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Improvement of Bacteriocin Production by Natural Zeolite and Detection of Antibacterial Activity of *Leuconostoc carnosum* Purified Carnosin

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Abstract: The purpose of this study was the improvement of bacteriocin production due to importance of bacteriocin in biotechnology. Zeolite was used for the first time in this study to support and improvement of bacteriocin production through the ability to increase the bacterial biomass. Zeolite is a mineral that consists mainly of aluminosilicate. One –hundred eighty isolates belonging to the lactic acid bacteria were obtained from 120 samples of different parts of cauliflower and broccoli .The vegetable samples were taken from different markets in Baghdad city for 3 months. Lactic acid bacteria (LAB) isolates were identified by standard diagnostic methods. Bacteriocin production was detected by measuring the inhibition zone towards indicator pathogenic bacterial isolates. The isolates were screened to determine the most efficient producer which caused the largest inhibition zone and it chosen. The most efficient producer was *Leuconostoc carnosum* after confirmed identification using the VITEK®2 System. The results showed the maximum bacteriocin production with a specific activity of 1093 $AU.mg^{-1}$ protein when added 1.5%. The active peptide was purified from the cell-free supernatant of *Leuconostoc carnosum* in three processes: (1) Ammonium sulfate for precipitation with 40-60% saturation (2) ion-exchange chromatography (3) gel filtration chromatography. The purified carnosin was characterized by determining the molecular weight using SDS PAGE (8KDa) in size .Carnosin had lost antimicrobial activity with different protease treatments (pepsin, trypsin and proteinase K). Carnosin was maintained its activity at 100ºC but lost 13% of it at 121ºC after 15 min.; Carnosin resistance for changed in PH range of 2.0-11.0, while lost 15% of its activity at pH 12.0. Also the organic solvent, surfactant and metal ion salt do not effect on its activity. The purified carnosin appeared a board range of antimicrobial activity against many pathogenic and food-borne spoilage bacteria such as *Escherichia coli, Pseudomonas aeruginosa, salmonella typhi*, *Listeria monocytogenes* and *Staphylococcus aureus.*

Keywords: Bacteriocin Carnosin, Lactic Acid Bacteria, *Leuconostoc carnosum,* Zeolite.

Introduction

Lactic acid bacteria (LAB) are a type of Gram-positive bacteria. It can ferment carbohydrates leading to acidification (lower than pH 4.0), due to the production of lactic acid. Currently they are widely used in the fermented food industry. Because of their useful effect based on their biologicalproperties improve the value of nutritional compounds, safety, organoleptic and preservative characteristics (Otunba *et al*.,

2021; Wang *et al*., 2021; Al-Salhi *et al*., 2022). The genus *Leuconostoc* is placed within Lactobacillaceae; All species within this genus are heterofermentative; It was metabolizes the carbohydrates during pentose phosphate and phosphoketolase pathway, yielding lactic acid, CO2, ethanol and/or acetic acid (Candeliere *et al.*, 2021). Lactobacilli, are generally produce a wide range of primary and secondary metabolites, including lactic acid, hydrogen peroxide, bacteriocins, biosurfactants, enzymes and other related compounds (Al-Seraih *et al*., 2022). Bacteriocins of LAB have significant applications in biotechnology. Since they are simple to produce, constant at low pH, non-toxic to human, and susceptible to proteases (Todorov *et al*., 2019). Bacteriocins are naturally occurring antimicrobials, which have been unknowingly consumed through fermented foods for thousands of years. Which have proven to reinvigorate efforts in detecting novel bacteriocins and producing sources, as food bio-preservatives and for further application (Patel *et al*., 2017). Bacteriocins can kill or inhibit bacterial strains that are related or unrelated to the bacteria that produces them. However, it will not harm the bacteria that are producing because synthesize self-immunity proteins that protect them from being killed by their own bacteriocins. Immunity proteins are thought to protect producing cells by scavenging bacteriocins or by antagonistic competition for the bacteriocin receptor (Verma *et al*., 2022).

