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Effect of *Bacillus subtilis* FPTB23 on Behaviour of *Enterococcus faecalis* in Vacuum-packed Asian Sea-BassFillets at 5±1^{O}C

S. Nath[1], S. Chowdhury[2] and K.C. Dora[3]

[1,2]Department of Fish Processing Technology Department of Fish Processing Technology
Faculty of Fishery Sciences, WBUAFS, Kolkata, India
[3]Department of Fish Processing Technology, Faculty of Fishery Sciences, WBUAFS, Kolkata, India
E-mail: [1]swarnadyutinath@gmail.com, [2]supratimchowdhury@yahoo.co.in, [3]kc_dora@yahoo.co.in

Abstract—*Biopreservation is a process where non-pathogenic microorganisms and/or their metabolites are used to improve microbiological safety and extend the shelf life through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of raw materials and food products. Bacillus sp. have a broad antimicrobial spectrum against various Gram positive and Gram negative spoilage and pathogenic bacteria as well as fungi. USFDA certified the carbohydrase (amylase) and protease enzymes produced by B. subtilis, as GRAS in 1960. In the present study, an isolate from curd designated as FPTB23 showed wide spectrum of antimicrobial activity against S. aureus (ATCC 25923), Enterococcus faecalis (MTCC 2729), Vibrio cholera and Vibrio parahaemolyticus, with the strongest inhibition against Enterococcus faecalis. The isolate FPTB23 was found to be Gram positive and catalase negative. 16S rRNA gene sequencing followed by BLAST analysis for homology searches showed that FPTB23 was B. subtilis. (Accession no. KF556680). The E. faecalis population in inoculated fillet was inhibited by 1.2 log cycle in the presence of B. subtilisunder normal packaging conditions (T_1) at the end of 15 days as compared to C_1. Application of vacuum, however, had a synergistic effect in combination with Bacillus strain resulting in reduction of E. faecaliscount ($p<0.05$). Packing of inoculated fillets (C_2) in vacuum resulted in nearly1.3 log cycle reduction in E. faecalis count on day 15. Similar trend was observed in case of T_2 samples, with 0.5 log cycle reduction in final count of E. faecalis when compared to T_1. The reduction in final count of E. faecalisin treated samples after 15 days at 5±1^{O}C suggested a bacteriostatic rather than bacteriocidal impact of B. subtilis FPTB23 in combination with vacuum.*

1. INTRODUCTION

Conventionally, drying, salting, fermentation, canning, freezing and food additives are commonly used to preserve food and enhance food quality. Present day food preservation is viewed as a 'convenience' product with the consumer preference for minimally processed foods. This has led to the increasing consumption of precooked food, prone to temperature abuse, thus increasing the likelihood of food related illness and product spoilage [1]. The food related illness is frequently caused by the contamination of pathogens such as *Salmonella, Campylobacter jejuni, Escherichia coli* 0157:H7, *Listeria monocytogenes, Staphylococcus aureus*, *Enterococcus faecalis*and *Clostridium botulinum*[2]. Therefore, there is a need to search for an attractive and alternative approach to food preservation without the use of chemical preservativesor harsh physicochemical treatments. Such an alternative approach may be achieved by using biological antimicrobial compounds, either alone or in combination with mild physicochemical treatments and low concentrations of traditional and natural chemical preservatives. This approach of food preservation may be an efficient way of extending shelf life and food safety through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of raw materials and food products. Biopreservation is a process where non-pathogenic microorganisms and or their metabolites are used to improve microbiological safety and extend the shelf life. [3].

