to 921.8 ± 52 nm respectively. On comparison with the protein sodium caseinate, the lesser extent of droplet size increase of the polysaccharide gum Arabic on raising temperature could be due to the fact that unlike protein, polysaccharides do not unfold to expose non-polar groups (Charoen et al., 2011). From figure 9, it can be inferred that there was no influence of heat on the ζ-potential of evaluated emulsions of gum Arabic. This was consistent with the results of Charoen et al. (2011). No significant change in ζ-potential was observed in emulsions stabilsed with either polysaccharide or protein on heating to temperatures up to 90 °C.

4. CONCLUSION

In this study, the comparison of the properties of sodium caseinate stabilised emulsions and gum Arabic stabilised emulsions reveal in a concise way that protein stabilised emulsions are more vulnerable to environmental stresses than carbohydrate stabilised emulsions. Gum Arabic has proved to be effective in providing steric protection against the degradative factors of the aqueous phase. Though sodium caseinate formed smaller emulsion droplets with fairly large charge, it was susceptible to coagulation on subjecting to freeze-thaw and addition of sodium chloride salt. Further, the polysaccharide had provided an improved stability to stress factors such as varying pH, salt concentration and freeze-thaw cycling. Though the proteins are labile to heat damage, the heat stability of both gum Arabic and sodium caseinate were comparable. Therefore, both proteins and polysaccharides can be used to prepare emulsions but the choice of emulsifier has to be made depending on the conditions they may be subjected during processing, transit and storage.

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FROM AGROFORESTRY TO COFFEE ECO-CERTIFICATION: AN EXAMINATION OF SUSTAINABILITY OF SMALLHOLDER FARMERS IN SEKAMPUNG WATERSHEDS, SUMATRA-INDONESIA

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Keywords: Ecosystem services, Land and soil degradation, Agroforestry, Coffee eco- certification, Sumatra-Indonesia

1. Background and Research Objectives

Food security, closely linked with environmental problems, has become one of the most important contemporary issues in Asia. Especially in recent decades, ecological degradation such as sedimentation, water quality degradation and frequent flood occurrence, many of which are enhanced by climate change impacts, is expanding in Indonesia and many other Southeast Asian countries. Such degradation of natural resources is affecting negatively food supply and food safety conditions. The basic issue here is whether and how Asian agriculture and fisheries can supply sufficient amount and quality of food to meet with the rapidly growing population, without jeopardizing natural and environmental resources.

While agriculture supplies wide-ranged ecosystem services including marketed goods such as food and fiber, it also provides non-marketed services to the society such as flood regulation, disease control, water purification and scenic view. People generally.

Assume that such non-marketed ecosystem services have no economic significance (Daily, et.al 2002). In recent years, however, as modern agriculture has been causing significant land and soil degradation due to urbanization, deforestation and overexploitation of natural resources, we are learning that the four major categories of ecosystem services have been drastically reduced by human activities, causing higher ecological risks in Indonesia and other Asian countries (see Tab. 1).

Based on an international collaborative research by RIHN (Research Institute for Humanity and Nature, Kyoto), Shiga University and University of Lampung (UNILA), we try to examined the recent changes of such environmental degradation in Indonesia. Based on this empirical research, the present paper has two major objectives: (1) one is to clarify and assess the roles of eco-certification, by examining small-holder coffee farmers in Sumatra; and (2) to estimate farmers' preferences on sustainable coffee certification by use of best-worst scaling methods (BWS) and examine necessary conditions for the potential expansion of such eco-certification scheme.

Our study has demonstrated that non-marketed ecosystem services from sustainable land use in agriculture would provide significant economic and ecological values, and developing mechanism for the payment for ecosystem services is crucial in enhancing sustainable agricultural development. It is also demonstrated that developing.

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Mechanism for the payment for ecosystem services is crucially important for enhancing sustainable agricultural development.

2. Characteristics of Research Site and Methodology

As a typical example of this natural resource degradation, we have conducted an empirical research on smallholder coffee farmers in the Upper Sekampung watershed, in Lampung, Indonesia. For this, we conducted a thorough household survey, by interview- ing 408 coffee farmers in the above-stated watershed in 2016. Many of those farmers have been adopting agroforestry system since the 1980s, especially in the degraded-prone watershed in coffee production centers, by planting shade trees, fruit trees, timber and other multi-purpose tree species. Smallholder farmers are the majority (over 90 percent) in coffee production system in Indonesia, controlling land area of 1-2 hectares, with limited access to technology, market information and financial schemes. At the same time, the coffee economy is also a sector with several competing global eco-certifying entities, some of which have a partnership structure of smallholder farmers, coffee traders, roasters, exporters, and coffee industry in the global value chain system.