The bacteriocins of lactic acid bacteria are classified into four classes depending on their molecular weight, heat resistance and the presence of modified amino acids (Niamah, 2018). Class I: called is lantibiotics which contains modified amino acids after DNA translation such as nisin (Jung, 1991). Class II: defined as peptide of small molecular weight (<10 kDa), heat-stable, non-modified amino acids and that shows strong anti listerial activity Pediocin is the most characterized component of the class II (Eijsink *et al*., 2002; Rodríguez *et al*., 2002). Class III: Large molecularweight (>30 kDa), unstable heat proteins, predominantly it is produced by enzymatic activity (Nilsen *et al*., 2003). Class IV: complex or globular proteins, composed of peptides with carbohydrate or lipid (Maqueda *et al*., 2004; Gabrielsen *et al.*, 2014). Bacteriocins produced by *Leuconostoc* spp. including mesentericin Y105, produced by *Leuconostoc mesenteroides* spp. *mesenteroides*; leucocin A-VAL 187, produced by *Leuconostoc gelidum;* carnosin 44A, produced by *Leuconostoc camosum*; and leuconocin S, produced by *Leuconostoc parameseneteroides* (STILES,1994).

Leuconostoc carnosum frequently occurs in the microbiota of foods, mostly meat-based products and has been isolated from processed vegetables; It has been able to produce bacteriocins, that can inhibit the growth of pathogenic and food spoilage microorganisms, therefore dealing with food preservation. It has been confirmed in LAB, and *Leuconostoc* thereby asserting the safety of the product (Raimondi *et al*., 2021; Silva *et al*., 2022).

Most *L. carnosum* isolates can produce bacteriocin effective against *Listeria monocytogenes* that grow in food. Which causes infection in humans, called listeriosis, a severe infection with a high rate of case-fatality (Raimondi *et al*., 2021; Tönz *et al*., 2024). Recently, the failure of treatment after a high or long-term use of antibacterial drugs by patients has increased emergence of strains resistant to these drugs. A drugs resistant warning that the world is “running out of antibiotics”, raising fears about global antibiotic resistance reaching new heights. All of these resulting in a gradual increase in the frequency of nosocomial infections. This prompted scientists to re-evaluate alternate therapeutic options as the discovery of new antibiotics with diverse

mechanisms of action has slowed considerably (Zhu *et al*., 2022). Zeolites are materials that can be synthesized or found in natural rock deposits with a basic composition consisting in Al, Si, and O. There are many applications of mesoporous ordered zeolites. Such as the cracking of oil to produce liquid hydrocarbon fuel, for the valorization of agro-industrial waste, and the immobilization of enzymes for biocatalytic processes (Vasconcelos *et al*., 2023).

The purpose of this study was the improvement of bacteriocin production due to importance of bacteriocin in biotechnology. Zeolite was used for the first time in this study to support and improvement of bacteriocin production through the ability to increase the bacterial biomass. It was led to enhance the manufacturing bacteriocin to expand applications of the produced bacteriocins as antimicrobial agent.

Materials & Methods

Screening of bacteriocin-producing isolates

One hundred twenty of small pieces were obtained out of 30 samples of fresh Vegetables (cauliflower and broccoli). The samples were collected randomly from Baghdad markets, Iraq during the period from November 2022 to January 2023. These samples were transported in a box to the food microbiology lab. It was gently washed with distilled water and cut into small pieces. 1.0 g of vegetable was suspended in a tube containing 1.0 mL sterile saline. Then these were incubated at room temperature for 24 hours. The Lactic acid bacteria were isolated by using tubes containing 9 ml of de Man, Rogosa and Sharp broth (MRS) were inoculated with 1 ml of the upper layer of saline containing samples separately and incubated anaerobically at 30°C for 48 hours. After incubation, serial dilutions were made for each culture using the spread-plate method on MRS agar. Then, after incubation, large and small pure colonies were selected (Junnarkar *et al*., 2019). Lactic acid bacteria was cultured in MRS medium for 24 hours at 30 °C. Cell-free supernatant was obtained by centrifuging the cultures at 4 °C, 4000 rpm for 30 minutes. The supernatants were sterilized by filtering through a 0.22 µm filter (Millipore filter).The supernatant was adjusted to pH 6.0 using 1N NaOH and used as crude bacteriocin (Al-Rawi *et al*., 2023). The antimicrobial activity of bacteriocin produced by lactic acid bacteria against some pathogenic bacteria was evaluated by using the agar well diffusion method (Al-fekaiki *et al*., 2017). Pathogenic isolates were cultured in nutrient broth, followed by incubation at 37°C overnight. 0.1mL (Approximately 10^6 CFU.mL^{-1}) of bacteria was streaked by L-shape on of the Mueller Hinton agar (Himedia-India). When made a 6 mm diameter well by cutting a hole with sterile cork borer were filled with 100 µl of the culture supernatant. The plates were kept in the refrigerator for two hours and incubated at 37°C for 24 h. Inhibition was detected by a zone of clearing around the supernatant well.