Bacillus and Lactic Acid Bacteria are bacterial genera well known to produce a diverse array of antimicrobial compounds and more than 20 different types of antimicrobial substances including bacteriocin which makes these bacteria potential agent for biopreservation. *Bacillus* sp. have a broad antimicrobial spectrum against various Gram positive and Gram negative spoilage and pathogenic bacteria as well as fungi. USFDA (United States Food and Drug Administration) certified the carbohydrase (amylase) and protease enzymes produced by *B. subtilis*, as GRAS in 1960. The resistant properties of *Bacillus* spores raise the possibilities that they can be incorporated in a number of food products such as beverage, chocolate, baked cake and muffin as probiotic additives [4]. Several bacteriocinogenic species of *B. subtilis* have been reported to be capable of producing subtilin and subtilosin. *B. coagulans*is reported to produce coagulin, *B. thuringiensis* strains produce Bacthuricin F4, thuricin S and thuricin 7 [5]. Subtilin and subtilosin are active against many strains of gram positive bacteria and these are the only

bacteriocins of *Bacillus sp.* to be characterized at the amino acid and DNA sequence level, thus making *Bacillus sp.* a potential strain for using as biopreservative[6].

Although Lactic acid bacteria (LAB) are known to be inhibitory towards various pathogenic bacteria and spoilage microorganisms during growth and refrigerated storage in food products [7], enough literature on the application of *Bacillus sp.* as a biopreservative agent determining the extent of inhibition and helping to increase the shelf life of the product is not available. Thus, the objective of the present study is to isolate *Bacillus* sp., screen them for antimicrobial activity and assess the antagonistic activity of the isolated *Bacillus* strain/s by applying the culture directly to food system (Asian Sea Bass fillet) pre-inoculated with *Enterococcus faecalis* (MTCC 2729), the latter being used as a strain to indicate the inhibitory activity (indicator strain) of*Bacillus* strain during refrigerated storage.

2. METHODOLOGY

2.1 Isolation and screening of bacteriocin-producing strain

10 grams each of seven local food samples such as: traditional curd, cheese, two samples of whey, milk and fishes (gut portion of mrigala and tilapia) was added to 90 ml of sterile peptone (0.1%) water and then thoroughly homogenized for 1 min in a Laboratory Blender Stomacher and serially diluted with 0.1% peptone water. From appropriate 10-fold dilutions, 100 µl was spread on modified MRS agar (containing 0.2% w/v glucose and supplemented with 50 µl/ml of cyclohexamide) plates and incubated anaerobically at 37^{o}C for 24-48 hours. After anaerobic incubation, the plates that provided separated bacterial colonies (with about 300 colonies) were overlaid with 10 ml of Nutrient soft (0.7% agar) agar containing approximately 10^6 cells of indicator organism (*S. aureus* ATCC 25923) and were anaerobically incubated at the optimal growth temperature of the indicator organism. The plates were then checked for inhibition zones around the colonies [8]. Colonies exhibiting inhibition zones were selected for further study. The spectrum of antimicrobial activity was tested by method described by Hernandez et al. [9].

Screening of isolate for bacteriocin production was done through well diffusion assay (WDA), as described by Barefoot and Klaenhammer[10] against a maximum number of bacteria used as indicator organism (Table 1) by preparing neutralized cell-free supernatant (NCFS) as described by Nath et al. [8].

Table 1: List of indicator organisms used with culture media and incubation temperature.

Indicator organisms	Specific medium	Incubation temperature °C
Staphylococcus aureus (ATCC 25923)	**Baird Parker**	37
Enterococcus faecalis (MTCC 2729)	**CAE (Citrate azide Enterococcus) Agar**	37
Vibrio cholerae	**TCBS agar**	37
Vibrio parahaemolyticus	**TCBS agar**	37
Escherichia coli(MTCC 1563)	**Tergitol 7 agar**	37
Bacillus subtilis (FPTB 13)	**Luria-Bertani medium**	37
Bacillus amyloliquificans (FPTB 16)	**Luria-Bertani medium**	37
Lactobacillus brevis (MTCC 1750)	**Modified de Man Rogosa Sharpe (MRSm) medium**	32

2.2 Identification of isolate

The identification of isolate was done based on results of Gram staining, catalase activity, biochemical tests using API 50 CHL test strip (BioMerieux, France) and 16S rRNA gene technique followed by NCBI BLAST Analysis.

2.3 Protein concentration measurement

The protein concentration was measured by Biuret Method [11].

