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Production and characterization of bacteriocin-like peptide produced by *Bacillus amyloliquefaciens* B10

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Anahtar Kelimeler: *Bacillus amyloliquefaciens* B10, bakteriyosin benzeri peptit, antimikrobiyal aktivite, kısmi saflaştırma

ÖZET

Topraktan izole edilen *Bacillus amyloliquefaciens* B10 morfolojik, biyokimyasal ve 16S rRNA sekans analizi sonuçlarına göre belirlenmiştir. *Bacillus amyloliquefaciens* B10'un ürettiği bakteriyosin benzeri peptit (BLP) *Bacillus cereus* ve *Listeria monoctogenesis* gibi patojenik Gram pozitif ve *Candida albicans* gibi fırsatçı patojenleri içeren mayalara karşı geniş antimikrobiyal aktivite gösterir. BLP katalaz hariç proteolitik proteinaz-K, lipaz, tripsin, α-amilaz enzimlerine hassastır (5 mg/ml). BLP aktivitesi 90°C'de 30dk. da stabil iken aktivite 90°C 2 saatde ve 121°C'de 20 dk. da kaybolmaktadır. BLP pH 3-9 arasında aktivite göstermesine rağmen, optimum aktivite pH 7'dir. BLP ethanol, hekzan, etil eter ve tween-80 gibi bazı organik çözücü ve deterjanlara karşı hassastır. Amonyum sülfat çöktürmesi (%70) ile yapılan kısmi saflaştırma sonucu BLP'nin yaklaşik 5 kDa ağırlığında olduğu tespit edilmiştir.

Production and characterization of bacteriocin-like peptide produced by *bacillus amyloliquefaciens* b10

Key Words: *Bacillus amyloliquefaciens* B10, bacteriocin like peptide, antimicrobial activity, partial purification

ABSTRACT

Bacillus amyloliquefaciens B10 that was isolated from soil was identified according to data of morphological, biochemical and 16S rRNA sequence analyses. The bacteriocin like peptide (BLP) produced by *B. amyloliquefaciens* B10 shows extendend antimicrobial activity against gram-positive bacteria including some pathogenic bacteria such as *B. cereus* and *L. monocytogenesis* and yeast including opportunistic pathogen such as *Candida albicans*. The BLP was sensitive to proteolytic enzyme proteinase K, lipase, tripsin, α-amylase except for catalase (5 mg/ml). While the activity of BLP of B13 was stable at 90°C for 30 min, the activity was completely lost at 90°C for 2 h and 121°C 20 min. Although activity of BLP of B10 was stable pH range 3-9, optimum pH was 7.0. It was sensitive against some organic solvent and detergent such as ethanol, hexane, ethyl ether and tween-80. Partial purification of BLP of B10 performed 70% ammonium sulphate precipitation was shown that it has approximately 5 kDa molecular weight.

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Introduction

Bacteriocins are ribosomally synthesized bacterial proteins or peptides. They are produced by different groups of bacteria, which inhibit strains and species that are usually, but not always, closely related to the producing bacteria (Cherif *et al.*, 2003). Several classification criteria have been used to group different antimicrobial compounds produced by gram-positive bacteria. Bacteriocin of *Bacillus* sp. are classified into three different groups by Abriouel *et al.,* (2011). Class I includes antimicrobial peptides that undergo different kinds of post-translational modifications. Class II bacteriocins include small proteins (0.77–10 kDa), ribosomally synthesized, nonmodified and linear peptides, which are heat and pH stable. Class III includes large proteins (430 kDa) with phospholipase activity such as megacins A-216 and A-19213 produced by *Bacillus megaterium* strains.

Naturally occurring antimicrobial compounds, such as bacteriocins, received increasing attention for food preservation due to consumers' demand for minimally processed foods, without chemical preservatives, still safe and able to maintain good shelf life. On the other hand, the emergence of bacterial resistance and multiple resistance to antibiotics represents a major public health problem and intensive efforts have been devoted to the development of alternative antimicrobial agents (Grosu-Tudor *et al*., 2014).

