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ORIGINAL ARTICLE

Chromosomal genes-mediated inhibition of intestinal and foodborne pathogens by *Lactobacillus acidophilus* AA11

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ABSTRACT. Approximately 63 strains of *Lactobacillus acidophilus* were isolated from Egyptian home-made cheese and examined for production of antagonism. Only eight strains demonstrated inhibitory activity against spoilage microorganisms (i.e. *Staphylococcus aureus* and *Bacillus cereus*) and pathogens (i.e. *E. coli*, *Salmonella* sp. and *Shigella* sp.). *Lactobacillus acidophilus* AA11 produced a more antimicrobial activity with a wide range of inhibition. The agent AA11 was sensitive to proteolytic enzymes and retained full activity after 30 min at 100 ºC. Activity against sensitive cells was bactericidal but not bacteriolytic. The compound was produced during growth phase and can be extracted from the culture supernatant fluids with n-Butanol. 12 % SDS-PAGE analysis of 40% ammonium sulphate precipitated agent showed two peptides with molecular weights of ~36 kDa and ~29 kDa. No plasmid was identified in *Lactobacillus acidophilus* AA11 indicating that the genes encoding the inhibitory agent located on the chromosome. These characteristics identify the inhibitory substance as a bacteriocin, designated acidocin AA11 and confer the agent an application potential as a biopreservative.

Key words: *Lactobacillus acidophilus*, acidocin AA11, plasmid, SDS-PAGE.

RESUMEN. Se aislaron aproximadamente 63 cepas de *Lactobacillus acidophilus* de queso egipcio elaborado en casa y se examinó la producción de antagonismo. Sólo ocho cepas demostraron actividad inhibitoria contra microorganismos que echan a perder el queso (i.e. *Staphylococcus aureus* y *Bacillus cereus*) y patógenos (i.e. *E. coli*, *Salmonella* sp. y *Shigella* sp.). *Lactobacillus acidophilus* AA11 produjo una mayor actividad antimicrobiana con un mayor rango de inhibición. El agente AA11 fue sensible a enzimas proteolíticas y retuvo actividad completa después de 30 min a 100º C. La actividad contra células sensibles fue bactericida pero no bacteriolítica. El compuesto se produjo durante la fase de crecimiento y puede ser extraído de cultivo de fluidos del sobrenadante con n-Butanol. Análisis con 12% SDS-PAGE del agente precipitado en sulfato de amonio al 40% mostró dos péptidos con pesos moleculares de ~36 kDa y ~29 kDa. No se identificó ningún plásmido en *Lactobacillus acidophilus* AA11 indicando que los genes que codifican al agente inhibidor están localizados en el cromosoma. Esas características identifican a la sustancia inhibitoria como un bacteriocín, designado acidocin AA11 y confieren al agente una aplicación potencial como un biopreservador.

Palabras clave: *Lactobacillus acidophilus*, acidocin AA11, plásmido, SDS-PAGE.

INTRODUCTION

Lactobacilli play an important role in suppressing undesirable intestinal microflora.[27] Organic acids and hydrogen peroxide produced by *Lactobacillus acidophilus* have demonstrated broad-spectrum inhibition.[15,44] Previous studies suggested that bacteriocins either mediate or facilitate inhibitory activity produced by *L. acidophilus*.[15-17,28,37,47] Crude lactocidin produced by *L. acidophilus* demonstrated antimicrobial effect against *Proteus*, *Salmonella*, *Escherichia*, *Staphylococcus*, *Bacillus*, *Streptococcus*, and *Lactobacillus*.[47] Criteria for bacteriocin identification include a little potential for broad-spectrum inhibition restricted to closely related species, a bactericidal mode of action, and a proteinaceous nature.[39] Previous studies reported that bacteriocins produced by lactobacilli showed antimicrobial activities restricted to the *Lactobacilliaceae*[6,8,19,45,46] although a broader range of inhibition has been observed for a number of bacteriocins produced by gram-positive bacteria. Hurst[18] and Klaenhammer[23] reported that inhibitory activity by *L. acidophilus* does not specifically confirm the involvement of bacteriocins. Production of bacteriocin by *L. acidophilus* and *L. casei* has been reported.[7,21] Bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria.[14,25] It may contribute to the colonization resistance of the host and its protection against gastrointestinal pathogens.[5,34] The bacteriocin produced by *L. acidophilus* IBB 801 isolated from dairy products displays antibacterial activity against *E. coli* and *Salmonella* suggesting that it may have potential as a probiotic.[48] Bacteriocin may facilitate the establishment of probiotic strains in the competitive environment of the gut;[25] however, the importance of bacteriocin production *in vivo* remains under discussion.[32] Finally, bacteriocins produced by probiotic lactic acid bacteria may contribute to an increased stability of the food product during its storage and shelf-life.[22,31,35] As increasing demand to decrease the use of chemical addi-

