J. Terr. Mar. Res. Vol. 1(2) 2017:36-40.
Journal of Terrestrial and Marine Research

Waveland Scientific Publications
wavelandscientificpublications.com/journal-of-terrestrial-and-marine-research-jtmr/

ISSN:2456-7639

Research Article

Isolation, Screening and Characterization of Extracellular Enzyme Producing Bacteria Associated with Gills and Gut of Fresh Water and Marine Fishes

A.VIJAYAKUMAR, E. TAMILSELVI, K.ANANDARAJ AND A. JAYAKUMAR

PG & Research, Department of Microbiology,
Shanmuga Industries Arts and Science College, Tiruvannamalai-606603

Abstract

Enzyme producing bacteria from gills and gut of fresh water Nile tilapia, (*Oreochromis niloticus*) and marine fish (*Rastrelliger kanagurta*) have been investigated. A total of 8 bacteria were isolated and identified based on the phenotypic characterization. All the isolates were screened for the production of extracellular enzymes Agarase, Gelatinase and Amylase. Six bacterial isolates in this study produced extracellular enzymes, particularly genus *Aeromonas* and *Vibrio* were found to be excellent enzymes producers. This study proves that, gut and gill associated bacteria are having the potential of enzyme production for various industrial applications.

Keywords: *Aeromonas*, Enzymes, *Rastrelliger kanagurta, Vibrio*,

Introduction

Intestinal microorganisms associated with fishes have a beneficial effect on the digestive process of fish, such as microbial breakdown of cellulose (Saha and Ray, 1998).Such associated microorganisms produce secondary metabolites and enzymes to protect the host from the pathogenic microorganisms. Marine microbial enzymes are significantly different from terrestrial origin, as the marine environment ranges from nutrient rich to nutrient sparse regions with properties such as high salt tolerance, hyperthermostability, barophilicity, and cold adaptability (Zhang and Kim, 2010). Marine environment is a complex niche comprising of various organisms micro algae to larger plants, whose external body is majorly comprised of various Polysaccharides such as chitin in case of fungi, zooplankton and crustaceans; agar in agarophytes; alginates in alginophytes and carrageenan in case of carrageenophytes, besides pectin and cellulose in case of phytoplankton and other plant components such as mangroves and sea grass. Along with polysaccharide components marine organisms are rich in secondary metabolites. These organic biopolymers act as substrate for the microbial communities to produce various substrate specific extracellular enzymes helping in the remineralisation of organic matter and recycling of nutrients in the marine environment (Murthy et al., 2016).

Studies on fish-associated microorganisms involved culture-dependent population within the gastrointestinal (GI) tract of marine and freshwater fish species has been widely investigated by various authors from different geographical locations (Austin, 2002; Ghosh et al., 2010; Askarian et al., 2012). The nutrient-rich GI tract of fish is a favorable growth environment for bacteria (Kar et al., 2008). The gut microorganisms have been categorized as either autochthonous (indigenous) or allochthonous (transcient) depending upon their ability to colonize and adhere to the mucus layer in the digestive tract (Ringo et al., 2003). Bacteria within the gastrointestinal tract of fish have showed broad and variable enzymatic potential, and these enzymatic masses may interfere positively in the digestive process of fish (Ray et al., 2010).

The bacterial flora associated with the intestine of tropical estuarine fish species *Tilapia guineensis* has been reported (Ariole and Kanu, 2013).Extensive research on the gut microbiota in both marine and freshwater fish has confirmed that the gastrointestinal (GI) tract of fish is an abode of dense microbial population (Austin, 2002), different enzymes produced by gastro intestinal bacteria could be a contributing source of enzymes in fish (Ray et al.,2012). Fish gut bacterial isolates have been demonstrated to break down chitin (Itoi et al., 2006), p-nitrophenyl-b-N-acetylglucosamine and protein (MacDonald et al., 1986; Belchior and Vacca, 2006), cellulose (Saha and Ray, 1998; Bairagi et al., 2002; Ghosh et al., 2010; Saha et al., 2006; Mondal et al., 2008),

Manuscript Information

Received: 01st April 2017 / **Revised:** 20th April 2017 / **Accepted:** 22nd April 2017 / **Published:** 26th April 2017 /
Corresponding Author: vijaymicrobes@gmail.com

amylase (Sugita et al., 1997; Ghosh et al., 2010), phytate (Li et al., 2008; Khan et al., 2011; Khan and Ghosh, 2012, 2013), and tannin (Mandal and Ghosh, 2013).