2.4 Test of isolate for haemolytic activity

Haemolytic activity of the isolate was tested by spot inoculating the cultures on sheep blood agar plates. Plates were incubated at 37^{O}C for 24 hours and results were determined, as described by Luis-Villasenor et al. [12], as: α-hemolysis (slight destruction of hemocytes and erythrocytes with a green zone around the bacterial colonies); β-hemolysis (hemolysin that causes a clean hemolysis zone around the bacterial colonies); and γ-hemolysis (without any change in the agar around the bacterial colonies. Observation of the clear zone surrounding the spot inoculation reflects positive haemolytic activity.

2.5 Elimination of haemolytic activity

The fillets of Asian Sea Bass (10g) were surface inoculated with an overnight culture of the haemolytic positive strain at a level approximately 1X10^{7}cfu/ml. The concentration of the bacterial cells was determined using McFarland Standard. For all treatments, surface inoculation of the products was achieved by aseptically dipping the fillet samples into the previously prepared diluted mixture of the culture for 7 minutes. Excess fluid was drained into a sterile container and the samples were allowed to dry in air for 15 minutes under a laminar flow hood to allow bacterial cells to attach [7]. Each set of finely ground meat of inoculated fillets (10g) was dissolved in 90 ml of sterile distilled water and then exposed to temperatures of 90^{O}C (15 minutes), 100^{O}C (10 minutes), 120^{O}C (10 minutes), 140^{O}C (10 minutes), 160^{O}C (10 minutes) and 180^{O}C (10 minutes). Dilutions without any exposure to high temperature were kept as control. The determination of haemolytic activity on sheep blood agar plates was done by spot inoculation from appropriate dilution. Plates were

incubated at 37^{O}C for 24 hours. Presence of clear zone surrounding the inoculums indicates haemolytic positive.

2.6 Inhibition of *Enterococcus faecalis* (MTCC 2729) in Asian Sea Bass (L*atescalcarifer*) fillets by culture of isolated strain

Fresh Asian Sea Bass (*Latescalcarifer*) with an average weight of 3-4kg was purchased from the fish market in South Kolkata and were transferred to the laboratory in iced condition, decapitated and filleted by hand. Cubes were cut from the fillets such that the final weight of each piece was approximately 10g.

The study was conducted as a completely randomized design, with four treatments viz. only *E. faecalis*treatment, under normal packaging condition (C_1), Only *E. faecalis*treatment, under vacuum packaging condition (C_2), *E. faecalis* + FPTB23 treatment, under normal packaging condition (T_1) and *E. faecalis* + FPTB23 treatment, under vacuum packaging condition (T_2) stored under 5±1^{O}C. The fillets were subjected in triplicate to microbial analyses during the storage periods. The samples were analyzed at three days' interval starting from day 0 for *E. faecalis*(MTCC 2729) count by spread plating appropriate dilutions on CAE (Citrate azide Enterococcus) Agar Plate (Hi-media) [13]and results expressed as log cfu/g.

An overnight culture of *E. faecalis*(MTCC 2729) was diluted approximately in sterile peptone buffer to obtain a viable cell population of approximately 10^6 CFU/ml. The cell population was determined by using Mcfarland Standard. For all treatments, surface inoculation of the products with *E. faecalis*was achieved according to the method of Amezquita and Brashears [7].

For the cubes of treatment T_1 and T_2, 1ml of the fresh concentrated culture of FPTB23 containing a population of $1X10^7$ CFU/ml was added and distributed with a dropper onto the surface of the fillets, as described by Amezquita and Brashears [7] and stored at 5±1^{O}C. All steps involved in the preparation of this culture were done aseptically maintaining good hygienic practices.After inoculation, the fillets of treatment C_2 and $T_2$of each set were vacuum packed by using INDVAC packaging machine. Packaging material was a film bag of 15X20 cm vacuum package bags having low gas permeability at 23^{O}C (oxygen: 10 cm^3/m^2 day bar, nitrogen: 6 cm^3/m^2 day bar, carbon dioxide: 35 cm^3/m^2 day bar and water vapour: <2g/m^2 day).