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tives in food and for more natural and microbiologically safe food products, bacteriocins may have considerable potential for food preservation.[1] Therefore, this study was purposed to screen a number of lactic acid bacteria in an attempt to discover bacteriocins with wide range of inhibition spectra. In this study 63 strains of *L. acidophilus* were examined for antigonism of gram-positive and gram-negative bacteria under conditions eliminating inhibition by hydrogen peroxide and organic acids. Spoilage microorganisms (i.e. *Staphylococcus aureus* and *Bacillus cereus*) and pathogens (i.e. *E. coli*, *Salmonella* sp. and *Shigella* sp.) were sensitive to *L. acidophilus*. The inhibitory compound, designated acidocin AA11, was identified as a bacteriocin.

MATERIAL AND METHODS

Bacterial cultures and cultivation conditions

Lactobacillus acidophilus strains were isolated from different sources of Egyptian home-made cheese under anaerobic conditions (BBL Gas-pak system) using MRS agar (Oxoid)[10] at 37 ºC. The identification of the strains was performed according to their morphological, cultural, physiological and biochemical characteristics by the procedures described in the Bergey's Manual.[4] The indicator strains (Table 1) were grown in LB medium (1% tryptone, 1% yeast extract, 0.5% NaCl) and on LA agar (LB with 1.5% agar).[36] The *Lactobacillus acidophilus* strains were subcultured and maintained by bi-weekly transfer in MRS agar.

Detection of antimicrobial activity

An agar spot test was preliminary used for this purpose.[13] Overnight cultures of the *Lactobacillus acidophilus* strains were spot-inoculated on the surface of MRS agar plates and incubated, under anaerobic conditions (BBL Gas-pak system), overnight at 37 ºC to allow colonies to grow. Ten ml of LA soft agar (LB with 0.75% agar) inoculated with 75 µl of the organism (grown overnight at 37 ºC in LA) to be tested for sensitivity was poured on the plates. After incubation, a clear zone around the colonies was scored as a positive inhibition.

Preparation of culture supernatant

The bacteriocin producing strains were grown in MRS broth under anaerobic conditions (BBL Gas-pak system) at 37 ºC for 48 hours. The supernatant fluids were separated from the cells by centrifugation at 2,555 Xg for 20 min. The pelletted cells were kept at 4 ºC for plasmid and protein examination. The supernatant was adjusted to pH 6.5 with 1 M NaOH and treated with catalase (5 mg/ml final concentration) to eliminate antagonism by organic acid and hydrogen peroxide respectively. The supernatant fluid was concentrated using a disposable ultrafiltration device, Vivaspin (Sartorius AG).

Antimicrobial activity assay

Well diffusion agar was used to demonstrate antimicrobial activity.[38] Fifty µl of overnight culture of each target

Table 1. Inhibition of different target organisms by culture supernatant fluids of *Lactobacillus acidophilus* strains according to agar well diffusion assay.

