Study the Antibacterial Activity of Bacteriocin Produced from the Locally Isolated *Cronobacter sakazakii*

Hameed M. Jasim*, Nedhaal S. Zbar and Mahaba R. Al-Roubaiee
Department of Biotechnology, Al-Nahrain University, Baghdad-Iraq.
*Corresponding Author:-maysmays9510@gmail.com.

Abstract

Cronobacter sakazakii was isolated from food samples and identified using morphological, biochemical tests and VITEK-2 system to produce bacteriocin by induction with mitomycin-C. C.sakazakii isolates were screened for their ability for bacteriocin production. Result showed that all these isolates were bacteriocin producers, but the most efficient isolate was MA7. Antibacterial activity of bacteriocin against some pathogenic bacteria was tested using well diffusion method. Bacteriocin was partially purified by ammonium sulfate precipitation, ion exchange using (DEAE)-Cellulose. After purification steps, three peaks were obtained. quantitative screening of bacteriocin involving the estimation of protein concentration and estimation of antibacterial activity showed that the large first peak was 1.6 mg/ml, 640 U/ml. [DOI: 10.22401/JNUS.21.2.17]

Keywords: *Cronobacter sakazakii*, Bacteriocin, Purification.

Introduction

The rapid increase in drug-resistant infections has presented a serious challenge to antimicrobial therapies. The failure of the most potent antibiotics to kill "superbugs" emphasizes the urgent need to develop other control agents. Natural antimicrobial peptide can be found in prokaryotes such as bacteria and eukaryotes such as fungi, insects, plants, protozoan and animals [1]. They are of great interest to medicine, pharmacology, and the food industry. These peptides are capable of inhibiting pathogenic microorganisms. They can attack parasites, while causing little or no harm to the host cell. A substance to be deemed as a useful chemotherapeutic agent must have toxic selectivity for the parasite, i.e., at an effective concentration in tissue the substance must have low toxicity to host cells and high toxicity to the infective agent, it also should not alter the natural defense mechanisms of the host, such as phagocytosis and synthesis of antibodies [2]. *C. sakazakii* belongs to the genus *Cronobacter*, and as most species in this genus, it is considered an opportunistic pathogen. *C. sakazakii* is Gram-negative, oxidase-negative, non spore forming, non-acid-fast, straight, rod-shaped bacteria and 1 µm x 3 µm in size, it is motile by peritrichous flagella, non-halophilic, facultative anaerobic [3]. Bacteriocins are antimicrobial peptides with different sizes, microbial target and mechanisms of action produced by large variety of bacteria. They are characterized by a bactericidal or bacteriostatic activity against strains of the same species or closely related species and differ from most therapeutic antibiotics due to their narrow activity spectrum and their proteinaceous nature[4]. Many bacteriocins have been described and classified, but only a few of them have been studied in detail, therefore this study was aimed to Isolate and identify *C. sakazakii* from different food sources, partially purify bacteriocin produced by *C. sakazakii* and test its antibacterial activity against different strains of *C. sakazakii* and some pathogenic bacteria.

Materials and Methods

Test microorganisms

Pathogenic bacteria (*Escherichia* coli, *Staphylococcus aureus, Klebsiella penumoniae, Shigella dysenteriae, Proteus vulgaris, Serratia marcescens*) were supplied from Al-Zafaraniyah General Hospital.

Isolation and identification of *C. sakazakii*

Isolation of *C. sakazakii* was done according to the method described by [5]. Seventy six samples of food materials were collected from Iraqi local markets. Food samples (1, 10, 100) g were added to (9, 90, 900) ml of peptone water respectively and mixed thoroughly and incubated for 1 hr at 37˚C. Then 10 ml of each sample was

resuspended in 90 ml of Enterobacter Enrichment broth (EE) and incubated overnight at 37˚C. Then a loopful of each culture broth was streaked onto Violate Red Bile Glucose Agar (VRBGA) plates and incubated overnight at 37˚C. Dark pink or purple colonies were streaked on tryptic soy agar and incubated for 48-72 hrs at 25˚C. Yellow pigmented colonies were selected for identification, streaked on *Enterobacter sakazaki* Agar (DFI) and incubated at 44˚C for 24 hrs**.**