Production of antimicrobial peptides is widespread among diverse bacteria (Bizani *et al.,* 2005). Most known bacteriocin producing *Bacillus* strains are either from soil or food isolates such as Cerein 8A produced by *Bacillus cereus* 8A isolated from soil (Bizani and Brandelli, 2002) and bacteriocin produced by *Bacillus cereus* isolated from food (Naclerio *et al., 1*993). The aim of this study was to isolate novel *Bacillus* strains with strong antimicrobial activities from soil and characterize the antimicrobial substances for the possible applications in food industry and medicine.

Material methods

Isolation and identification of *Bacillus* strains

Bacillus strains in this study were isolated from soil in different area of Rize, Turkey. Morphological, biochemical, physiological tests and 16S rRNA sequence were used for identification fifteen isolates.

The genomic DNA's were isolated from strains with genomic DNA purification kit (Promega). The 16S rRNA gene was selectively amplified from purified genomic DNA by using primers of UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTCA-3') and UNI16S-R (5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3') (Weisburg *et al.,* 1991). PCR reactions contained 5 µL 10× Taq DNA polymerase reaction buffer, 1.5 µL 10 mmol/L dNTP mix, 1.5 µL 10 pmol each of the opposing primers, 1 µL 5 U/µL of Taq DNA polymerase(Fermentas), 3 µL MgCl2, 2 µL genomic DNA, and 34.5 µL dH2O. The PCR was performed under the following conditions: 2 min initial denaturation at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing(60 s at 55 °C), and extension (60 s at 72 °C); a final extension at 72 °C for 10 min. Finally, PCR products were analyzed by electrophoresis through 1 % agarose gels and then visualized under UV light by staining with ethidium bromide. Approximately 1400 bp PCR product was cloned to pGEM-T Easy (Promega) vector system and then the right products were sent to Macrogen (The Netherlands) for sequencing. The obtained sequences were analyzed by BLAST searches using the NCBI GenBank database (Altschul *et al.,* 1990; Benson *et al.,* 2002). Finally, the sequences were used to construct a phylogenetic tree to verify isolates identification.

Bacteriocin activity

Fifteen isolates were screened to produce bacteriocin like antimicrobial activity by agar spot test (Cherif *et al.,* 2001). The test was performed as follows: 200 µl of each *Bacillus* cultures at early exponential growth phase (OD 600 of 0.2-0.3) in Mueller Hinton Broth was mixed with 4 ml of Mueller Hinton soft (0.6%) agar and poured on the Mueller Hinton agar plate. Then, the 5 µl of each cell free supernatant (CFS) was dropped onto the solidified soft agar. The plates were incubated at 35ºC overnight. Inhibition was indicated by a clear zone in the soft agar layer.

Determination of bacteriocin activity in different media

B. amyloliquefaciens B10 was growth in different media as Brain Heart Infusion Broth (BHI), Mueller Hinton Broth (MHB), Nutrient Broth (NB) and Triptic Soy Broth (TSB) at 35°C for different time (8, 16, 24, 32 hours). During the incubation period, 1 ml culture samples were taken at the specified time. The cells were removed by centrifugation (8000Xg for 10 min.) and cell-free supernatant (CFS) was obtained. CFS's were used to determine the best condition for production of antimicrobial activity by using the agar-well diffusion method (Izquerdo *et al.,* 2008). The amount of indicator isolate B13 was adjusted to 0.5 MacFarland and this suspension was spread on MHB agar and allowed to dry. A cork borer was used to open well of 5mm diameter on the agar plates. The wells were filled with 50 µl of cell-free supernatant of *B. amyloliquefaciens* B10. The inhibition zones were measured after incubation at 35°C for overnight and the best condition was determined according to inhibition zone.

Antimicrobial spectrum of BLP of B10

The antimicrobial activity of BLP of B10 was tested against several Gram-positive, Gram-negative and yeast organism using the agar well diffusion method as described above. All microorganism were obtained from the Refik Saydam Hygiene Institute (Ankara, Turkey) and were as follows: *E. coli* ATCC25922, *Y. pseudotuberculosis* ATCC911, *P. aeruginosa* ATCC43288, *S. aureus* ATCC25923, *E. faecalis* ATCC29212, *B. cereus* 709 Roma, *Klebsiella pneumonia* ATCC 13883, *Listeria monocytogenesis* ATCC 43251, *C. albicans* ATCC60193, *C. tropicalis* ATCC 13803 and *S. cerevisiae* RSKK 251. While the bacterial cultures were grown in MHB at 37°C, yeast cultures were grown in PDA at 30°C.

