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High Potential Probiotic *Bacillus* Species from Gastro-intestinal Tract of Nile Tilapia (*Oreochromis niloticus*)

[1,2]K. Chantharasophon, [3]T. Warong, [3]P. Mapatsa and [3,4]V. Leelavatcharamas
[1]Graduate School, Khon Kaen University, Khon Kaen, Thailand
[2]Department of Microbiology, Faculty of Science, Ubon Ratchathani Rajabhat University, Ubon Ratchathani, Thailand
[3]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand
[4]Fermentation Research Center for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand

Abstract: In study to obtain a safe *Bacillus* probiotic for Nile tilapia (*Oreochromis niloticus*) cultivation, hemolytic activity on blood agar medium was used in isolation of *Bacillus* probiotic species from gastro-intestinal tract of Nile tilapia. One hundred and three isolates of *Bacillus* sp. which showed no hemolytic activity were obtained from 2 sampling sites of the Nile Tilapia net-cage culture farms. Among these 103 isolates, however, there was only 1 isolate, named as *Bacillus* UBRU4 which showed the inhibitory effect on *Aeromonas hydrophila* growth. The results of physiological and biochemical test and molecular identification (99.90% identity) showed that *Bacillus* UBRU4 was similar to *Bacillus brevis*. This was possibly the first report of isolation of *Bacillus brevis* in aquaculture. The optimum pH and temperature for *Bacillus* UBRU4 growth on Tryptic soy broth were 6.5 and 37°C, respectively. The maximum cell numbers of *Bacillus* UBRU4 in modified broth culture medium was obtained when using the medium contained 30 g L^{-1} of Nile tilapia commercial feed and 20 g L^{-1} of molasses. The bioactive compound production of *Bacillus* UBRU4 showed the growth associated characteristic. Partial purified bioactive compounds by 80% saturated ammonium sulfate could increase the activity to 6,400 AU mL^{-1}. The specific activity of the bioactive compound was increased from 1,298 to 5,807 AU mg^{-1}. These results suggested that the *Bacillus* UBRU4, thus, could possibly be used as high potential probiotic in Nile tilapia feed.

Key words: Nile tilapia, net-cage culture, hemolytic activity, blood agar, *Bacillus brevis*

INTRODUCTION

Disease is a major problem for the fish farming industry which currently is the fastest growing food-protein producing sector, with an annual increase of approximately 9% (Deeseenthum *et al*., 2007). Tilapia (*Oreochromis* sp.) is a group of fish which is growing importance in the aquaculture industry. Nile tilapia is the most commonly cultured species among Tilapias in many countries around the world (Mehrim, 2009), including Thailand. Only in Ubon Ratchatani province, there are more than 2,000 net-cage cultures. Disease outbreak is an important problem for Nile tilapia farmers and antibiotics are used as effective agents to control disease and growth promoters in domestic animals. Nevertheless, the continuous use of antibiotics has contributed to the occurrence of antibiotic-resistant bacteria population (Rahman *et al*., 2009) and to an increase in more virulent pathogens. The fear for the spread of this resistance to human pathogens has recently led to the banning of several antibiotics as so-called growth promoters in animal husbandry within the European Union (Naviner *et al*., 2006; Deeseenthum *et al*., 2007). Probiotics are viable cell preparation which must have the ability to survive passage through the intestinal tract which has beneficial effects on health of the host by improving its intestinal balance via producing nutrients, enhancing immune response (Song *et al*., 2006). Probiotics have been used in aquaculture as a means of disease control, digestion aids and immune booster or even in some cases replacing the use of antimicrobial compounds (Irianto and Austin, 2002; EL-Haroun, 2007; Mehrim, 2009). In order to make Nile tilapia products more acceptable to consumers, it is necessary to find the effective probiotics to prevent diseases and improving growth performance with a particular attention to

Corresponding Author: Vichai Leelavatcharamas, Fermentation Research Center for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand Tel/Fax: 66-43-362122

Bacillus spp. because bacilli are spore-forming bacteria and can grow rapidly. However, some *Bacillus* spp. could be pathogenic bacteria also, such as *B. cereus*, *B. anthracis*. Hemolytic activity on blood agar plate is generally used for identifying pathogenic microorganism and rarely use for screening a useful bacteria. Thus, the aims of the present work were isolation and screening of the high potential bacilli probiotic from the gut of Nile tilapias by using blood agar plate. Bioactive compound production, optimizations of the cultivation condition and the modification of the cheap medium for producing of the selected *Bacillus* sp. were also reported.