Target organisms	Inhibition by supernatant from *Lactobacillus* strains							
(source, Lab stock)	AA06	AA11	AA15	AA36	AA41	AA52	AA55	AA64
Gram-negative bacteria								
Escherichia coli	++	++	++	++	++	++	++	++
Salmonella paratyphi	+	++	++	+	+	+	+	+
Shigella dysenteriae	+	++	++	++	++	++	++	+
Shigella sonnei	++	++	++	++	++	++	+	+
Shigella sp.	+	++	-	-	+	-	+	++
Gram-positive bacteria								
Staphylococcus aureus	++	++	+	++	+	++	+	+
Staphylococcus epidermidis	+	++	+	-	+	+	+	+
Bacillus cereus	+	++	+	+	+	+	+	+
Bacillus subtilis	+	++	+	+	+	+	+	+
Micrococcus luteus	+	+	+	+	+	++	+	+
Micrococcus roseus	+	++	+	++	+	+	+	+

The data represent the diameter of inhibition zone (mm) obtained by agar well diffusion assay. ++, large inhibition zone (> 10 mm); +, small inhibition zone (5-10 mm); -, no inhibition zone; diameter of well is 5 mm.

Rev Latinoam Microbiol 2006; 48 (1): 24-30

strain (Table 1) was distributed on LA plates. Fifty µl of pretreated supernatant fluids of the *Lactobacillus acidophilus* strains were put in each 5 mm wells formed in LA plates. The plates were left at 4 ºC for 2 hours for supernatant diffusion and then incubated aerobically at 37 ºC overnight. Finally, the diameter of the inhibition zone was determined. Antimicrobial activities were assayed in duplicate.

Mode of action

To investigate whether the antimicrobial compound acts as a bactericidal or bacteriolytic peptide, the 20% (v/v) of the concentrated *L. acidophilus* AA11 supernatant were added to a mid-log phase of *E. coli* growing in LB at 37 ºC. At appropriate intervals during incubation, samples were taken and the optical densities at 600 nm and the number of cfu/ml on LA were determined.

Heat and proteases sensitivity

Heat stability was determined by holding the *L. acidophilus* AA11 supernatant at 37 ºC, 60 ºC, 100 ºC for 30 min and 120 ºC for 15 min, and assayed for the residual activity using well diffusion assay. Sensitivity to proteolytic enzymes was achieved by treatment the *L. acidophilus* AA11 supernatant with 0.5 mg/ml of lysozyme, catalase, papain, trypsin and pepsin and incubated at 37 ºC for 2 hrs. The enzymes were deactivated at 70 ºC for 20 min. The remaining antimicrobial activity was assayed

Stability of crude acidocin AA11 during storage

The supernatant fluids were stored at different temperatures, 37 ºC, 4 ºC and -20 ºC. At different time intervals samples were assayed for the antimicrobial activity of acidocin AA11 using well diffusion assay.

Acidocin AA11 production

To study the production of acidocin AA11 *vs* time during growth, *Lactobacillus acidophilus* AA11 was grown under anaerobic conditions (BBL Gas-pak system) in MRS broth at 37 ºC. At interval times, optical densities were measured at 600 nm and cell-free supernatant fluids were prepared to test inhibitory activity against *E. coli*.

Extraction of acidocin AA11 with organic solvents

Supernatant fluids were mixed thoroughly with different organic solvents such as n-butanol, n-hexane, i-amylalcohol, chloroform and di-ethyl ether by ratio 1:1. The mixtures were centrifuged at 2,555 X g for 10 min to achieve phase separation. The organic phase and the aqueous phase were collected. The solvent removed from the organic phase by evaporation at 45ºC. The residue from the organic phase was resuspended in an amount of saline (0.85 % NaCl) equal to the starting volume of the original supernatant fluid.[41]

Isolation of plasmid DNA

Plasmid DNA was isolated from *Lactobacillus acidophilus* AA11 using the method described by Sambrook et al.[36] of alkaline lysis with 10 mg/ml lysozyme. 1% agarose gel electrophoresis was used to detect the presence of plasmids.