Effect of enzyme, heat, pH, organic solvents and detergents

To determine the effect of enzymes on the antimicrobial activity of B10, B10 strain was grown in MHB at 35°C overnight. After incubation, CFS was obtained by centrifugation (8000Xg for 10 min.).

Table I. Morphological, biochemical and physiological characterization of *Bacillus* strains*

	***Bacillus* strains**														
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**
Colonie color	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Colonie shape	R	R,M	R	R	R	R,M	R,M	R	R,M	R,M	R	R	R	R	R
Gram strain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore strain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape of spore	Cen.	Cen.	Cen.	Cen.	Cen.	Cen.	Cen.	Ter.	Ter.	Cen.	Ter.	Cen.	Ter.	Cen.	Ter.
Motility	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Methyl red test	+	-	+	-	-	-	-	+	-	-	+	+	+	+	+
Voges Proskauer	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Kligler iron agar	A	Bc	A	Bc	Bc	Bc	A	A	Bc	Bc	A	A	A	Bc	A
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of urea	+	-	+	-	-	-	-	+	-	-	+	+	+	+	+
Gelatin hydrolysis	-	+	+	-	-	-	+	+	-	-	+	+	-	+	+
Coagulase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lesitinase	-	+	-	-	-	+	-	-	+	-	+	+	+	-	+
pH															
3,0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4,0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5,0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6,0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7,0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8,0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9,0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10,0	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
11,0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12,0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NaCl (%)															
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	W	-	+	-	W	-	+	-	W	-	-	-	-	-
12	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature (°C)															
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
55	+	+	-	+	+	-	+	+	+	-	+	+	+	-	+

* C, Cream; R, Rough; M, Mucoid; Cen., Central; Ter., Terminal; A, Acid; Bc, Basic; W, Weak positive

CFS was treated for 1 hour with several enzymes (proteinase K, lipase, α-amylase, trypsin, catalase) at a final concentration of 0.5 mg/ml and 5 mg/ml. Inactivation of this enzymes were performed by incubating at 95°C for 5 min. The effects of these enzymes were tested using B13 strain as indicator organism by agar-well diffusion method.

To determine the heat stability of BLP of B10, CFS was incubated at 30°C, 45°C, 60°C, 90°C for 30 min. and 2 h. and 121°C for 20 min. After cooling to room temperature, the effects of heats were tested using B13 strain as indicator organism.

Effect of pH on antimicrobial activity of B10 was determined chancing pH of CFS's. The pHs of CFS's were adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 with HCl and NaOH. Then, all samples were incubated at room temperature for 2 hours. After incubation, pH of CFS's was adjusted again to 7.0 and tested using B13 strains as indicator organism.

To determine the effect of organic solvents and detergents on the antimicrobial activity of B10, ethanol, hexane, ethyl ether, formaldehyde, chloroform, acetone, propanol, methanol, tween-80 and triton X-100 were used. B10 strain was grown in MHB at 35°C overnight, after incubation CFS was obtained by centrifugation (8000Xg for 10 min.). CFS was treated for 1 hour with organic solvents and detergents at a final concentration of 10% (v/v). The effects of organic solvents and detergents were tested using B13 strain as indicator organism by agar-well diffusion method. As positive control, organic solvents and detergents without CFS was used. All the experiments were done in duplicated.

Growth and bacteriocin like peptide production

Bacillus amyloliquefaciens B10 was inoculated (1/100 v/v) in 500 ml nephelo-erlenmayer containing 200 ml MHB and incubated on a shaker at 35°C for 50 hours. Every 30 min, optical density of culture was measured spectrophotometrically (A600) and 1 ml culture sample was aseptically removed. The culture supernatant was obtained by centrifugation and tested against indicator strain *Bacillus cereus* B13 by the agar well diffusion method.

Partial purification of BLP of B10

Bacillus amyloliquefaciens B10 strain was incubated at 35°C overnight in 1 lt erlenmayer containing 300 ml MHB. Cells were harvested by centrifugation at 8000 rpm for 30 min at 4°C. Supernatant was collected and precipitated with ammonium sulfate at 70% saturation for overnight. The precipitate was collected by centrifugation 9000 rpm for 30 min at 4°C. Pellet was resuspended in 20 mM Tris-HCl buffer (pH 7.5) and dialysed against 2 lt 20 mM Tris-HCl buffer (pH 7.5) for 20 hours in dialysis tubing.