Induction and extraction of bacteriocin production

Brain heart infusion broth (BHI) was distributed in test tubes then inoculated with fresh culture of *C. sakazakii* and incubated at 37˚C for 24 hrs with shaking at 200 rpm. One ml of overnight culture was transferred to 500 ml conical flasks containing 100 ml of BHI broth medium, incubated at 37˚C with shaking at 200 rpm for 2-3 hrs. Optical density of each culture was recorded by spectrophotometer at 600 nm to reach between 0.3 and 0.4. One ml of Mitomycin-C stock solution was added to each 100 ml culture to have 1 µg/ml as a final concentration of Mitomycin-C. Culture were incubated for 2-5 hrs at 37˚C with shaking (200 rpm), then 700 µl of chloroform was added to 100 ml of each culture, transferred to sterile tube and vortexed for 15 s followed by then centrifugation at 4˚C (7000 rpm/ 10 min). Supernatant was filtered by Millipore filter and transferred to a sterile vial and stored in refrigerator at 4-0˚C.

Antagonistic interactions between *Cronobacter sakazakii* isolates

In order to select the more efficient isolate of *C. sakazakii*, well diffusion method [6] was used to test the ability of *C. sakazakii* isolates to inhibit the growth of each other. Brain heart infusion broth was used for propagation of all isolates identified as *C. sakazakii* and incubated at 37°C for 24 hrs. Then 100 µL of culture of *C. sakazakii* isolates were taken and spread on BHI agar plates. Immediately wells was made in the medium using sterile cork borer and wells were filled with 100 µl of crude filtrate of the *C. sakazaki* isolates. Plates were incubated at 37°C for 24 hrs. The inhibitory effect of bacteriocin was measured using the diameter of clear circular inhibition zones surrounding bacteriocin extract.

Antagonistic reactions between *Cronobacter sakazakii* and pathogenic bacteria

The antibacterial activity of bacteriocin produced by selected isolate of *C. sakazakii* was studied by detection the inhibitory effect of bacteriocin against *E. coli, S. aureus, K. penumoniae, S. dysenteriae, P. vulgaris* and *S. marcescens*, grown on BHI agar plates. The antagonistic effect against the test microorganism was done according to the well diffusion assay method described by [6] as follows: Brain heart infusion broth was inoculated with fresh culture of test microorganism (*E. coli, S. aureus, K. penumoniae, S. dysenteriae, P. vulgaris* and *S.marcescens*) and incubated at 37°C for 24 hrs. Then 100 µL of overnight culture of test microorganisms was taken and spread on BHI agar plates by sterile cotton swabs. Immediately wells were made in the medium using sterile cork borer and filled with 100 µl of crude filtrate of the *C. sakazakii*. Plates were incubated at 37°C for 24 hrs. The inhibitory effect of bacteriocin measured using the diameter of inhibition zones around bacteriocin extract.

Partial purification of bacteriocin.

The first step of bacteriocin purification was achieved by precipitation with ammonium sulphate. This was done by production of bacteriocin by *C. sakazakii* MA7 under the optimum conditions, then cultures were centrifuged at 7000 rpm for 20 min at 4^{o}C. Ammonium sulfate was added to the supernatant (crude bacteriocin) with gradual saturation ratios ranging between 20 to 60%. After the addition of ammonium sulfate to crude bacteriocin at each saturation ratio, the mixture was mixed gently on magnatic stirrer at 4^{o}C for 40-60 min, then centrifuged at 4˚C (10000 rpm/15 min). Supernatant was discarded and precipitated proteins were dissolved in a suitable volume of 0.05 M Tris buffer at pH7. Bacteriocin activity and protein concentration were estimated before and after ammonium sulfate precipitation, then dialyzed at 4^{o}C for overnight against the same buffer solution with three increments of substitutions. The dialyzed bacteriocin was passed through

DEAE-cellulose column, then eluted with NaCl (0.1,0.2,0.3,0.4, and 0.5 $mg.ml^{-1}$). Protein concentration in each fraction was monitored spectrophotometrically at 280 nm. Fractions of the peaks were assayed for bacteriocin activity. Fractions containing bacteriocin activity were collected and stored in refrigerator for future application. Bacteriocin activity, concentration and specific activity were estimated before and after each steps of purification according to Mesa-Pereira *et al*. [7], Bradford [8], and Whitaker and Bernard [9] respectively. Finally, antibacterial activity of partially purified bacteriocin against test microorganisms was tested by well diffusion method.