SDS-PAGE analysis

The supernatant fluids were precipitated with 40 % ammonium sulphate and subjected to SDS-PAGE (12%) according to the method of Laemmli.[26] The gel was stained with Coomassie blue (Sigma) after electrophoresis at 30 mA for 45 min. The protein markers and their molecular weights were Dalton Marker Vll-LTM standard mixtures 14,000-66,000 (Sigma) which include BSA (66 kDa), Ovalbmin (45 kDa), Glyceraldehyde-3-phosphate dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa), Trypsinogen PMSF treated (24 kDa), Trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.2 kDa).

RESULTS AND DISCUSSION

Inhibitory spectra

Sixty eight Strains of *Lactobacillus acidophilus* were isolated from Egyptian home-made cheese. Extracts from these strains were examined for inhibition of the indicator strains listed in Table 1. A total of eight strains (AA06, AA11, AA36, AA41, AA52, AA55, AA64) displayed a wide range effect of antimicrobial activity toward Gram-positive and Gram-negative bacteria. Gram-positive foodborne pathogens such as *Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus* were strongly inhibited by the antimicrobial agent AA11. Interestingly, the inhibitory compound AA11 strongly inhibited several gram-negative bacteria including foodborne pathogens such as *Salmonella paratyphi, Shigella sonnei, Shigella dysenteriae, Escherichia coli* and *Shigella* sp. Because of the interesting inhibition spectrum of AA11 it was studied in more details. Various bacteriocins produced by *Lactobacillus acidophilus* strains have been described before, such as lactacin B,[2,3] lactacin F,[29,30] aci-

Rev Latinoam Microbiol 2006; 48 (1): 24-30

dophilucin A,[42] acidocin 8912,[20,40] acidocin B[41] and acidocin D20079.[12] However, a broad spectrum inhibition of acidocin AA11 against foodborne and intestinal pathogens has not been reported for of any these bacteriocins, and their inhibitory effect appears to be limited to some (often closely related) lactic acid bacteria.

Characteristics of acidocin AA11

As shown in Table 2, the antagonism produced by *Lactobacillus acidophilus* AA11 was resistant to lysozyme and catalase but was completely absent after treatment with pepsin, trypsin or papain. Acidocin AA11 is moderately heat-stable, all antimicrobial activity was retained after incubation at 100 ºC for 30 min while heat sterilization by autoclaving (121 ºC for 15 min) led to a loss of activity. The temperature stability of acidocin AA11 differs from that of the above mentioned bacteriocins, acidophilucin A and acidocin B (80 ºC for 20 min)[41] are less heat-stable, whereas lactacins B (121 ^{0}C for 3 min)[2] and F (121 ºC for 15 min) are more heat-stable.[30] The antimicrobial compound of *Lactobacillus acidophilus* AA11 could be stored at 4 ºC or – 20 ºC for at least 100 days without loss of its activity (Fig. 1). However, storage at 37 ºC resulted in a loss of activity possibly attributed to the action of proteolytic enzymes which might be present in the supernatant fluids. The antimicrobial agent was produced continuously during growth phase as demonstrated in Fig. 2. However, the level of inhibition reached a maximum at the beginning of mid-log phase and remained constant at stationary phase. These results showed that the agent AA11 is heat-stable and strongly sensitive toward proteolytic enzymes. On the basis of the above mentioned results, the antimicrobial agent produced by *Lactobacillus acidophilus* AA11 was classified as a bacteriocin which was designated acidocin AA11.

Table 2. Effect of different heat and enzymatic treatments on the activity of crude Acidocin AA11. *E. coli* was used as an indicator strain in well diffusion assay.

Treatments	Diameter (mm) of Inhibition zone
Temperature (for 30 min)	
Control (4 ºC)	15
37 ºC	15
60 ºC	15
100 ºC	15
120 ºC (autoclaving for 15 min)	9
Enzymes	
Control (without treatment)	15
Lysozyme	15
Catalase	15
Pepsin	-*
Trypsin	-
Papain	-

* No inhibition zone, diameter of well is 5 mm.