In this context, the search for extracellular enzyme producing beneficial gut bacteria to be used as probiotics for the brackish water fish culture may be of interest. Microorganisms are the most important sources for enzyme production. Selection of the right organisms plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. In the present study, an attempt has been made to isolate the potential bacteria from freshwater fish (*Oreochromis niloticus*) and marine fish (*Rastrelliger kanagurta*).

Materials and Methods

Sample collection and processing

In the present study Nile tilapia (*Oreochromis niloticus*) freshwater and (*Rastrelliger kanagurta*) marine fish were purchased from local fish market in Tiruvannamalai and were transferred to the laboratory in a sterile polythene cover. Fishes were dissected with sterile knife and small portion of the gills and gut samples were transferred separately to the labelled vial containing 20 ml of Alkaline Peptone Water (APW-pH 8.5, Hi media) and incubated for 6 h at 37°C for enrichment of bacteria. After the incubation, 100 µl of inoculum were plated onto Nutrient agar plates containing 0.5% sodium and incubated at 37°C for 24 h (Murthy et al., 2016). After incubation each isolated colony was further purified on Nutrient agar plates (Himedia, India) incubated at 37°C for 24 h. After successive purifications pure strains were obtained. All these isolates obtained were then stabbed in Nutrient agar with 0.5% NaCl and 0.8% agar, sealed and stored at room temperature.

Extracellular enzyme production

Isolates obtained were subjected to various substrates such as Agar, Amylase, Gelatin to know the potential of these isolates to produce extracellular enzymes such as Agarase, Amylase and Gelatinase respectively (Baumann et. al., 1971; Hu et. al., 2009; Tropeano et. al., 2012). All these assays were conducted on solid media by providing the respective substrate at 0.5 to 1% w/v for each enzyme. The results were recorded based on the methodology adopted by Murthy et al. (2016) as grades average, good, very good, excellent and negative based on their appearance and zone of hydrolysis of the substrate surrounding the bacteria on solid media.

Phenotypic characterization of the isolated bacteria

Bacterial isolates obtained obtained were subjected to phenotypic characterization for the identification by following the protocol described in Bergey's manual of systemic Bacteriology (Brenner et al., 2005). ABIS online software was also used for identification.

Result

In the present study, 8 bacterial isolates were obtained, 4 isolate each from both fresh water Nile tilapia (*Oreochromis niloticus*) and marine fishes (*Rastrelliger kanagurta*) respectively. All the 8 isolates were screened for the production of extracellular enzymes such as agarase, gelatinase and amylase. Among the 8 isolates, 6 isolates 3 from marine MG2, MGL1, MGL2 and 3 from fresh water LG1, LGL1, LGL2 produced agarsase, gelatinase and amylase and 1 isolate LG2 from fresh water produced gelatin and amylase and one from marine MG produced gelatinase enzyme (Table 1). Bacterial isolates were phenotypically characterized and identified by Gram stain, Biochemical characterization and sugar fermentation tests and they were also cross checked by ABIS, online software for identification confirmation (Table 2).

Table 1: Screening of enzyme producing bacteria from fresh water and marine fish purchased from the local fish market Tiruvannamalai.

S.No	Sample	Agarose	Gelatin	Amylase
1	LG 1	+	++	++
2	LG 2	-	+	++
3	LGL 1	+	++	++
4	LGL 2	++	+	+
5	MG 1	-	++	-
6	MG 2	+++	++	++
7	MGL 1	+++	++	++
8	MGL2	++	++	+

Discussion

Microbial communities from different gastrointestinal tracts of freshwater or marine carnivorous, herbivorous, and omnivorous fish species have been reported by Ray et al. (2012). Various enzymes have been isolated from plants and animals but recent findings revealed that the microbial origin enzymes were reported to be an excellent alternative due to their diverse biochemical properties, plasticity for easy genetic manipulation and large-scale production. Marine microbial enzymes are unique in nature when compared to the enzymes of terrestrial based origin due to their adaptability of marine bacteria towards an array of diverse environmental parameters like high salt concentration, extreme temperatures, acidic and alkaline pH, extreme barometric pressure, and low nutrient availability (Zhang and Kim, 2010).Digestive tracts of endotherms have been reported as obligate anaerobes (Finegold et al., 1983), while aerobes or facultative anaerobes have been isolated as the predominant bacterial genera from most of the fish guts (Bairagi et al., 2002; Ghosh et al., 2002).