Results and Discussion

Out of seventy six samples , eighty six bacterial isolates were obtained and streaked on Violate Red Bile Glucose Agar (VRBGA) and incubation at 37˚C for 24 hrs. Result showed that fifty one bacterial isolates were grown on VRBGA and gave dark pink colonies surrounded by pink halo [10]. Violate Red Bile Glucose Agar medium promote the growth of Enterobacteriaceae only and inhibits the growth of other bacteria because this medium contains Crystal Violet and blie salt in addition to Brilliant green. Fifty one bacterial isolates were streaked on Tryptone soy agar (TSA) and incubated at 25 ˚C for 48-72 hrs. Results showed that thirty bacterial isolates gave yellow colonies which identified on Enterobacter sakazaki Agar (DFI) and gave blue-green pigmented colonies and these result compatible with Iversen *et al*. [11]. After microscopic examination, results also showed that these isolates were gram negative, rod shaped, non spore forming, bacteria which coincided with result of Kandhai *et al*. [3]. Final identification of tested isolates were confirmed by using VITEK-2 identification system. Only seven isolates were identified as *C. sakazakii* and one isolate was identified as *C. universalis* which was obtained from Novalac powder milk. The other seven isolates identified as *C. sakazakii* were obtained from Dialac powder milk, and one from each source: mixed salad vegetables, Raw meat, Nactalia powder milk, Celia powder milk and Novalac Powder milk. These seven isolates were designated as MA4, MA7, MA16, MA28, MA31, MA45, MA67.

Antagonistic reactions between *C. sakazakii* isolates

In order to select efficient isolate of *C. sakazakii*, Well diffusion method was used to test the ability of *C. sakazakii* isolates to inhibit the growth of each other. The ability of these isolates in bacteriocin production was assayed after culturing in BHI broth medium at 37°C and incubated until optical density was reached (0.3-0.4). Crude bacteriocin in culture filtrate was used to study the antagonistic interaction against each other by measuring the inhibition zones according to the well diffusion method. Results in Table (1) showed that MA7 was most efficient isolate of *C. sakazakii* in bacteriocin production according to the inhibition zones against other *C. sakazakii* isolates. Which gave the maximum diameter zone of inhibition (13mm) against MA16 isolate. This result agreed with De Graaf and Klaasen, [12] who found that cloacin DF13 that produced from *Enterobacter cloacae* was very effective killing activity against sensitive strains of the same bacterial species.

Table (1)
Ability of local isolats of C. sakazakii in bacteriocin production specified by inhibition zones against each others.

C. *sakazakii* isolates	MA4	MA7	MA16	MA28	MA31	MA45	MA67
MA4	-	5mm	6mm	5mm	-	5mm	5mm
MA7	7mm	-	13mm	-	8mm	6mm	9mm
MA16	-	-	-	-	-	-	-
MA28	-	-	5mm	-	-	5mm	-
MA31	5mm	-	5mm	7mm	-	-	-
MA45	-	-	5mm	-	6mm	-	-
MA67	5mm	-	5mm	6mm	5mm	-	6mm

mm (diameter of inhibition zone) ***-(non-detectable)***

According to these results, MA7 isolate was selected to study antagonistic reaction against different pathogenic bacteria in addition to study the optimum condition for bacteriocin production.

Antagonistic reactions between *Cronobacter sakazakii* and pathogenic bacteria

Screening for bacteriocin production by *C. sakazakii* was achieved by detection the antagonistic effect of these isolate against test microorganisms. Results in Fig.(1) showed that bacteriocin produced by *C. sakazakii* have the antibacterial activity according to the inhibition zones against *E. coli, S. aureus, K. penumoniae, S.dysenteriae, P. vulgaris* and *S.marcescens*. The maximum inhibition zone was observed in *S. aureus* as shown in Fig.(2). Diameters of inhibition zones were ranged between 15 and 27mm. Graaf *et al*. [13] found that *Enterobacter cloacae* produces a bacteriocin with killing action on a sensitive strains or related bacterial species.