Bactericidal action of acidocin AA11

To test for a bactericidal or bacteriolytic mode of action, the effect of acidocin AA11 on the lysis of *Escherichia coli* was investigated. The lysis of *E. coli* was monitored in the presence of the acidocin AA11. As presented in Fig. 3, Exposure of *E. coli* to acidocin AA11 resulted in a decrease in the number of viable cells, the viability was reduced at ~ 21-fold during 2 hours. However, the optical density remained constant, indicating that lysis did not occur. Clearly, the antimicrobial compound exhibited a bactericidal effect on the target strain. A similar result obtained by ten Brink *et al.*[41] with acidocin B. The general mechanism of action which has been investigated for other

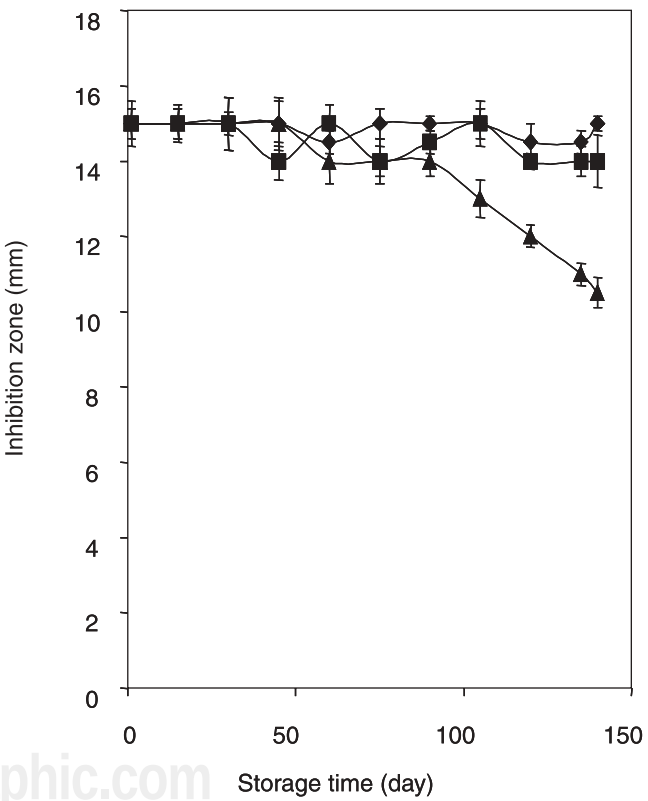

Figure 1. *Effect of time and temperature on acidocin AA11 during storage. Cell-free supernatant fluid was stored at -20 ºC (◆), 4 ºC (■) or 37 ºC (▲). The inhibitory activity was determined by well diffusion assay using* E. coli *as indicator strain.*