MATERIALS AND METHODS

The isolation and screening for the safe *Bacillus* probiotic from Nile tilapia's intestinal tract took almost 2 years (from February 2009-November 2010) to obtain the targeted *Bacillus* probiotic. This was because most of the isolated *Bacillus* showed the hemolytic activity or no antagonistic activity.

Isolation and screening of the bacilli probiotics from the intestinal tract of Nile tilapia: The Nile tilapia net-cages culture farms located in Moon River at Ban Kudua, Jaramae sub-districts and Moon-noi River at Ban Pakmoonnoi, Bungkasael sub-districts, Maung district, Ubon Ratchatani province, Thailand were the two study sites of this work. The bacilli probiotics from the intestinal microbiota in Nile tilapia were screened over 4 months with regular sampling of 90 fishes in total from the two study sites. Five randomly live Nile tilapia from each site were collected and transferred to the laboratory within 1 h for microbiological analyses. The collected fish were anesthetized and cleaned with alcohol to remove any external microbial contamination. Their gastrointestinal tracts were then dissected, grinded and boiled at 100°C for 5 min in order to isolate for bacilli group on blood agar medium by cross-streak technique. Colonies of spore forming bacteria without clear-zone on blood agar were considered as the isolated bacilli and all isolates were purified by spread plate technique 10 times for further use. All isolated bacilli were screened for the ability of growth inhibition against *Aeromonas hydrophila* by spotted on lawn technique on tryptic soy agar plates. The plates were swabbed with 24 h cultured *A. hydrophila* and were spotted on lawn with 24 h cultured bacilli. All plates were incubated at 37°C for 24 h. If clear inhibitory zones formed around, the spots were considered as antagonistic against the growth of *A. hydrophila*.

Identification of the isolated bacilli probiotics from the intestinal tract of Nile tilapia: *Bacillus* isolates were characterized by biochemical test and molecular biology assays at the genus or species level. The selected *Bacillus* were determined on the criteria of Gram reaction, spore shape and position, susceptibility to penicillin, streptomycin and kanamycin, catalase activity, motility, starch digestion, nitrate reaction, acid and gas formation from glucose, growth at temperature of 10 and 45°C, growth in medium with 4, 6.5% salt and 0.2% bile salt. The nucleotide sequencing, 16S rDNA (Full sequence; 1,400 bp), was also used for *Bacillus* identification by the service at BIOTECH Culture Collection, BIOTECH Central Research Unit, 113, Thailand Science Park, Paholyothin Road, Klong 1, Klong Luang, Prathumthani, Thailand.

Optimization of cultivation conditions and culture medium for *Bacillus* cells production: The selected *Bacillus* was cultured in nutrient broth at 37°C for 24 h and used as inoculum. The initial pH of nutrient broth was adjusted to 4, 5, 6 and 7 prior to sterilize at 121°C for 15 min. All conditions of culture medium were inoculated with 10^8 CFU mL^{-1} of inoculum and incubated at 30 and 37°C for 24 h on rotary shaker (150 rpm). Samples were withdrawn during the cultivation process for cell growth determination by total plate count method. For cheap medium optimization, the isolated *Bacillus* was cultured in modified liquid medium which was contained of molasses at the concentrations of 20 g L^{-1}. The effect of varying Nile tilapia commercial feed CP No. 895 (Charoenpokapan Company, Samutsakhon, Thailand) at the concentrations of 10, 20, 30 and 40 g L^{-1} was then examined after incubation at 37°C for 24 h. Samples were withdrawn during the cultivation process for cell growth measurement by total plate count method.

Bioactive compound production of the selected *Bacillus* sp. probiotic: The selected *Bacillus* sp. was cultured in 1 l Erlenmeyer flasks containing 500 mL nutrient broth and incubated at 37°C in incubation shaker (150 rpm). Samples were withdrawn during the cultivation process for cell growth, protein concentration and antibacterial activity determinations by total plate count method, Lowry method (Waterborg and Matthews, 1994) and two-fold-dilution method (De Carvalho *et al*., 2006), respectively. The activity of the partial purified bioactive compound, obtained from 80% saturated ammonium sulfate precipitation, was also examined by two-fold-dilution technique. The partially purified substance from the selected *Bacillus* sp. was serially diluted into 50 mM sodium acetate buffer (pH 5.5) and tested against *Aeromonas hydrophila*. The antibacterial activity

(AU mL^{-1}) was calculated by the following formula: The highest dilution which still showed the positive result×1,000/sample volume.