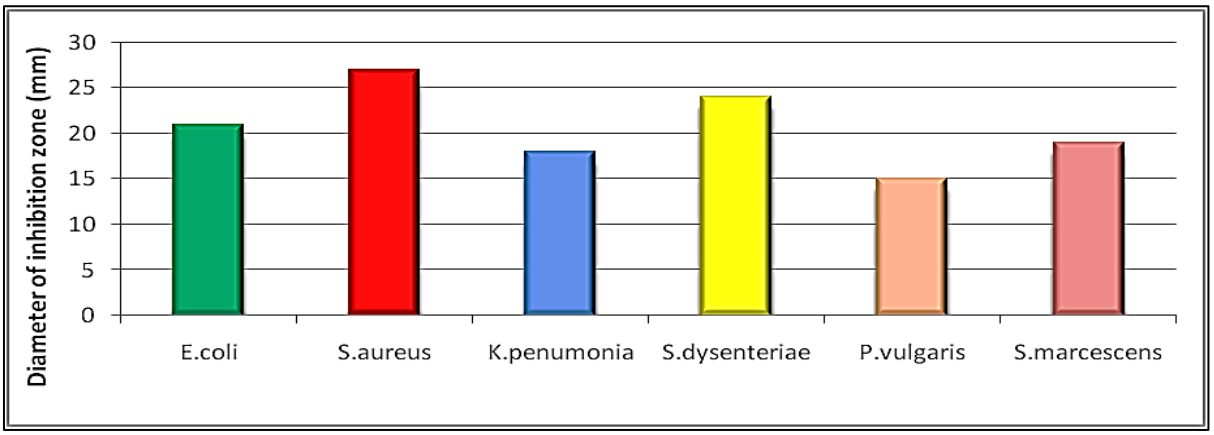

Fig.(1): Ability of C. Sakazakii in bacteriocin production specified by inhibition zones against test microorganisms.

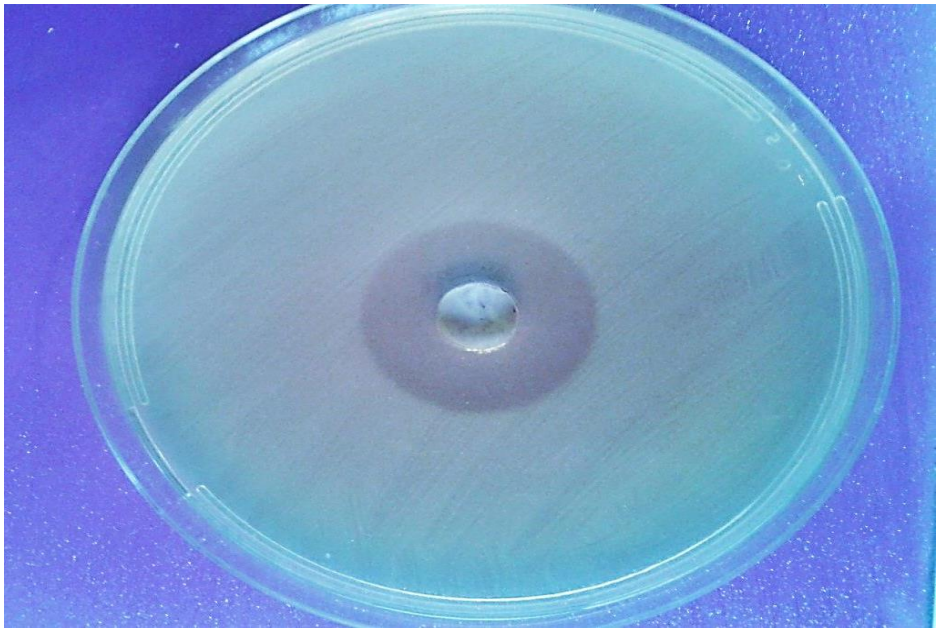

Fig.(2) : Inhibitory effect of bacteriocin produced by locally isolated C.Sakazakii MA7 against S. aureus on brain heart infusion agar medium after incubation at 37°C for 24 hrs.

Partial purification of bacteriocin.