Rev Latinoam Microbiol 2006; 48 (1): 24-30

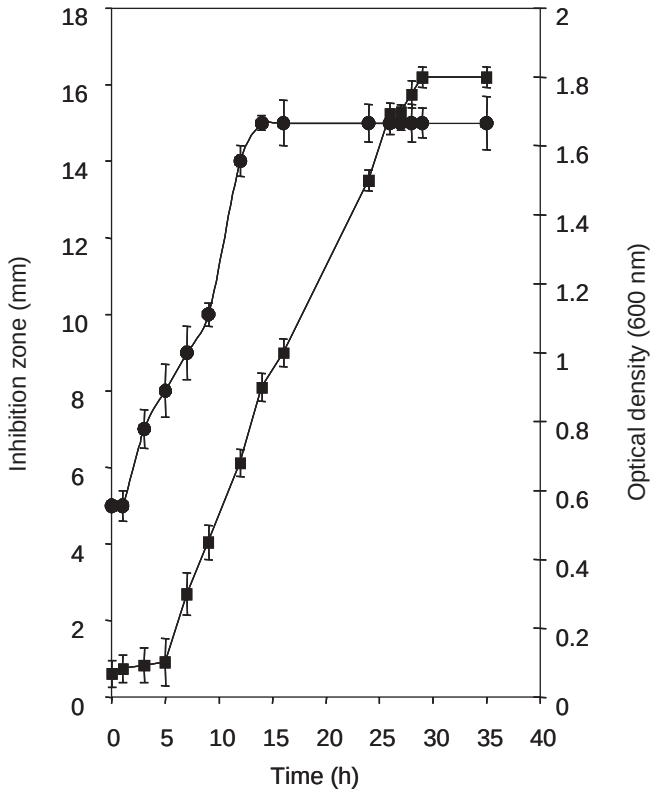

Figure 2. *Bacteriocin production and growth of* Lactobacillus acidophilus *AA11. Production of bacteriocin (●) is detected by well diffusion assay in the presence of* E. coli *as an indicator strain grown in LA aerobically at 37 °C. The growth of* Lactobacillus acidophilus *AA11 (■) was grown anaerobically in MRS medium at 37 °C.*

bacteriocins of lactic acid bacteria is disruption of the electrochemical gradients across the plasma membrane by pore formation.[24]

Extraction of acidocin AA11 with butanol

Organic solvents were investigated for the extraction of acidocin AA11 from culture supernatant fluids of *Lactobacillus acidophilus* AA11 (Table 3). Extraction with solvents such as i-Amylalcohol and di-ethyl ether did not show the complete removal of acidocin AA11 from the aqueous phase. Hexane did not result in any removal of the agent from the aqueous phase. However the best extraction was achieved with butanol and chloroform. Extraction with butanol demonstrated that acidocin AA11 was removed completely from the aqueous phase and could be recovered from the organic phase. This result suggests that at least part of acidocin AA11 has a hydrophobic character and shares this property with other bacteriocins.[24,41]

Chromosomally genes encoded acidocin AA11

To determine whether plasmid or chromosomal genes are responsible for the production of acidocin AA11, samples of *Lactobacillus acidophilus* AA11 grown anaerobically in MRS broth at 37 °C were withdrawn during logarithm phase for plasmid isolation and analysis. 1% agarsoe gel electrophoresis of plasmid preparations indicated that no plasmids were detected in *Lactobacillus acidophilus*