Although Sekampung is the main watershed in Lampung Province, serving as major food baskets, such as rice, maize and other crops, it also produces major export

agricultural commodities such as coffee, cocoa, palm oil, coconut, etc. The current land use system, however, has led to serious soil degradation, where 49 percent of land area is degraded, 34 percent potential to degrade and 17 percent non-degraded. The average rate of soil erosion is 67.5 ton per hectare per year, higher than the 25 ton per hectare tolerable soil loss. Non-sustainable land use system would increase ecological risks, posing serious threats to the livelihood deterioration of the poorer segment of rural and suburban people in the Province. Such ecological risks have caused significant impacts eventually on soil and water quality, agricultural production and productivity, food and water supply on which public health heavily depends.

The case study of coffee farmers' decision-making in the two sub-districts of Tanggamus in Upper Sekampung Watersheds provides clearly some lessons of how smallholders could contribute to such an economic-driven transition. Applying the best- worst scaling (BWS) method, originally proposed by Jordan Louviere (2000), alternative land-use system in the study sites was analyzed and compared, and then the farmers' preferences on sustainable coffee certification were estimated. Also examined are the management of land-use system and the effectiveness of eco-certification for the coffee agroforestry system in the catchment area, as it is considered to affect significantly the ecosystem health and resilience of the watershed.

3. Economic and Ecological Impacts of Adopting Coffee-Agroforestry System

We have first examined the economic and ecological impacts of coffee agroforestry system in Upper Sekampung Watershed that serves as conservation strategy in the catchment area (Hanung, et.al. 2016). Simple criteria of agroforestry system are applied in the study, i.e. where farmers grow at least 100 shade trees per hectare in their coffee farms, taking consideration that one tree could provide some functional shade (light, nutrient, etc.) for about for coffee trees. Additional analysis is also focused on the performance of farmers joining coffee eco-certification and not joining the certification.

The findings show that agroforestry systems have no significant negative impacts on economic benefits. Farm income of agroforestry adopters and non-adopters has no statistical difference.

As shown in Tab. 2, coffee eco-certificates have positive significant impacts on improving economic benefits, represented by higher farm income, and environmental benefits, represented by less chemical fertilizer use. Farmers joining RFA certification has higher economic and environmental benefits than those who join 4C certification. Farm gate price of coffee closer to the premium market price has more short-term attention from coffee farmers, although environmental risks are higher.

Coffee eco-certification scheme has emerged from growing concerns over global environmental governance, as an alternative to serve as new vehicles of corporate control over global food production, trade and consumption. Eco-certification generally connects consumers and business in the so-called developed countries with producers and small farmers in developing countries. In the face of this development, the Southern actors from business, civil society and government need to redefine their position and the supply chain of export commodities need to change the structure of commodity value chains.

The coffee eco-certification generally requires establishment of farmers' organizations and locally adopted conducts. However, many of these standards provide no guarantee that direct benefits, particularly price premiums, would reach farm laborers or local communities. The social and economic effects of eco-certification in agricultural commodity chains are still to be analysed and examined more extensively.

Some important findings from our study include that farmers have adopted coffee agroforestry system since the 1980s using shaded fruit trees and multi-strata coffee system to secure their own household income and to contribute to conservation practices in the catchments area of the watershed. Average coffee production in Upper Sekampung is 394 kg/ha, which is far away below the national average of 645 kg/ha, mostly due to traditional farming practices and simple processing techniques. Coffee crops remain profitable in the watershed, but ample opportunities could be explored to improve the coffee yield and quality to fulfill increasing demand for coffee. Agroforestry system has provided additional income sources, mostly from tree species, shown by significant higher B/C ratio than the coffee farm only.

Moreover, since early 2000s, some farmers practicing agroforestry system have adopted global coffee eco-certification, such as Rainforest Alliance, 4C, Utz certified, and Organic Certification. These coffee eco-certification programs have attempted to create price premiums at the farm level, where Rainforest Alliance and 4C certificates have been around in the study sites for about 5 and 2 years respectively. The roles of intermediaries in ensuring the sustainability principles are very important in providing links between sellers (smallholder farmers) and buyers (roasting companies, research institute, civil society organizations or international agencies) of the services. Farm gate price of coffee closer to the premium market price has more short-term attention from coffee farmers in the study sites, although environmental risks are higher.

In short, the new global initiatives of coffee-eco certification have, unfortunately, a small impact on the farm-gate coffee price in Indonesia, mostly because the price transmission elasticity of global coffee price is also very small. However, coffee-Eco certification has impact potentials on strengthening social capital and improving the community-cooperative governance in the producing regions as the partnerships generally require establishment of farmers' organizations and locally adopted conducts.