RESULTS

Isolation and screening of the bacilli probiotics from the intestinal tract of Nile tilapia: One hundred and sixty randomly live Nile tilapias from two net-cage culture sites were collected for 8 months. The samples were transferred to the laboratory within 1 h for microbiological determination. One hundred and three isolates of bacilli without clear-zone on blood agar (no hemolytic effect) were obtained from both study sites. However, there was only 1 from 103 isolated *Bacillus* exhibited both no hemolytic activity on blood agar medium (Fig. 1a) and the inhibitory activity against the growth of *A. hydrophila* by spot on lawn technique (5-6 mm of inhibition zone) (Fig. 1b) and swab paper disc method (5-7 mm of inhibition zone). This isolated *Bacillus* was found from Nile tilapia of site I and it was designed as *Bacillus* sp. UBRU4.

Identification of the bacilli probiotic from the intestinal tract of Nile tilapia: The results of Physiological property and Biochemical test of *Bacillus* sp. UBRU4 were shown in Table 1 and 2. Colony morphology of *Bacillus* sp. UBRU4 was evaluated after complete expression of the phenotypes on LBY agar, the resulting colony was described in terms of their most distinct features (Table 1). The full phenotype typically became manifest after 1 week of cultivation (2 days at 37°C, followed by 5 days at room temperature). The developed phenotype was exhibited whitish or pale yellow color, circular form, convex elevation, convex elevation in the center, glistening surface, rough, translucent colonies, 3 to 4 mm in diameter and undulated irregular edges (Table 1). Micro-morphology of *Bacillus* sp. UBRU4 was 1×1.5-2.5 μm of cell size, bacilli shape, gram positive, spore forming bacteria in swollen sporangium which were ellipsoidal shape, central or subterminal endospore position.

It was exhibited that the Biochemical properties of *Bacillus* sp. UBRU4 (Table 2) were resistance to the antibiotic penicillin and kanamycin but susceptible to streptomycin, catalase positive, motility positive, starch digestion negative, nitrate reducing positive, acid and gas

Table 1: Physiological property of *Bacillus* sp. UBRU4

Parameters	*Bacillus* sp. UBRU4
Macro-morphology	Colony characterization on LBY
	:2 to 3 mm in diameter
	:Whitish or pale yellow color
	:Circular form
	:Convex elevation
	:Convex elevation in the center
	:Rough and glistening
	:Undulated irregular edges
Micro-morphology	:1×1.5-2.5 μm of cell size
	:Bacilli shape
	:Swollen sporangium
	:Ellipsoidal endospores
	:Central/subterminal endospore position

Table 2: Biochemical characteristics of *Bacillus* sp. UBRU4

Parameters	*Bacillus* sp. UBRU4
Susceptible to: Penicillin	0 mm
:Kanamycin	0 mm
:Streptomycin	16 mm
Gram	+
Catalase activity	+
Motility	+
Starch digestion	-
Reducing of nitrate	+
Acid and gas from glucose	+
Growth at :10°C	+
:45°C	-
:4% salt	+
:6.5% salt	-
:0.2% bile salt	+

Note: *Bacillus* sp. *UBRU4* was tested for susceptibility to some antibiotics (inhibition zone in mm), Gram straining, productions of catalase, amylase and nitrate reductase, motility, formation of acid and gas from glucose, the ability to grow at different temperatures and salts; +was positive result (with activity) and -was negative result (no activity)

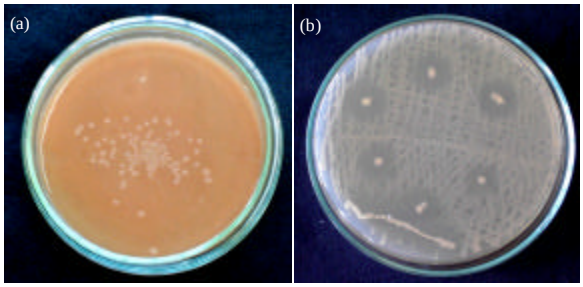

Fig. 1(a-b): *Bacillus* sp. UBRU4 colonies on Blood Agar (a) and inhibition zone of *Bacillus* sp. UBRU4 against the growth of *A. hydrophilla* by spot on lawn method (b)