In this study, ammonium sulphates was used in a gradual saturation ratio ranging between 20 and 40%. Precipitated bacteriocin reaches its maximal activity (36 mm) at the saturation of 40% of the ammonium sulphate as shown in Fig.(3). Protein precipitation using ammonium sulphate depends on the salting out phenomenon. Salting out removes proteins that easily aggregate from those that are very soluble making it a good initial purification step for small soluble proteins. Ammonium sulfate, $(NH_4)_2SO_4$, is often used for salting out because of its high solubility, which allows for solutions of very high ionic strength, low price, and availability of pure material. Additionally, $(NH_4)_2$ and SO_4 are at the ends of their respective Hofmeister series and have been shown to stabilize protein structure [14].

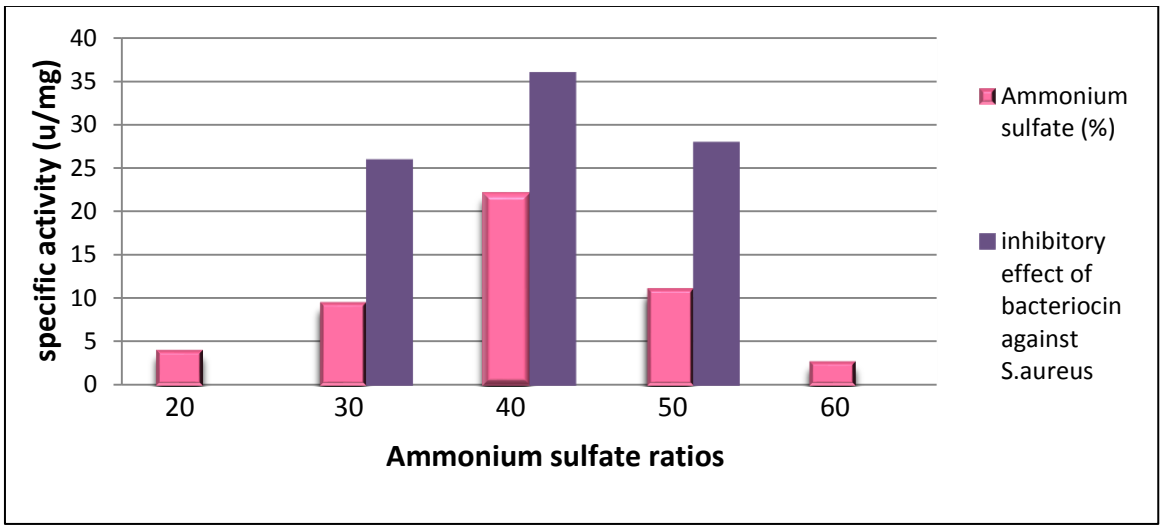

Fig.(3): Specific activity of bacteriocin produced by C.Sakazakii MA7 isolate after precipitation with different ammonium sulfate saturation ratios.

Ion exchange chromatography technique was used to purify bacteriocin produced by locally isolated *C. sakazakii* after ammonium sulfate precipitation and dialysis step. In this technique, the dialyzed bacteriocin was applied to DEAE-cellulose column, then the column was equilibrated and washed with an equal volume of 0.05 M Tris-buffer solution (pH 7) to wash uncharged and positively charged proteins in bacteriocin sample. The bound proteins (negatively charged) were then eluted using gradient concentrations of sodium chloride which ranged between 0.1 and 0.5 M. Results in Fig.(4) showed that there was one protein peaks appeared in washing step, while there were three protein peaks appeared by the gradient concentrations of sodium chloride. All these four protein peaks were detected by measuring the absorbance at 280 nm of each eluted fraction. Results in Table (2) showed that the first peak of the three eluted protein (fraction number 22 to 34) have bacteriocin activity reached to 640 U/ml while the other fraction were pooled and activity were measured. Antibacterial activity of partially purified bacteriocin was measured by well diffusion method. Results in Fig.(5) showed that the activity of bacteriocin increased when it partially purified. The maximum inhibition zone was observed in *S. aureus*. Diameters of inhibition zones were ranged between 21 and 41 mm.