4. Decision Making and Preferences of Coffee Farmers on Eco-Certification

Coffee certification is considered to be a viable economic scheme for making agriculture more sustainable and eco-friendly. It is a financing mechanism in the private sector, bridging the producer of coffee (as supplier) and the consumer of it (as demander), not financed by the public body. For this reason, there exist various requirements in eco-certification system, such as reduction of agrochemicals, adoption of multi-story agroforestry systems, and conservation of biodiversity and indigenous local species.

The present study is considered as an appropriate and typical case for such certification with respect to agroforestry system.

As a next step, we have analyzed to estimate coffee farmers' preferences on sustainable certification (K. Tanaka, 2016). As already stated, the best-worst scaling (BWS) method is adopted, which is a choice-based conjoint analysis for valuing environmental amenities. By this method, we will be able to find the motivations of farmers as to the willingness to participate in the scheme, and clarify the necessary conditions for the certification system to be expanded more in the region

The questionnaire-based farm survey was conducted to 408 local coffee farmers in the Upper Sekampung Watershed, in July 2016. In the questionnaire, we asked in-depth questions of current production practices, input-output patterns, preference on agroforestry and participation into various eco-certification, etc.

Out of 408 samples, about 10% of farmers (n = 41) have adopted eco-certification, of which 4C is the majority (63%), Organic (17%) as second and Rainforest Alliance (7%). The following eight (8) factors have been selected carefully for BWS questions, after literature search and intensive interviews with farmers. They are: ① price level in international market; ② environmental benefits; ③ price premium (price difference with non-certified coffee); ④ social cohesiveness with group members; ⑤ technical assistance to improve yield and quality; ⑥ price difference by size of production; ⑦ expanding market access; and ⑧ improving access to credits and other inputs. In the questionnaire interview, each respondent was asked to choose "best" and "worst" among 4 factors which wewe randomly selected from the above 8 factors. This process was repeated for 4 times for each responded.

With the use of mixed (random parameter) logit model, we have been able to estimate the following results with respect to each factor above listed (see Tab. 3). As shown in Table 3, 7 out of 8 factors are found to be statistically significant. In particular,

⑤Technical assistance to improve coffee yield and quality (coefficient as 2.098) and ③ price premium (1.054) are considered relatively more important than others. On the other hand, farmers do not consider those factors of ⑧, ②, ④, and ⑦ as important. It should be noted that all the values of SD (standard deviation) are found to be statistically significant, which may indicate that farmers' preferences on coffee certification are relatively heterogeneous.

As one important policy implications of this analysis is that market price and price premium are certainly important for farmers' decisions, but technical assistance (to enable agroforestry management more sustainable) appears to be more important for the coffee farmers. To provide better access to technical assistance would be a key to the success of wider expansion of coffee eco-certification schemes in the region.

5. Summary and Policy Implications

In summary, the present study calls for more empowerment programs for coffee farmers to improve coffee yield and coffee quality, by encouraging better farming practices at the farm level. Structural problems facing the smallholder coffee farmers need to be solved by providing technical assistance, extension services and empowerment actions at the field level. Here, some concluding remarks should be addressed with respect to interdisciplinary approach in managing environmental risks, as shown in the case of Sekampung Watershed of Sumatra, Indonesia. The issues of environmental quality need to be addressed properly as it would have consequences on endangering food security and health security. Environmental risks could constitute huge social cost in the long run if not managed and solved properly.

In economic theory, the so-called polluters pay principle (PPP) should be adopted in environmental incidences, although in practice no one would dare to pay, because there is no market existing to take care of environmental quality and social cost.

Strategies to tackle environmental deterioration include developing alternative or eco- friendly technology, institutional arrangement or social-economic setting, and trans- disciplinary approach with economic incentives, community-based and participatory decision making. Schemes of payment for environmental services (PES) and eco- certification schemes where coffee farmers can voluntarily choose to comply with eco- friendly practices. However, since most certifications are originally from North America and European countries, more research has to be conducted to measure the social- economic benefits to local farmers, especially smallholders.

An important key for policy advocacy in the future is by building up partnership the above-mentioned tasks with locally organized leaders and major stakeholders.

Involved in land use changes affecting ecological risks at the landscape and regional level. It should include (1) awareness on ecological degradation, complex, diverse and specific local conditions, (2) observation based on scientific data collection and reporting system partly collected by local people, (3) identification of potential risks, analysis and evaluation, and (4) action by governments, legislative actions, and science for community.

Finally, whether to expand the implementation of PES mechanisms in Southeast Asian countries, it should be noted that PES are mostly local specific and not necessarily applicable to the whole nation, since there is no legal foundation to execute such mechanisms. Some initiatives on characteristics between farming practices in private land and state-owned land might differ in terms practicing agroforestry system and adopting eco-certification. More detailed and rigorous analysis is required to reveal strong and solid conclusion based on field data and observation.