SDS-PAGE

SDS-PAGE analysis was performed by Laemmli, (1970) procedure. To detection of molecular weight and direct activity, BLP of B10 was load SDS-PAGE as two copies. After electrophoresis, the gel was pieced two part, one part was stained by Coommasie Brillant Blue R-250 for detection molecular weight. Another part was washed sterile distile water for 20 min. And then, the gel was load on MHA and covered with soft agar (% 0.5) including indicator strain B13. After incubation for 35°C at overnight, inhibition zone was compared with stained proteins bands and molecular weight of B10 BLP was determined.

Table II. Antimicrobial activity spectrum of the BLP of *B. amyloliquefaciens* B10.

Indicator organisms	Inhibition diameter (mm)	Reference
Gram negative bacteria		
E.coli ATCC 25922	0	RSHI
Y. pseudotuberkulosis ATCC 709	0	RSHI
P. auroginosa ATCC 43288	0	RSHI
K. pneumonia ATCC 13883	0	RSHI
Grampositive bacteria		
Bacillus cereus Roma 709	20	RSHI
S. aureus ATCC *25923*	15	RSHI
E. fecalis ATCC 29212	17	RSHI
L. monocytogenesis ATCC 43251	9	RSHI
Bacillus pumilus B1	11	This study
B. subtilis B2	8	This study
Bacillus sp. B3	10	This study
B. subtilis B4	7	This study
B. subtilis B5	7	This study
Bacillus sp. B6	-	This study
B. subtilis B7	18	This study
B. mojavensis B8	10	This study
Bacillus sp. B9	9	This study
B. thuringiensis B11	10	This study
B. thuringiensis B12	15	This study
B. cereus B13	21	This study
B. thuringiensis B14	14	This study
Bacillus sp. B15	5	This study
Yeast		
C. tropicalis ATCC 13803	13	RSHI
C. albicans ATCC 60193	13	RSHI
S. cerevisia ATCC 96581	0	RSHI

RSHI: Refik Saydam Hygiene Institute

Results and Discusion

Isolation and identification of BLP-Production *Bacillus* strains

Bacillus genus is gram positive, aerobic, and spore-forming bacteria. This genus is characterized by their rod-shape cell morphology, catalase production and ubiquitous distribution. They are found in environments such as soil and clays, rocks, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals (Nicholson, 2002). Fifteen *Bacillus* isolates were isolated from soil in Rize, Turkey. Strains were identified using morphological, biochemical tests and 16S rRNA sequences analyses. The results of morphological, biochemical and physiological test were given in Table I.