The bacterial isolate that caused inhibition zone was chosen and identification was confirmed with VITEK®2 System by using Identification-Gram positive bacteria (ID-GBB) cards were used according to manufacturer's instructions. A bacterial suspension of 0.5 McFarland turbidity standards was generated for diagnosis. The reagent cards comprise 64 wells, each of which can contain a different test substrate.

Bacteriocin activity assay

The bacteriocin activity was measured by the arbitrary unit (AU). The reciprocal of the highest dilution produced a distinct inhibition zone against the growth of the isolate.

The AU was computed as follows:

$$AU/ml = (D \times 1{,}000)/V$$

Where 1000: is constant and D: is the highest dilution exhibiting inhibition V: the volume of the filled sample into each well (Parente *et al*.,

1995)

Determination of protein concentration

According to Bradford (1976) the protein concentration was determined.

Estimation of specific activity of bacteriocin

The specific activity of bacteriocin was estimated according to the following equation (Whitaker, 1994).

$$SA=B/P$$

AS: Specific activity unit mg^{-1} protein

B: Bacteriocin activity unit ml^{-1}

P: Protein concentration mg ml^{-1}

Using natural zeolite for improvement bacteriocin production

The natural zeolite was used to support the production of bacteriocin. It was analyzed by an Iraq geological survey. The protocol used for the quantitative determination of zeolite-group mineral compositions was electron probe microanalysis (wavelength-dispersive spectrometry) under ambient conditions. (Campbell *et al*., 2016). Then, it was studied at various concentrations (0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0) %. It was added separately to tubes containing MRS broth. All tubes were incubated anaerobically at 30°C for 48 hours after being inoculated with the bacterial isolate's 18-hour-old culture. Protein concentration and bacteriocin activity were determined (Silva *et al*., 2021; Tekeşoğlu & Ergün, 2021).

Purification of bacteriocin

The bacteriocin was precipitated from supernatant by using ammonium sulfate with specific weight. It was gradually added to the crude bacteriocin in an ice bath with continuous stirring for 60 min to get saturation percentages of (20-40, 40-60, 60-80 and 80-100%). The mixture was then centrifuged at 10,000 rpm at 4ºC for 30 min. After that, the precipitate was taken and dissolved in a volume of phosphate buffer saline. The bacteriocin activity assay to evaluate the best percentage of saturation (Banerjee *et al*., 2016).The dialysis process was carried out in dialysis tube with 3500 MW cutoff after precipitation step using 0.1 M phosphate buffer pH 7.0. Subsequently, the sample was dialyzed against distilled water several times at 4°C with continuous stirring. The solution was then concentrated using sucrose until it reached a volume of 12ml and stored in the refrigerator for further purification (Asenjo & Andrews, 2009; Walker, 2009). Twelve-milliliter samples from the dialyzed bacteriocin were applied to the ion exchange column (DEAE-Cellulose). The flow rate was organized to be 1 $ml.min^{-1}$, and elution was collected as a 5 ml fraction. Protein that bound the gel was eluted by elution buffer and gradient NaCl in a concentration of 0.1-0.5 M. The absorbance for each fraction was measured at 280 nm. Estimation of bacteriocin activity and protein concentration was done for peaks of all the active fractions. Then, pooled and dialyzed against sucrose for further purification steps (Zhou *et al*., 2008). Sephacryle S-200 gel filtration chromatography had been used as a third step in the purifying of the bacteriocin. Partially purified bacteriocin was loaded slowly over the gel. The bacteriocin was eluted from the column by 0.05 M potassium phosphate buffer pH7.0. The flow rate was adjusted to give 30 ml per hours at 5 ml for each fraction was collected. Activity and absorbance at 280nm of each fraction were determined and active fractions were pooled (Hong *et al*., 2022).

Characterization of purified bacteriocin

Molecular weight determination by SDS-PAGE

The molecular weight and purity of the bacteriocin have been determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis device received the resolving gel with butanol for 45 to 60 min to polymerize. Then butanol was eliminated and the gel surface was washed with distilled water. After that, stacking gel