Agar is a polysaccharide which exists as a primary cell wall component of red algae (Rhodophyceae) such as *Gelidium* and *Gracilaria* species (Fu and Kim, 2010). Several *Vibrio* derived agarases were reported from the marine environment that displays an excellent agarolytic activity (Macian et. al., 2001) and sediments (Liao et.al., 2011). Sugano et. al. (1993) isolated *Vibrio* sp. strain JT0107, as an agar degrading bacteria that decomposes the cell walls of some seaweeds, including a *Laminaria* sp. and *Undaria pinnatifida* in Japan. In this study, family Vibrionaceae were observed with excellent agarase activity, among the isolates (MGL2) obtained from the gills of *Rastraliger kanagurta* showed higher range agarase activity. *Aeromonas bestarum* is a gut associated bacteria which also exhibited

agarase activity. Bacteria LG1, LGL1, LGL2, obtained from gut and gills of freshwater fish *Oreochromis niloticus* also showed agarase activity ranging from moderate to average activity.

Gelatin is a polypeptide derived by hydrolytic degradation of collagen, the principal component of animal connective tissue which is traditionally been extracted from the skin and bone collagens of certain

Table 2: Phenotypic characterization of bacteria isolated from fresh water and marine fish purchased from the local fish market Tiruvannamalai.

Isolates	**LG 1**	**LG 2**	**LGL 1**	**LG L2**	**MG 1**	**MG 1**	**MGL 1**	**MGL 2**
Morphology								
Grams stain	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve
Motility	+	+	+	+	+	+	+	+
Colonies	Grey	White	Translucent	Gray	Cream white	Translucent	Translucent	Translucent
Shape	Short rod	Rod	Long Rod	Short rod	Straight rod	Rod	Rod	Curved rod
Growth at 45ºC	-	+	+	-	+	-	-	-
Growth at 3% Nacl	-	+	+	-	+	-	+	+
Growth at 5% Nacl	-	+	-	-	+	-	-	+
Growth at TCBS	-	-	-	-	-	+	+	+
Biochemical tests								
Indole	+	+	+	+	+	+	+	+
Methyl Red	-	-	-	-	-	-	-	-
Voges Poskauer	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	+	+	+	+
Catalase	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+
Sugar Utilization								
Sucrose Su	+	+	+	+	+	+	+	+
Lactose La	-	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+
Mannitol	+	-	+	+	-	-	+	-
Identified isolates	***Citrobacter koseri***	***Bacillus licheniformis***	***Bacillus niacin***	***Citrobacter murliniae***	***Panenibacillus cellulositrophicus***	***Aeromonas fluvialls***	***Aeromonas bestarum***	***Vibrio* sp**

mammalian species, primarily cows and pigs and gelatin from marine sources especially from the fin and shellfish waste e.g. skin, bones, scales and fins (Venkateshwarlu, 2013). In the present study all the bacterial isolates 8 LG1, LG2, LGL1, LGL2 (*Oreochromis niloticus*) MG1, MG2, MGL1, MGL2 obtained from (*Rastrelliger kanagurta*) had shown gelatinase activity but the enzyme production has been ranged between less and average activity.

Amylases are enzymes that hydrolyze amylase molecules to give diverse products, including dextrins and progressively smaller polymers which composed of glucose units. These enzymes are one of the great significance in the present-day biotechnology with many industrial applications such as food, fermentation, textile, and paper industries. In this study, 7 bacterial isolates obtained from *Oreochromis niloticus* and *Rastrelliger kanagurta* showed gelatinase activity except MG1 strain identified as but the range of enzyme activity was observed average activity.