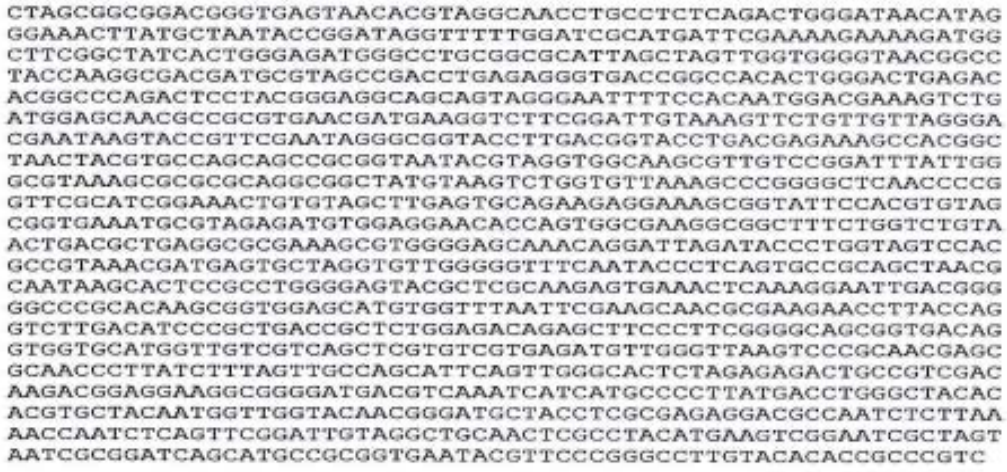

Fig. 2: Nucleotide sequence of *Bacillus* sp. UBRU4 by 16S rDNA sequence (full sequence; 1,400 base pairs)

productions from glucose positive, growth at 10 but not at 45°C, growth in medium with 4 salt but not at 6.5% salt, growth in medium with 0.2% bile salt. When comparing these results with those reported by Gordon (1989), it was found that *Bacillus* sp. UBRU4 was similarly to *Bacillus brevis*. This result was coincided with the result of molecular biology assays which indicated that the nucleotide sequencing (16S rDNA) of *Bacillus* sp. UBRU4 (Fig. 2) was similar to *B. brevis* (99.90% similarity).

Optimization of cultivation conditions and culture medium for *Bacillus* cell production: In order to obtain the high cell concentration, the effects of pH and temperature on *Bacillus* sp. UBRU4 growth were studied. It was observed that the highest growth of *Bacillus* sp. UBRU4 occurred at pH 6.5 and 37°C which the highest cell number was $3.2+0.13\times10^{9}$ CFU mL^{-1}. However, to facilitate the farmer to produce the probiotics by themselves, the modified low cost culture medium for *Bacillus* sp. UBRU4 cell production was examined. The selected carbon source used in this study was molasses which was a byproduct from sugar cane industry. The nitrogen source was the Nile tilapia commercial feed which was easily for the farmer to use. The results showed that the highest growth of *B. brevis* UBRU4 occurred at 3% commercial feed which the obtained cell numbers were $5.7\pm8.6\times0^{8}$ CFU mL^{-1}

Bioactive compound production of the selected *Bacillus* sp. probiotic: Cell growth, protein content and bioactive compound production of *Bacillus* sp. UBRU4 were showed in Fig. 3. It was found that the bioactive compound production of *Bacillus* sp. UBRU4 was growth associated. The activity of the bioactive compound of *Bacillus* sp. UBRU4 reached the maximum (1600 AU mL^{-1}) at 8 h of cultivation and stayed constant until 17 h of cultivation before declining at the stationary growth phase. Partial purification of bioactive compound by ammonium sulfate precipitation could increase the activity up to 6, 400 AU mL^{-1}, which the specific activity was increased from 1,298 to 5,807 AU mg^{-1}, as shown in Table 3.

Table 3: Activity and specific activity of the partial purified bioactive compound of *Bacillus* sp. UBRU4

Ammonium sulfate saturation (% w/v)	Activity (AU mL^{-1})	Volume (mL)	Protein content (mg mL^{-1})	Specific activity (AU mg^{-1})
0	800	500	0.616	1298.70
80	6,400	50	1.102	5807.62

DISCUSSION

Probiotics, live microorganisms which may serve as dietary supplements to enhance the growth and health of the host, have received some attentions in aquaculture as a means of diseases control, digestion aids, immune booster, supplementing or replacing the use of antimicrobial compounds (Irianto and Austin, 2002; EL-Haroun, 2007; Abdelhamid *et al*., 2009; Denev *et al*., 2009). The probiotic, in addition, may prevent or complete with the potential pathogens in colonizing the gut by production of antimicrobial compounds, or by out-competing them for nutrients or mucosal space (Robertson *et al*., 2000; Denev *et al*., 2009; Fernandez *et al*., 2011). Some of probiotics which are naturally occurring micro-organisms, are able to promote the growth and survival of aquaculture animals by inhibiting the activity of other bacteria that flourish in hatchery cultures. The *Bacillus* genus has not been