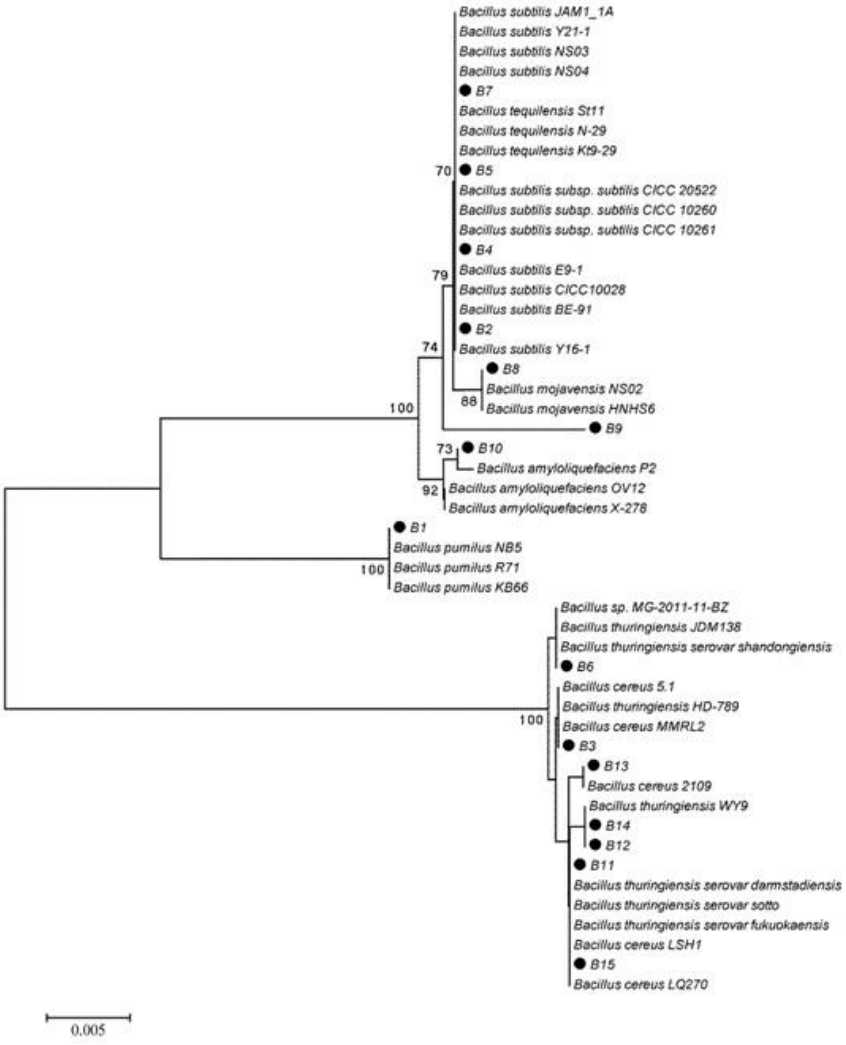

Fig. 1. Neighbor-joining analysis with the p-distance method was used to construct the dendrogram. Bootstrap values shown next to nodes are based on 1.000 replicates using MEGA 5.0. Bootstrap values of C≥70% are labeled. *Bacillus* isolates from soils in Rize, Turkey were indicated by the black dot. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

An approximately 1,400-bp fragment of the 16S rRNA gene region was also sequenced for further characterization of the isolates. *Bacillus pumilis* (B1), *Bacillus subtilis* (B2, B4, B5, B7), *Bacillus mojavensis* (B8), *Bacillus cereus* (B13), *Bacillus amyloliquefaciens* (B10), *Bacillus thuringiensis* (B11, B12, B14) and *Bacillus* sp. (B3, B6, B9, B15) are identified stains (Table II). This identification was also supported by the phylogenetic analysis (Fig. 1).

All *Bacillus* strains were screened for their antibacterial activity, but only one strain *Bacillus amyloliquefaciens* B10 was selected as the good candidate for bacreriocin like peptide producer. *Bacillus amyloliquefaciens* B10 strain that showing high antimicrobial activity as it inhibited the growth of 13 (92 %) of tested strains (Table II). Cell free supernatant of the B10 showed the antagonistic activity to all *Bacillus* strains except for *Bacillus* sp. B6. *Bacillus cereus* B13 was chosen indicator organism because of it was most sensitive. Members of *Bacillus* genus are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics and bacteriocins. The production of antimicrobial substances and sporulation capacity are an advantage for keep life in different habitats (Abriouel *et al.*, 2011).

The best antimicrobial activity was determined in MHB medium for 24 h (Table III).Antimicrobial activity was very low in other medium. Different media such as BHI, LB, TSB, MHB were used in *Bacillus* species to produce bacteriocin (He at al., 2006; Lucas *et al.*, 2006). The best bacteriocin activity was determined different medium in studies (Lisboa *et al.*, 2006; Benitez *et al.*, 2011).

Antimicrobial activity spectrum

BLP of B10 was tested for determinate antimicrobial activity on several gram positive and negative bacteria and yeast. The results were shown in Table II. Antimicrobial activity was observed towards several gram positive bacteria (including pathogenic bacteria such as *B. cereus* and *L. monocytogenesis*) and yeast (including opportunistic pathogenic yeast such as *Candida albicans*). Any inhibition activity was not observed on gram negative bacteria.

Bacteriocins produced by gram-positive bacteria in generally effects gram positive organism. They tend to be active against a wide range of gram positive bacteria. The outer membrane of gram-negative bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents, and dyes from reaching the cytoplasmic membrane (Stevens *et al*., 1991). But some bacteriocins have been reported to inhibit gram negative microorganisms (Cherif *et al.*, 2003; Lisboa *et al.*, 2006; Xie *et al.*, 2009; Ayed et al., 2014).