Bacterial amylase are produced widely from a range of temperature by *Bacillus* sp particularly *Bacillus amyloliquefaciens*, *Bacillus subtilis* and Bacillus *lichniformis* are among the most commonly used *Bacillus* species for enzyme α-amylase production at temperature ranging from 37ºC to 60ºC (Mishra et al., 2005). Enzyme producing *Bacillus* sp. was not capable of producing enzyme at temperature below 25ºC on other hand, a progressive decline of enzyme production was observed at 45ºC and no enzyme production was observed at 50ºC (Ashwini et al., 2011).

Bacillus cereus isolated from the gut of *Catla Catla* produced amalyse under controlled condition (Chovatiya et al., 2014). Amylase producing *Bacillus licheniformis* was isolated from brackish water fish *Mystusgulio* (Das et al., 2014). In this study two *Bacillus* species have been isolated from gut and gills of *Oreochromis niloticus* LG2 (*Bacillus licheniformis*), LGL1 (*Bacillus niacini*) and they produced Agarase, Gelatin and Amylases at 37 ºC among these two strains *Bacillus licheniformis* was found to showed their enzyme production at 45 ºC and similarly the production was not observed above 45ºC. Numerous studies reported that *Bacillus licheniformis* was found to be potent enzyme producing strain and in this study also we observed that *Bacillus licheniformis* was isolated from fresh water *Oreochromis niloticus* produced different enzymes at various temperature.

Aeromonas and *Citrobacter* sp are the most prevalent bacterial species present in the freshwater sting ray mucus and they produced gelatinase enzyme (Domingo et al., 2011). *Aeromonas* sp isolated from the cultured fish of *Clarias gariepinus* showed different enzyme activity (Ariole et al., 2014). In this study two *Citrobacter* species *Citrobacter koseri* and *Citrobacter murliniae* isolated from fresh water *Oreochromis niloticus* produced agarase, gelatinase and amylase enzymes at 37ºC. Genus *Aeromonas* is prevalence in marine fish *Rastrelliger kanagurta* (Sudha et al., 2017).

Genus *Vibrios* are known to produce gelatinase enzyme and it is one of the key biochemical or phenotypic characteristic to differentiate between species of *Vibrios* (Noguerola and Blanch, 2008). Twenty isolated of marine *Vibrio* produced six types of enzymes under controlled condition (Murthy et al., 2016). Gelatinase production is recognized as a virulence factor in human beings and marine animals (Vergis et al., 2002). In this study single isolate *Vibrio* sp. isolated from marine fish *Rastrelliger kanagurta* produced enzymes at optimal temperature.

Conclusion

Fish is one of the major food which containing both beneficial and pathogenic bacterial population. Outcome of the present study showed that the beneficial bacteria isolated from fresh water fish tilapia, *Oreochromis niloticus* and marine fish *Rastrelliger kanagurta* had produced different enzymes which are highly importance in the field of various industrial applications.