2.7 Statistical Analysis

All of the data were checked for normal distributions with normality plots prior to two-way analysis of variance (ANOVA), to determine significant differences among means at $\alpha = 0.05$ level, using statistical tools of R software. The Tukey HSD was used to determine significant differences between treatments and storage time.

3. RESULTS AND DISCUSSION

A total of 51 colonies displayed antibacterial activity against the indicator lawn of *Staphylococcus aureus* (ATCC 25923) by soft agar overlaying technique. Out of these, one isolate from curd designated as FPTB23 showed wide spectrum of antimicrobial activity against *S. aureus* (ATCC 25923), *Enterococcus faecalis* (MTCC 2729), *Vibrio cholera* and *Vibrio parahaemolyticus*. The strong antagonism against a number of serious and challenging foodborne pathogens/spoilage-causing microorganisms advocated the high possibility of using thisbacteria as an effective preservative in food. Sharma and Gautam [14] newly isolated bacteriocin from *B. mycoides* that can suppress the growth of *Listeria monocytogenes* and *S. aureus*. Similarly, paenibacillin (bacteriocin from *Paenibacillus sp*.), was found to be active against many bacteria including *Bacillussp*., *Clostridiumsporogenes*, *Lactobacillussp*., *Listeriasp*. and *S. aureus*[15]. Other bacteriocins are also known to exhibit antimicrobial activity which include entomocin 9, a bacteriocin produced by *B. thuringiensis* ssp. *entomocidus* HD9 [16] and lactosporin, an antimicrobial protein produced by *B. coagulans* ATCC 7050 [17]. A bacteriocin produced by *B. licheniformis* MKU3 inhibited *Escherichia coli*, together with many Gram-positive species [18]. In 2012, Kindoli et al. [5] reported that out of total 660 isolates from Cheonggukjang collected from different regions of Korea, 10 isolates exhibited strong antimicrobial activities against various indicator organisms. Among the isolates from strains of *Bacillus subtilis* was reported to be most inhibiting. In the present study, the isolate FPTB23 inhibited both the Gram positive and Gram negative pathogens inferring that the isolate may possibly be used as protective culture for extending the shelf life of food and ensure food safety through inhibition of pathogenic bacteria.

The reason for antimicrobial activity may be attributed to competitive inhibition and production of compounds such as bacteriocin [3]. To confirm that, the inhibition is due to bacteriocin, Neutralised Cell Free supernatant (NCFS) was prepared and WDA performed. Although the NCFS exhibitedstrong inhibitory effect against *E. faecalis* (MTCC 2729), the protein concentration in NCFS of FPTB23 was recorded 1.64 mg/dl, which makes the extraction of that target protein low and the process will not be economic for the mass production.

The isolate FPTB23 was found to be Gram positive and catalase negative. Carbohydrate fermentation test of the isolate using API 50 CHL test strip (BioMerieux, France) showed poor matching with *Lactococcuslactisssp*. *Lactis* (48.2%) or *Lactobacillus acidophilus* (36.0%). To confirm the identification of the isolate, 16S rRNA gene sequencing (Fig. 1) was done followed by BLAST analysis for homology searches (Fig. 2). The results showed that FPTB23 was *B. subtilis*. (Accession no. KF556680).