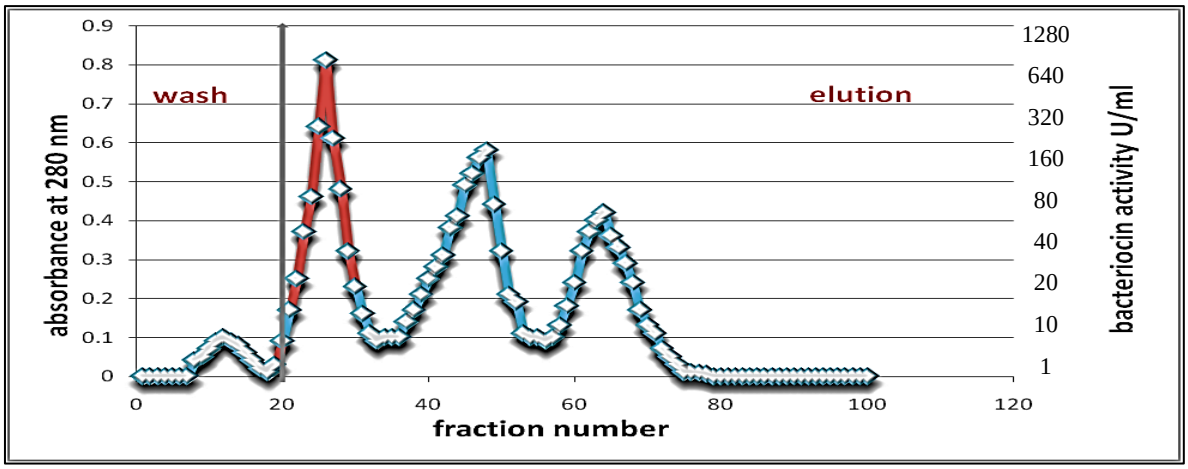

Fig.(4): Partial purification result of bacteriocin produced by Cronobacter sakazakii.

Table (1)
Partial Purification steps for bacteriocin produced by C. sakazakii

Steps of Partial Purification	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Yields (%)
Crude Extract	100	40	5.2	4000	7.69	100
40% Ammonium Sulfate Saturation	50	80	3.6	3200	22.2	80
Dialysis	17	160	2.7	2720	59.25	68
Ion exchanger DEAE-cellulose	13	640	1.6	8320	400	2.08

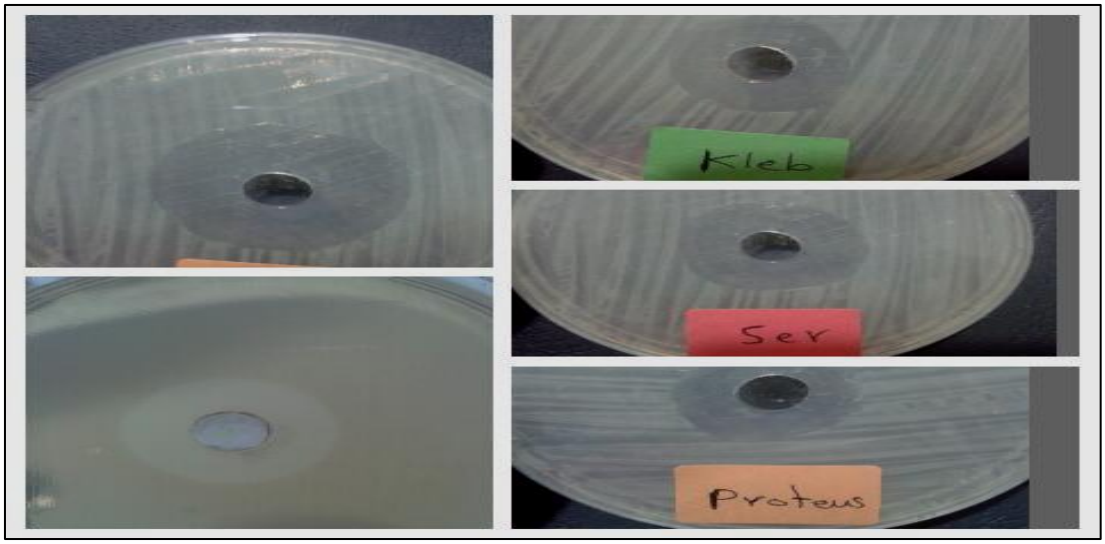

Fig.(5): Inhibitory effect of partially purified bacteriocin produced by locally isolated C.sakazakii MA7 against pathogenic bacteria.

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