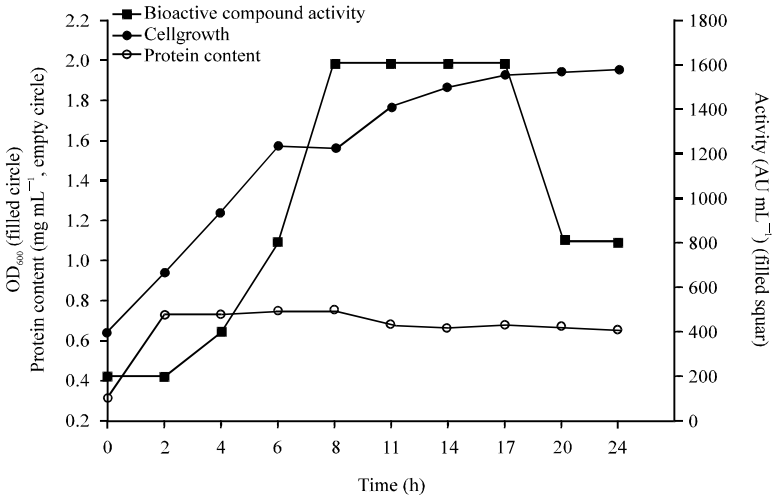

Fig. 3: Cell growth, protein content and bioactive compound activity during batch cultivation of *Bacillus* sp. UBRU4

reported as pathogens of the aquatic organisms (Moriarty, 1998). On the other hand, it is often antagonistic against some fresh water fish pathogenic bacteria (Vijayabaskar and Somasundaram, 2008). In addition, *Bacillus* species could be able to produce antibiotics, amino acids and enzymes (Sanders *et al*., 2003; EL-Haroun, 2007). Consequently, *Bacillus* probiotics may have positive nutritional effects on fish (Bagheri *et al*., 2008). Moreover, these *Bacillus* species could be able to survive through the pelletization process (Mehrim, 2009). Its application, thus, has been promoted and more widely accepted within the aquaculture industry (Gullian *et al*., 2004).

This present study, found that all isolates of bacilli from the gastro-intestinal tracts of Nile tilapia, except the *Bacillus* sp. UBRU4, showed hemolytic activity at days 7-14 of post-cultured on Blood agar medium. Not only exhibited the negative hemolytic activity, the *Bacillus* UBRU4 also showed the inhibitory activity against the growth of *A. hydrophila*. This result was agreed with Sanders *et al*. (2003) who reported that some of different antibiotics exhibiting antagonism against a broad spectrum of microbes have been identified from the *Bacillus* genus. Using hemolytic activity and antagonistic activity against the pathogenic growth in screening of the safe *Bacillus* probiotic in this study is one of a few reports at the present (Duc *et al*., 2004; Rahman *et al*., 2009; Thirabunyanon *et al*., 2009). This indicated that *Bacillus* sp. UBRU4 could possibly be safe for using as probiotic in Nile tilapia culture. Sanders *et al*. (2003) reported that normally *Bacillus* species were allochthonous microbes to the host intestinal tract and they were not normal colonizing inhabitants of the host intestinal tract. So in order to find the suspected probiotics, the screening of probiotic *Bacillus* from Nile tilapia's gut was spent a long time. This was corresponded to Al-Harbi and Uddin (2004) who reported that the intestine bacteria flora of hybrid tilapia (*O. niloticus*×*O. aures*) cultured in Saudi Arabia had some influences from the seasonal quantitative and qualitative and revealed that *Bacillus* spp. was present in some seasons of the year.

The physiological, biochemical and Molecular characters of the suspected probiotic bacterial which was *Bacillus* sp. UBRU4, was identified as *Bacillus brevis* by modifying the method of Gordon (1989) and 16S rDNA (Full sequence; 1,400 bp). Using *B. brevis* as probiotic in aquaculture in our study, if not the first, is possibly one of the pioneer works.