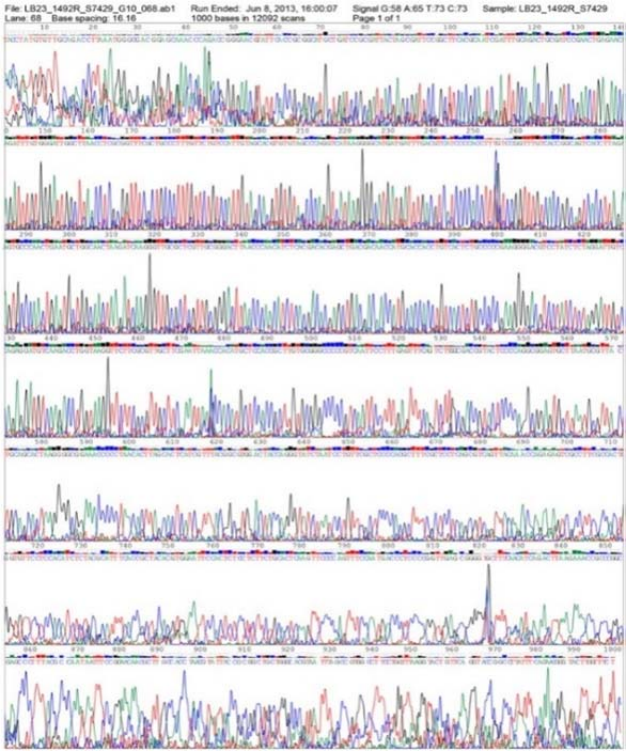

Fig. 1: 16S rRNA gene sequencing of FPTB23 *B. subtilis*

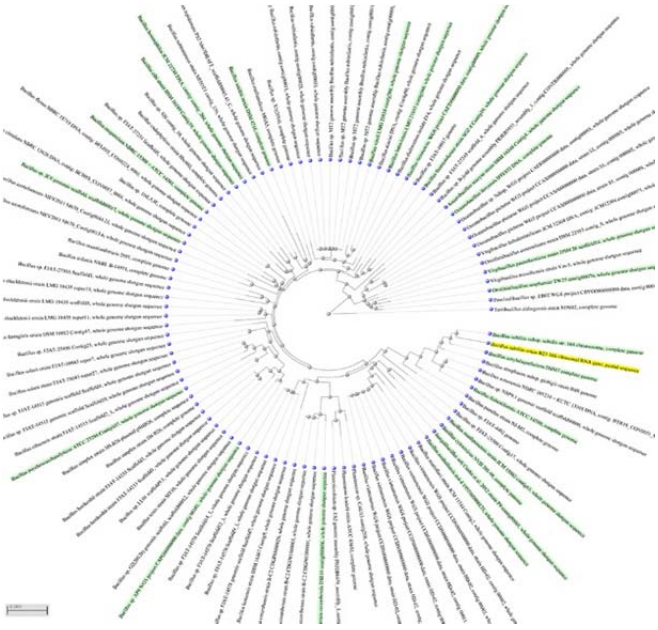

Fig. 2: Phylogenetic tree of FPTB23 *B. subtilis* after NCBI BLAST analysis

As the isolated strain,*B. subtilis* FPTB23 showed α-hemolysis characterized by slight destruction of hemocytes and erythrocytes with a green zone around the bacterial colonies, it was exposed to temperatures of 100^{O}C and above for 10 minutes to eliminate hemolytic activity. The exposure to high temperatures resulted in complete loss of haemolytic activity of *B. subtilis* FPTB23 strain. The result is supported by the findings of Mukry et al. [19], who reported that, the loss of haemolytic activity of S140(c) strain of *B. cereus* group on exposure to cholesterol, papain, acetone, pH 3 and high temperature.

In the present study, the inhibition of *Enterococcus faecalis* (MTCC 2729), by *B. subtilis* FPTB23 was studied on Asian Sea Bass fillet stored at 5±1^{O}C temperature. *Enterococcus faecalis* (MTCC 2729) was chosen as an indicator strain because *B. subtilis* FPTB23 showedstrongest inhibition against *Enterococcus faecalis*,as compared to other indicator strain. The presence of enterococci in food products has long been considered as an indication of poor sanitary conditions during production and processing. Presence of enterococci in food products indicates faecal contamination. Enterococci are important causes of nosocomial infections and the emergence of vancomycin-resistant enterococci (VRE) is a particularly serious problem. Molecular studies indicate that the horizontal transfer of resistance genes plays an important role in the persistence of VRE in animal environments and that the van-A gene can be transferred from animal to human isolates. Although there is little evidence of human infections being directly linked to the consumption of VRE-contaminated foods, the reservoir of VRE in food-producing animals presents a risk of vancomycin-resistance being transferred to virulent human strains through food and other routes [20]. Thus, the control of enterococci in foods has assumed a new level of importance in food processing and food microbiology [21].