Table III. The effects of enzymes, heat, pH, different medium and organic solvents/detergents on antimicrobial activity of bacteriocin like peptide of *B. amyloliquefaciens* B10.

Treatment	Resudial activity (%)*	
Enzymes	0.5 mg/mL	5 mg/mL
Proteinase K	57	-
Trypsine	57	-
α-amylase	43	-
Lipase	57	-
Catalase	97	95
Heat	30 min	2 h
30°C	100	95
45°C	85	71
60°C	85	47
90°C	76	-
120°C(20 min)	-	

Medium**	16h	24h	32h	48h
BHI	24	24	10	-
MHB	67	100	86	72
TSB	-	19	10	-
NB	10	24	14	10

Organic Solvents (%10)	
Ethanol	64
Hexane	64
Ethyl ether	48
Detergents	
Tween-80	91
Triton X-100	100
Ethyl ether	48
pH	
3	24
4	48
5	57
6	86
7	100
8	87
9	24
10	-
11	-

* Residual activity was measured by comparing the antimicrobial activity before the treatment.

** BHI, Brain Heart Infusion Broth; MHB, Mueller Hinton Broth; TSB, Triptic Soy Broth; NB, Nutrient Broth

Effects of enzyme, pH, heat, detergents and organic solvents on antimicrobial activity

Effects of enzyme, pH, heat, detergents and organic solvents on antimicrobial activity of BLP of B10 was evaluated by measuring residual activity against *Bacillus cereus* B13 strain using agar well diffusion method. Proteolytic enzyme proteinase K, tripsin, lipase, α-amylase and catalase were used for determination effects of enzymes on antimicrobial activity. After treatment with proteolytic enzymes that are concentration 0.5 mg/ml, the inhibitory activities was shown a significantly decrease. When the enzymes concentration was 5 mg/ml, antimicrobial activity was disappeared completely except for catalase enzyme (Table III). This result is closely indicated that BLP of *B. amyloliquefaciens* B10 is protein nature because of inactivated after proteinase K and tripsin treatment. The same results were given a lot of study (Lisboa *et al.*, 2006; Kamoun *et al.*, 2011; Kindoli *et al.*, 2012;). The antimicrobial activity of B10 was disappeared after lipase and α-amylase treatment. Based on this result, it is possible to say that the structure of BLP of B10 might contain lipidic or glicosidic structure. At the same time, catalase was not affected antimicrobial activity of BLP of B10, which indicates that antimicrobial activity was not due to H_2O_2. The same results were given for some bacteriocin such as Bacthuricin F103 (Kamoun *et al.*, 2011).

The results of heat effect on antimicrobial activity have shown that BLP of B10 was comparatively stable to heat treatment. While activity was maintained during treatment up to 90°C for 30 min, disappeared at 90°C for 2 hours. At the same time, activity was not maintained at 120°C for 20 min. BLP of B10 was also pH stable in the range of 3.0- 9.0. Lisboa *et al.*, (2006) reported the heat stable BLP of *B. amyloliquefaciens* LBM 5006. BLP of LBM 5006 is stable pH between 3.0 -8.0 (optimum pH is 4.0), like BLP of B10 is stable 3- 8 but optimum pH is 7.0.

Organic solvents (ethanol, methanol, acetone, chloroform, ethyl ether, formaldehyde, propanol) and detergents (Tween-80 and Triton X-100) were used at working concentrations of 10% (v/v). It was shown that inhibitory activity of B10 lost after ethanol, hexane and ethyl ether treatment, activity was not affected by treatment with other organic solvents and detergents used in study. The results are similar to many studies (Naclerio *et al*., 1993; Bizani and Brandalli, 2002; Ayed *et al*., 2014).

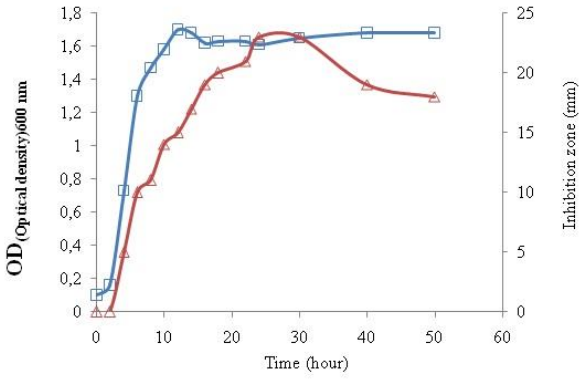

Fig. 2. Growth curve (square symbols) and the antimicrobial activity (triangle symbol) of *Bacillus amyloliquefaciens* B10 during cultivation at 35°C in Mueller Hinton Broth.