Reference

1. Ariole, CN, and Kanu. NA. (2013). Bacterial flora associated with intestine of tropical estuarine fish species, J. Chem. Biol. Phy. Sci. 4(1): 209-215
2. Ariole, CN., Nwogu, HA. and Chuku, PW. (2014) Enzymatic Activities of Intestinal Bacteria Isolated from Farmed *Clarias gariepinus*, Int. J. Aquacul. 4 (18): 108-112
3. Askarian, F., Zhou, Z, Olsen, RE, Sperstad, S and Ringo, E. (2012). Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. Aquaculture. 326:1-8
4. Ashwini, K., Kumar, G., Karthik, L. and Rao, KVB. (2011). Optimization, production and partial purification of extracellular α- amylase from *Bacillus* sp. marini, Arch. Appl. Sci. Res. 3(1): 33–42
5. Austin, B. (2002). The bacterial microflora of fish. Sci. World J. 2: 558–572
6. Bairagi, A.,Ghosh, KS., Sen, SK, and Ray, AK. (2002). Enzyme producing bacterial flora isolated from fish digestive tracts. Aqua. Int. 10: 109-121
7. Baumann, P., Baumann, L. and Mandel, M. (1971). Taxonomy of marine bacteria: the genus *Beneckea*. J. Bacteriol. 107:268-294.
8. Belchior, SG. and Vacca, G. (2006). Fish protein hydrolysis by a psychrotrophic marine bacterium isolated from the gut of hake (*Merluccius hubbsi*). Can. J. Microbiol. 52: 1266–1271.
9. Brenner, DJ., Krieg, NR. and Staley J.T. (ed.). (2005). Bergeys manual of Systematic Bacteriology, Springer, New York.
10. Chovatiya, S., Ingale, S., Rana, D. and Patel, P. (2014). Isolation, Characterization and Optimization of Amylase Producing Micro-Organism from Gastrointestinal Tract of *Catla Catla.* Int. J. Inn. Res. Sci. Eng. Techn. 3(12):17890-17899.
11. Das, P., Mandal, S., Khan, A., Manna, S.K. and Ghosh, K. (2014). Distribution of extracellular enzyme-producing bacteria in the digestive tracts of 4 brackish water fish species. Turk. J. Zool. 38:79-88
12. Domingosa, MA., Franzolin, MR., Anjos, MT., Franzolin, TMP., Albes, RCB., Andrade, GR.,Lopes, RJL. and Barbaro, KC. (2011).The influence of environmental bacteria in freshwater stingray wound-healing. Toxicon. 58 (2011): 147–153.
13. Finegold, SM., Sutter, VL. and Mathisen. GE (1983). Normal indigenous intestinal flora. In: Human Intestinal Microflora in Health and Disease (Ed. D.J. Hentgens). Academic Press, New York, NY, USA, pp. 3–31.
14. Fu, XT. and Kim, SM. (2010). Agarase: Review of Major Sources, Categories, Purification Method, Enzyme Characteristics and Applications. Mar. Drugs. 8: 200-218.
15. Ghosh, K., Sen, SK. and Ray, AK. (2002). Characterization of *Bacilli* isolated from rohu (*Labeo rohita*) fingerlings and its significance in digestion. J. App. Aqua. 12: 33-42.
16. Ghosh, K., Roy, M., Kar, N. and Ringo, E. (2010). Gastrointestinal bacteria in rohu, *Labeo rohita* (Actinopterygii: Cypriniformes: Cyprinidae):Scanning electron microscopy and bacteriological study. Acta Ichthyologica et Piscatoria 40: 129-135.
17. Hu, Z., Lin, B., Xu, Y., Zhong, MQ. and Liu, G. (2009). Production and purification of agarase from a marine agarolytic bacterium *Agarivorans* sp. HZ105. J. App. Microbiol. 106: 181–190.
18. Itoi, S., Okamura, T., Koyama, Y. and Sugita, H. (2006). Chitinolytic bacteria in the intestinal tract of