The temperature of 5±1^{O}C was chosen because; it is the minimum temperature where *Enterococci* can grow [22]. The Asian sea bass fillet was chosen to study the inhibitory effect of *Bacillus* strains on *E. faecalis*(MTCC 2729). During production and processing of fillet inside a processing establishment chances of faecal contamination exists. Therefore, application of suitable biopreservative agent is expected to inhibit the growth of faecal contaminants.

In the control (only *E. faecalis*, under aerobic condition) sample C_1 the population of *E. faecalis*ranged between 6.26±0.08 log cfu/g to 8.90±0.08 log cfu/g over a period of 15 days at 5±1^{O}C (Table 2). The density plot of *E. faecalis*

population in various samples (

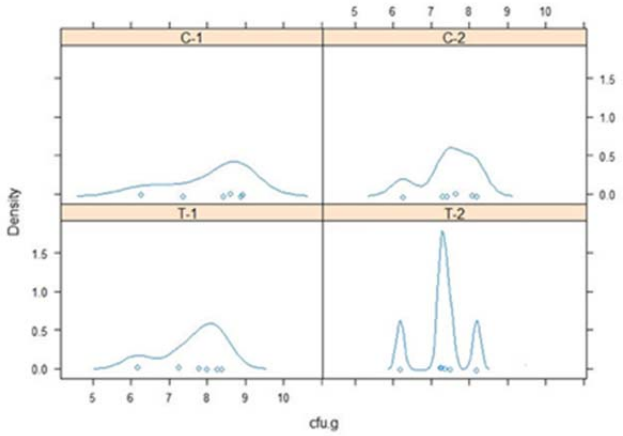

Fig. . 3) also reveals that the log cfu/g value of C_1 samples is dispersed widely as compared to C_2 and T_2. In C_2 and T_2 samples the counts are densely concentrated suggesting a significant ($p<0.05$) inhibition of *E. faecalis*as compared to C_1 and supported by the plot of Tukey HSD (**Error! Reference source not found.**4). In case of T_1, treated with *B. subtilis*, the final count of *E. faecalis*was recorded to be 7.87±0.12 log cfu/g at the end of 15 days which is 1.2 log cycle lower than C_1. Packing of inoculated fillets (C_2) in vacuum resulted in nearly1.3 log cycle reduction in *E. faecalis*count on 15th day. Similar trend was observed in case of T_2samples, with 0.5 log cycle reduction in final count of *E. faecalis* when compared to T_1(Table 2).

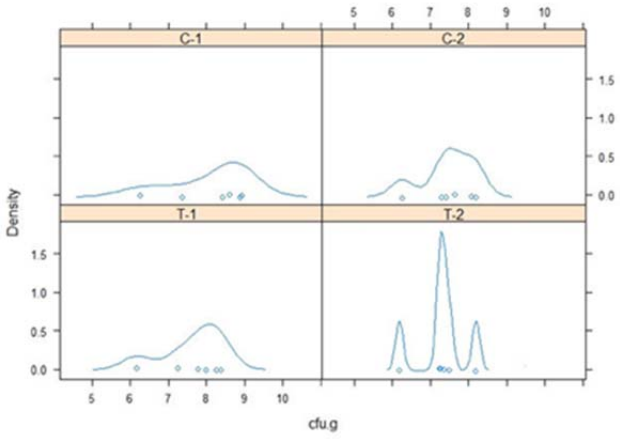

Fig. 3: Density plot of *E. faecalis* (MTCC 2729) count (log cfu/g) on fillet at 5±1^{O}C