Production of Bacteriocin B10

During *B. amyloliquefaciens* B10 growth, production of the bacteriocin like peptide was evaluated. Samples were taken at different times and activity was measured by using the agar well diffusion method. The BLP activity of B10 could not be detected during the early logarithmic growth phase, but was suddenly detected at the late logarithmic growth phase (Fig. 2). It is generally recognized that many *Bacillus* sp. produce various antimicrobial peptides as secondary metabolites. Members of the genus *Bacillus* have been reported to produce antimicrobial peptides in the late logarithmic or early stationary phase of growth in batch cultures (Slepecky and Hemphill, 2006; Stein, 2005).

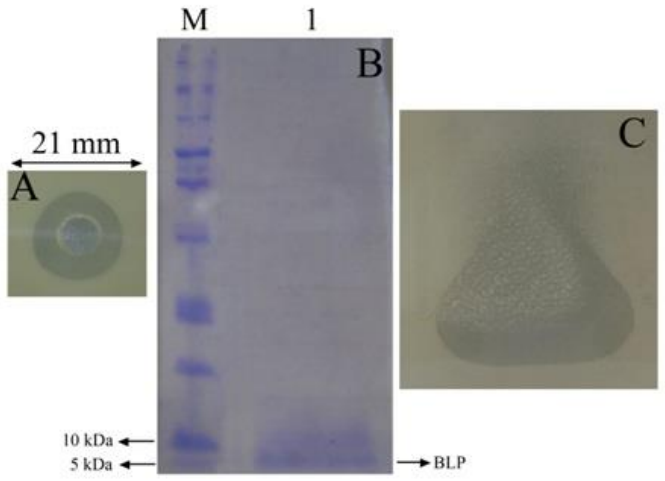

Fig. 3. Antimicrobial activity of partial purification of BLP on the agar and the gel. (A) The bacteriocin like peptide activity of *B. amyloliquefaciens* B10 after 70% ammonium sulphate precipitation on the agar (B) SDS-PAGE analysis of the BLP, 1 Molecular weight marker (Promega), 2 Partial purified BLP (C) Direct detection of the BLP. Part of the gel overlaid by the soft agar with indicator strain *B. cereus* B13.

Partial Purification and Activity on Gel

BLP of B10 was purified partially by 70% ammonium sulphate precipitation. After precipitation B10 precipitant was dissolved in 20 mM Tris-HCl buffer (pH 7.0) and presence of antimicrobial activity was tested by agar well diffusion method (Fig 3A). After electrophoresis, the gel was cut into two parts. One part of the gel was stained with Comassie Brillant Blue R-250 and other part was used to determine activity. A result of the test has shown that approximately 5 kDa weight protein, estimated by calculating the different *rf* (rela tive migration) values of standard proteins, shows bacteriocin activity (Fig 3B). Also, the gel results clearly showed the bacteriocin activity (Fig. 3C). The low molecular masses have been reported for several Bacilli bacteriocins, such as 4 kDa bacteriocin RS108 (Sevim et al., 2013) and 3.2 kDa licheniocin 50.2 (Beric et al., 2014).

In terms of many feature of *Bacillus amyliloquefaciens* is similar to *Bacillus subtilis* (Priest *et al.*, 1987). A many bacteriocin produced by *Bacillus subtilis* were studied and identified such as Subtilin (Banerje and Hansen, 1988), Subtilosin A (Zheng *et al.*, 1999). However, there are few studies for *Bacillus amyloliquefaciens* (Hiradate *et al.*, 2002; Lisboa *et al.*, 2006). Bacteriocin like peptide produced by *Bacillus amyloliquefaciens* B10 which isolated from soil was studied in this study. The results in this study indicated bacteriocin like peptide produced by B10 used for medicine, food safety, biological control of pathogenic and spoilage microorganisms.

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