Japanese coastal fishes. Can. J. Microbiol. 52: 1158–1163.
19. Kar, N., Roy, RN., Sen, SK. and Ghosh, K. (2008). Isolation and characterization of extracellular enzyme-producing bacilli in the digestive tracts of rohu, *Labeo rohita* (Hamilton) and murrel, *Channa punctatus* (Bloch). Asian Fish. Sci. 21(4): 421–434
20. Khan, A., Mandal, S., Samanta, D., Chatterjee, S. and Ghosh, K. (2011). Phytase-producing *Rhodococcus* sp. (MTCC 9508) from fish gut: a preliminary study. Proc. Zool. Soc. 64: 29–34.
21. Khan, A. and Ghosh, K. (2012). Characterization and identification of gut-associated phytase-producing bacteria in some freshwater fish cultured in ponds. Acta Ichthyol. Piscatoria. 42(1): 37–45.
22. Khan, A. and Ghosh, K. (2013). Evaluation of phytase production by fish gut bacterium, *Bacillus subtilis* for processing of *Ipomea aquatic* leaves as probable aqua feed ingredient. J. Aquat. Food Prod. Technol. 22(5): 508–519
23. Liao, L., Xu, X., Jian, X., Cao, Y., Yi, N., Huo, Y., Wu, Y., Zhu, X., Zhang, X. and Wu, M. (2011). Cloning, Expression, and Characterization of a New β-Agarase from *Vibrio* sp. Strain CN41. Appl. Environ. Microbiol. 77: 7077–7079.
24. Li, X., Chi, Z., Liu, Z., Yan, K. and Li, H. (2008). Phytase production by a marine yeast *Kodamea ohmeri* BG3. Applied Biochem. Biotechnol. 149: 183–193.
25. MacDonald, NL., Stark, JR. and Austin, B. (1986). Bacterial microflora in the gastrointestinal tracts of Dover sole (*Solea solea* L.), with emphasis on the possible role of bacteria in the nutrition of the host. FEMS Microbiology Letters 35: 107-111.
26. Macian, MC., Ludwig, W., Schleifer, KH., Pujalte, MJ. and Garay, E. (2001). *Vibrio agarivorans* sp. nov., a novel agarolytic marine bacterium. Int. J. Syst. Evol. Microbiol. 51:2031–2036.
27. Mandal, S. and Ghosh, K. (2013). Isolation of tannase-producing microbiota from the gastrointestinal tracts of some freshwater fish. J. App. Ichthyol. 29:145-153
28. Mishra, S., Noronha, SB. and Suraishkumar, GK. (2005). Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data. Process Biochem. 40(5): 1863–1870.
29. Mondal, S., Roy, T., Sen, SK. and Ray, AK. (2008). Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. Acta Ichthyol. Piscatoria. 38: 1–8
30. Murthy, KN., Mohanraju, R., Karthick, P. and Ramesh, CH (2016). Isolation, molecular characterization and screening of extracellular enzymes produced by *Vibrio alginolyticus* from the seaweeds of Andaman Islands. Seaweed. Res. Utiln. 38(1):67-74.
31. Noguerola, I, and Blanch, AR. (2008). Identification of *Vibrio* spp. with a set of dichotomous keys. J. Appl. Microbiol. 105: 175–185.
32. Ray, A.K, Roy, T., Mondal, S. and Ringo, E. (2010). Identification of gut-associated amylase, cellulase and protease producing bacteria in three species of Indian majorcarps. Aqua. Res. 41:1462-1469.
33. Ray, A.K., Ghosh, K. and Ringo. E. (2012). Enzyme-producing bacteria isolated from fish gut: A review. Aquaculture Nutrition 18:465-492.
34. Ringo, E, Olsen, RE., Mayhew, TM. and Myklebust, R. (2003). Electron microscopy of the intestinal microflora of fish. Aquaculture. 227: 395-415.
35. Saha, S. and Ray, AK. (1998). Cellulase activity in rohu Fingerlings, Aqua. Int. 6: 281-291
36. Saha, S., Roy, RN., Sen, SK. and Ray, AK. (2006). Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (P) and grass carp, *Ctenopharyngodon idella* (V). Aquacult. Res. 37: 380–388
37. Sudha, K., John, R. and Sivansankari, TR. (2017). Extracellular l-asparginase production by halotolerant strain of *Enterobacter hormaechei* isolated from marine fishes. Int. J. Adv. Res. 5(1), 2619-2625.
38. Sugano, Y., Terada, I., Arita, M. and Noma, M. (1993). Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. strain JT0107. Appl. Environ. Microbiol. 59: 1549–1554.
39. Sugita, H., Shibuya, K., Hanada, H. and Deguchi, Y. (1997). Antibacterial abilities of intestinal microflora of the river fish. Fisheries Science 63: 378-383.
40. Tropeano, M., Coria, S., Turjanski, A., Cicero, D., Bercovich, A., Cormack, WM. and Vazquez, S. (2012). Culturable heterotrophic bacteria from Potter Cove, Antarctica, and their hydrolytic enzymes production. Pol. Res. 1: 1–11.
41. Venkateshwarlu, G. (2013). Fish Gelatin: A Versatile Ingredient for the Food and Pharmaceutical Industries. In*:* Marine Proteins and Peptides: Biological Activities *and* Applications. S. K. Kim. (Ed.), John Wiley & Sons, Ltd., UK. pp. 271-298.
42. Vergis, EN., Shankar, N., Chow, JW., Hayden, MK., Snydman, DR., Zervos, MJP., Linden, K., Wagener, MM. and Muder, MM. (2002). Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis*. Clin. Infect. Dis.35: 570-575.
43. Zhang, C. and Kim, SK. (2010). Research and Application of Marine Microbial Enzymes: Status and Prospects. Mar. Drugs. 8: 1920-1934.