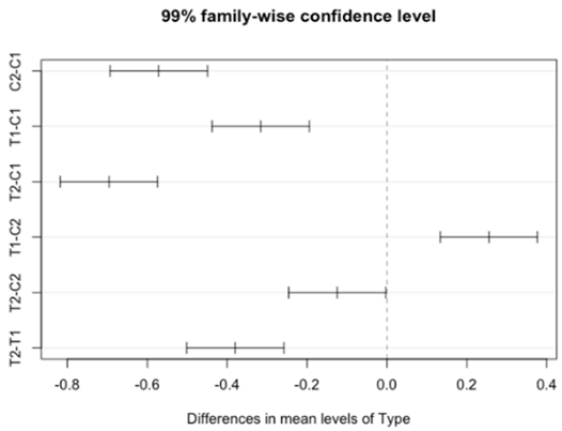

Fig. 4: Plot of Tukey HSD of inhibition of *E. faecalis* (MTCC 2729) by *B. subtilis* FPTB23 on fillet at 5±1^{O}C.

Table 2: Inhibition of *E. faecalis* (MTCC 2729) by *B. subtilis* FPTB23 on fillet at 5±1^{O}C.

Days	Log cfu/g of *E. faecalis* (MTCC 2729)			
	C-1	T-1	C-2	T-2
0	6.26±0.08	6.26±0.08	6.26±0.08	6.26±0.08
3	7.38±0.14	7.33±0.25	7.30±0.24	7.32±0.12
6	8.42±0.17	8.36±0.25	7.41±0.14	8.28±0.32
9	8.62±0.25	8.47±0.14	8.08±0.08	7.43±0.05
12	8.94±0.17	8.07±0.08	8.20±0.17	7.59±0.14
15	8.90±0.08	7.88±0.12	7.65±0.08	7.34±0.12

Results are mean of three determinations with SD

From Table 2 it is evident that, the *E. faecalis*population in inoculated fillet was inhibited by about 1 log cycle in the presence of *B. subtilis*under normal packaging conditions. Application of vacuum, however, had a synergistic effect in combination with *Bacillus* strain resulting in reduction of *E. faecalis*count ($p<0.05$) as compared to C_1. In case of fillets inoculated with *E. faecalis*and packed in vacuum (C_2), the *E. faecalis* count increased from an initial value of 6.26±0.08 (log cfu/g) to 7.65±0.08 (log cfu/g) at the end of 15 days. This final count was 1.3 log cycle less than the fillets stored under aerobic conditions (C_1).

Kindoli et al. [5] reported that W42 *B. subtilis* strain exhibited strong antimicrobial activity against *E. faecalis* (ATCC 29212) by agar well diffusion method. Fernandes et al. [23] reported that *B. subtilis* R14 showed antimicrobial activity against 12 strains of *E. faecalis* (viz. CI55671, CI55918, CI144, CI068, CI56671, CI56354, CI55995, CI295, CI222, CI55195, CI56288 and ATCC 29212) by the production of biosurfactant. Mersacidin like subtilin, the type B lantibiotic (1825 Da), produced by *Bacillus* sp., successfully inhibited in vitro and in vivo the growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* strains (MRSA) as well as enterococci expressing the VanA vancomycin resistance phenotype [24].

From the result of the present study it is evident that application of *Bacillus*on Asian sea bass fillet resulted in reduction in the final count of *E. faecalis*(MTCC 2729) after 15 days at 5±1^{O}C. The inhibition of *E. faecalis* in sea bass fillet may be attributed to the synergistic effect of several hurdles as described by Ananou et al.[1]. The principal hurdles employed in the present study are, low temperature (5±1^{O}C), vacuum and competitive flora (isolated *B. subtilis* FPTB23). During the period of study the growth of *E. faecalis*was encountered in both the samples but with varying rates suggesting a bacteriostatic rather than bacteriocidal impact of the hurdles. Application of vacuum, however, had a synergistic effect in combination with *Bacillus* strains resulting in reduction of *E. faecalis*count ($p<0.05$). Hence, from the findings of the present study it may be concluded that application of *B. subtilis* FPTB23 in combination with vacuum packaging can be a promising alternative to chemical preservatives for preservation of Asian sea bass fillet with a shelf life of 15 days stored at 5±1^{O}C.

4. ACKNOWLEDGEMENT

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