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1 Table 1. Degradation of Ecosystem Services and Ecological Risks in Asia

Reduction in Ecosystem Services (ES)	**Ecological risks due to a reduced level of ES**	**Responsible human activities**	**Countries in Asia where ecological risks are highly likely**
Supporting • Nutrient cycling • Soil Formation • Primary Production	Poor soil quality Destruction of genetical resources such as fish, frog, and earth worms in the paddy field	Use of chemical fertilizers Use of chemical insecticides Introduction of aquaculture	Bangladesh, India, Indonesia, Nepal
Provisioning • Food • Fresh Water • Wood and Fiber • Fuel	Food insecurity Water pollution Soil erosion	Conversion of agricultural land for human settlements Deforestation	Bangladesh, India, Nepal, Indonesia, Philippines, Sri Lanka
Regulating • Climate Regulation • Flood Regulation • Disease Regulation • Water Purification	GHGs emission Water purification Flood Public health Water Pollution	Extension of oil palm and sugar cane plantation	Indonesia, Malaysia, the Philippines
Cultural • Aesthetic • Spiritual • Educational • Recreational	Aesthetic Recreational Communication among people	Use of chemical fertilizers Use of chemical insecticides	Bangladesh, India

Table 2. Economic and Ecological Impacts of Eco-certification by Conversion from Coffee Plantation to Agroforestry

(1) Economic Impacts (Change of Income per ha. of land)

Comparison	Mean difference		t-value
Control - RA	-6,318,305	***	-6.747
Control - 4C	1,514,419		1.904
RA - 4C	7,832,723	***	8.715

(2) Ecological Impacts(Nitrogen Input per ha. of land)

Comparison	Mean difference		t-value
Control - RA	0.645	***	4.667
Control - 4C	-0.348		-1.661
RA - 4C	-0.992	***	-6.295

1.1 ***Source: Hanung et. al. (2016)***

Table 3: Estimated Results of Farmers' Preferences on Eco-certification

Variables	Coefficient		Std. Error
Price level in international market	0.138		0.164
Environmental benefits	-0.780	***	0.132
Price premium	1.054	***	0.117
Social cohesiveness with group members	-1.892	***	0.175
Technical assistance to improve coffee yield and quality	2.098	***	0.131
Expanding market access	-2.056	***	0.177
Improving access on credits and other inputs	-0.376	***	0.129
SD(Price level in international market)	2.365	***	0.182
SD(Environmental benefits)	1.457	***	0.158
SD(Price premium)	1.108	***	0.150
SD(Social cohesiveness with group members)	2.504	***	0.183
SD(Technical assistance to improve coffee yield and quality)	0.925	***	0.184
SD(Expanding market access)	2.203	***	0.162
SD(Improving access on credits and other inputs)	1.488	***	0.158
n	11067.000		
Log likelihood	-2983.730		

Source Tanaka K (2016)

This is the proceedings of 3rd AFSA conferences which was held on September 15-17, 2016 at KIIT University, Bhubaneswar, India. The 2nd AFSA conference was held on August 15 – 18, 2014 at Dong Nai University of Technology, Bien Hoa City, Vietnam. The first AFSSA Conference was held in Osaka on September, 2012 hosted by Osaka Prefecture University, JAPAN. However, the process of holding annual conferences started since 2005 and was limited between Japan and Bangladesh. The first annual general meeting of the Bangladesh-Japan Association for Science and Technology (BJAST) was organized by former BJAST chairman Professor Majibur Rahman and Professor Takashi Uemura, and was held at Dhaka in December 27-28, 2005. The BJAST was established with the hope to give a platform of Bangladesh students/researchers studding/working in related fields in Japan. The second BJAST international conference was held at the Kinki University in Nara, Japan in 2007. The third one at the University of Dhaka in 2008, the forth at Nara Advanced Institutes of Science & Technology in 2009 and the fifth annual meeting was held in Dhaka in 2010 and more than 11 Asian countries participants were presented their research work. The past presentations are summarized in two proceedings books published from The Bangladesh Academy Sciences entitled; Food Safety and Hygiene (ISBN: 984-300-001933-4) and Food, Health and Environments (ISBN: 397-984-334982-9). With the increasing participants from other countries in BJAST conferences, the 5th BJAST annual meeting decided to the formation of Asian Food Safety & Security Association (AFSSA). The AFSSA is a non-profitable voluntary organization aiming to provide a solution of food related challenges not for people in Asia but also for ever seven billion people of the world and its main office is located at the CARS (Centre for Advanced Research in Science), University of Dhaka, Bangladesh.