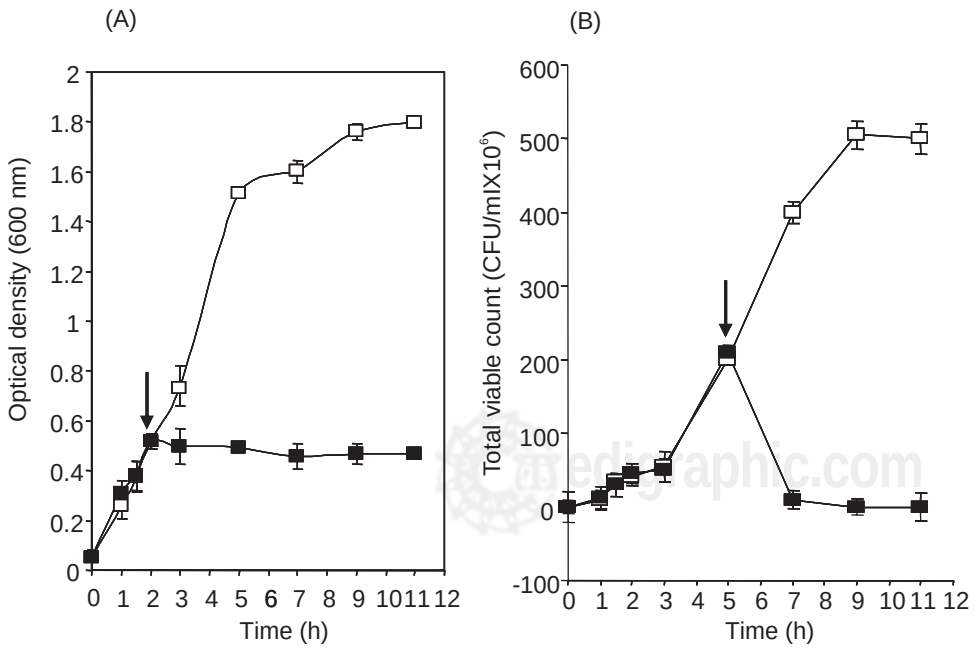

Figure 3. *Effect of* Lactobacillus acidophilus *AA11 bacteriocin on the growth of* E. coli *in LB at 37°C. The arrows indicate where the bacteriocin was added. Optical density at 600 nm (A) and viability (B) of* E. coli *culture were determined in the presence (■) and the absence (□) of AA11 supernatant fluid.*

Table 3. Extraction of acidocin AA11 from culture supernatant fluid of *Lactobacillus acidophilus* AA11 with different organic solvents. *E. coli* was used as an indicator strain in well diffusion assay.

Solvents	Diameter (mm) of inhibition zone present in solvents	
	Organic phase	Aqueous phase
None		15
n-Butanol	15	7
n-Hexane	–	9
i-Amylalcohol	12	10
Chloroform	13	8
Di-ethyl ether	9	–*

* No inhibition zone, diameter of well is 5 mm.

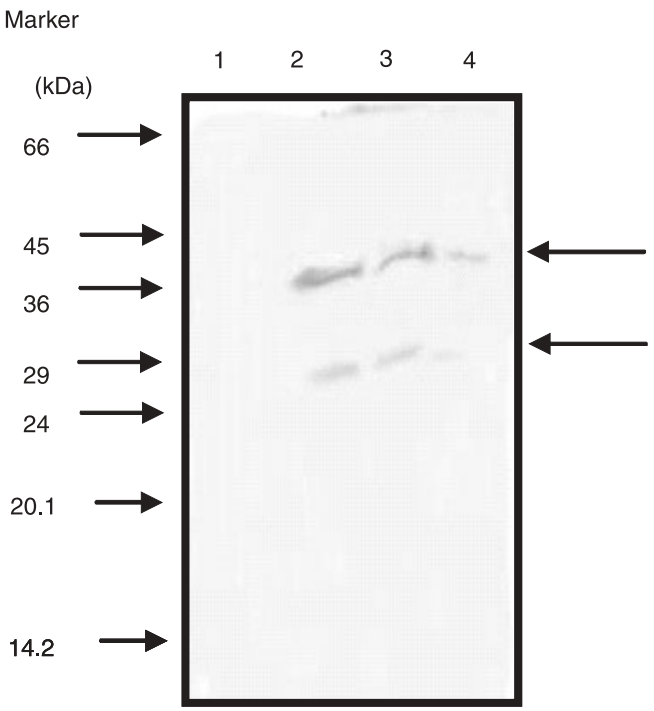

Figure 4. *12 % SDS-PAGE analysis of ammonium sulphate precipitated acidocin AA11 extracted from* Lactobacillus acidophilus *AA11 growing anaerobically in MRS at 37°C during growth curve. Lanes 1, 2, 3 and 4 represents the acidocin AA11 produced at lag, mid-log, late-log and stationary phases, respectively. The gel was stained with Coomassie blue. Arrows show respective bacteriocin bands. Molecular weight standards were Dalton Marker VII-LTM standard mixtures 14,000-66,000 (Sigma).*

AA11 suggesting that the genes responsible for acdiocin AA11 production are located on the chromosome. As shown in Fig. 4 that two major protein bands of apparent molecular sizes of ~36 kDa and ~29 kDa were distinguished in all samples. However, the intensity of these bands increased at mid-log and late-log confirming the previous result that the maximum level of the bacteriocin production was occurred during mid-log phase. Similar results were reported for two-peptide bacteriocins such as lactacin F and lactococcin G.[11,43] In conclusion, acidocin AA11 meets many of the requirements proposed by Piard and Desmazeaud[33] for an ideal antimicrobial compound. In addition to its inhibition spectrum, technological properties (heat and storage stability) provide the bacteriocin an application potential as a biopreservative.

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