Clear zone colony of *Bacillus* sp. UBRU4 showed the antagonism against *A. hydrophila* and indicated the production of antimicrobial which was considered to be a pathogen-inhibiting mechanism. This result was agreed with Sanders *et al*. (2003) who reported that dozens of different peptide antibiotics exhibiting antagonism against a broad spectrum of microbes had been identified from the *Bacillus* genus. Berditsch *et al*. (2007) reported that the production of antibiotic substance was involved in destroying pathogens. It was consistent with the previous studies that *B. brevis* could produce antibiotics substances, gramicidin and tyrocidine and these two antibiotics were peptide antibiotics. Moreover, these peptides exerted a wide range of antimicrobial effects, including activity against gram positive and gram negative bacteria, viruses, fungi and single-cell pathogenic eukaryotes. This was similar to the report of

Robertson *et al*. (2000) that a strain of *Carnobacterium* sp., isolated from the intestine of Atlantic salmon, was evaluated for potential use as a probiotic for salmonids. *In vitro* studies of this *Carnobacterium* sp. demonstrated antagonism against *A. hydrophila, A. salmonicida, Flavobacterium psychrophilum, Photobacterium damselae* subsp. *Piscicida, Streptococcus miller, Vibrio anguillarum* and *V. ordalii.* The original *B. brevis* strain which could produce antibiotic (Gramicidin S) was isolated from soil and was described as *B. brevis* var. G. B. Initially, *B. brevis* var. G. B. was delivered to the National Centre of Type Cultures (London, United Kingdom), from which it was obtained by the American Type Culture Collection (ATCC, Manassas, VA) where it was deposited as *B. brevis* ATCC 9999 (Berditsch *et al*., 2007). *B. brevis* Nagano caused complete inhibition of *Botrytis cinerea* germination. This spore concentration contained Gramicidin S (Edwards and Seddon, 2001). *Bacillus* species have antagonistic effect on other microorganisms and antibiotics have been recognized as the only means of effective microbial growth control (Kuta *et al*., 2009).

Optimum pH and incubation temperature for *Bacillus* sp. UBRU4 growth on Tryptic soy broth were 6.5 and 37°C, respectively. The maximum cell growth in modified broth culture medium was obtained when 30 g L^{-1} of Nile tilapia commercial feed, without urea adding and 20 g L^{-1} of Molasses were used. Dechmahitkul *et al.* (2007) reported that the use of Molasses caused a foaming problem in the fermentor culturation of *Bacillus subtilis* and defatted soybean meal was suitable for substitution. The modified solid medium for *Bacillus* sp. UBRU4, Grinded soybean, was performed and Bacillus sp. UBRU4 showed the highest live cell at 48 h (3.6×10^{10} CFU g^{-1}) of accelerated storage condition (37°C) and then was decreased to 4.5×10^{2} CFU g^{-1} at 192 h. Because of the potential of antibiotic producing, *Bacillus* sp. UBRU4 has got Feed-back inhibition activity which was exhibited short shelf-life in accelerated storage condition in Grinded soybean modified medium. The optimum modified medium of *Bacillus* sp. UBRU4 should be solid media and kept under 4°C in order to maintain alive cell and reduce the effect of antibiotic activity from itself in the surrounding liquid part from the medium.

The production of bioactive compound which produced by the *Bacillus* sp. UBRU4 was found to be growth association which was similar to other bacteriocins (Mojgani and Amirnia, 2007; Kumari *et al*., 2008; Sharma *et al*., 2010). It was well established that Gramicidin and Tyrocidine were produced by *B. brevis* (Foster and Woodruff, 2010). Thus, the bioactive compound of *Bacillus* sp. UBRU4 could possibly be one or both of these bacteriocins. The decline of the activity during stationary growth phase was possibly due to the hydrolysis of the protease from the dead cell lysate. The precipitation of the bioactive compound by ammonium sulfate could increase the activity up to 4 folds. These confirmed that the bioactive compound produced by Bacillus sp. UBRU4 was a bioactive peptide or bacteriocin (Bali *et al*., 2011).

CONCLUSION

Bacillus UBRU4 was only one isolate of *Bacillus* probiotic specie which was isolated from gastrointestinal tract of Nile tilapia by hemolytic and antagonistic activities. The physiological and biochemical test and molecular identification results indicated that this *Bacillus* UBRU4 was similar to *B. brevis*. This is possibly the first report of *B. brevis* isolation from aquaculture. The modified broth medium contained 3% of Nile tilapia commercial feed and 2% of molasses was the cheap medium for cultivation of this isolated *Bacillus* sp. probiotics which the farmers could prepare easily by themselves. The produced bioactive compound or bacteriocins of this safe probiotic *Bacillus* UBRU4 exhibited the growth associated character. The *Bacillus* UBRU4, thus, could possibly be safe and used as high potential probiotic in Nile tilapia cultivation.

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