was added and allowed to polymerize after being applied in the same manner. The stacking gel was allowed to stabiliz before placing and removing a comb between the glasses plates. The top and lower reservoirs of the electrophoresis cell were filled with electrode buffer. At a rate of 150 µL, the samples were added to the edges of every well. Electrophoresis was then carried out by employing currents of 2 $mA.gel^{-1}$ at 40 volts, for 45 min during the stacking stage. For the resolving stage, $5mA.gel^{-1}$ at 240 volts was applied over 5 hours after connecting the device to a power source. This gel was submerged in the fixing solution for just an hour. It was then immersed for one to three hours in Coomassie Brilliant Blue R-250 staining solution. Finally the stain was removed from the gel. De-staining solution was used to soak the gel until blue protein bands appeared, (it must be changed several times). Then, the connection between Rm and Log of the molecular weight of standard proteins and molecular weight of bacteriocin was calculated (Roy *et al*., 2012). Relative mobility (Rm) was computed to obtain the molecular weight of Bacteriocin as follows:

$$Rm = A/B$$

Rm: Relative mobility

A: Distance that bacteriocin has moved (mm)

B: Distance that bromophenol blue dye has moved (mm)

Effect of pH on bacteriocin

Bacteriocin solution was mixed with 10mM potassium phosphate buffer of pH values ranging from 2 to 12. It was re-adjusted to pH 6.5 with 1N NaOH or 1N HCl, after incubation for 15 min at 30°C. The samples were tested for remaining activity (Afrin *et al*., 2021). The following has been determined:

$$R\% = (A/B)\ X\ 100$$

R: Remaining units

A: Residual activity

B: Original units

Effect of temperature on bacteriocin

The bacteriocin solution was heated at various temperatures (4°, 20°, 25°, 30°, 37°, 40°, 45°, 50°, 60°, 70°, 80°, 90°, 100° and 121°C). The impact of temperature on bacteriocin activity was tested after fifteen minutes at each temperature. Bacteriocin residual activity was assessed (Djadouni & Mebrouk 2013).

Effect of enzymes on bacteriocin

Bacteriocin was incubated for 1 hr at 30˚C in the presence of enzymes. Then, the enzymes were inactivated by heating for 3 min at 100°C and the bacteriocin was tested for residual activity (Mahdi *et al*., 2014).

Effect of surfactants, organic solvents, and metal ion salts on bacteriocin

Solutions of surfactants, organic solvents and metal ion salts were added separately to the bacteriocin solution and the effects of these materials were investigated. After an hour of incubation at 30° C, all samples were checked for any residual activity (Otunba *et al*., 2022).

Antibacterial activity of crud and purified bacteriocin

The microorganisms studied are clinically important ones causing several infections and food spoilage. The Gram-positive bacteria studied were *Staphylococcus aureus* and *Listeria monocytogenes*. Gram-negative bacteria were *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhimurium*. The activity of crude and purified bacteriocin at the concentration (16 and 32) $\mu g.ml^{-1}$ against pathogens was determined by using the agar well diffusion method (Mahdi, 2017).

Statistical analysis

The obtained data are expressed as mean ± SD. Analysis of variance was performed by (ANOVA) test using the SPSS 20.0 software. Results are considered statistically significant at P-values < 0.05.

Results & Discussion

Screening of bacteriocin-producing isolates

From 15th November, 2022, to 15th February 2023, 180 lactic acid bacteria isolates were obtained from 120 samples of cauliflower and broccoli in Baghdad markets. Crude filtrate for the 180 isolates of lactic acid bacteria tested to detect the antibacterial activity against indicator bacterial isolates *(S. aureus, S. agalactiae E. coli* and *P. aeruginosa*). Only 20 isolates showed antibacterial activity. The result of identification these isolates with VITEK 2 Compact system was 5 (25%) isolates of *Leuconostoc mesenteroides* ssp. *mesenteroides*, 4 (20%) isolates of *Leuconostoc mesenteroides* ssp *dextranicum*, 3(15%) isolates of *Lactococcus garvieae*, 7 (35%) isolates of *Leuconostoc citreum* and 1(5%) isolate of *Leuconostoc carnosum*. The lactic acid bacteria from brocolli *Leuconostoc carnosum* (LabBr no. 28) had the highest activity against all indicators bacteria. So it was chosen as a bacteriocin producer isolate to be used in the next experiments. *Leuconostoc carnosum* which isolated first time from broccoli.

Improvement of bacteriocin production by natural zeolite

Analysis of the natural Zeolite by Iraq geological survey appeared in table (1). The components of this material were SiO_2, Fe_2O_3, Al_2O_3, TiO_2, CaO, Na_2O, K_2O and MgO. The percentages of these components were 39.82, 0.71, 27.88, 0.10, 0.25, 13.20, 0.25, 0.04 % respectively. The SiO_2 and Al_2O_3 have the highest ratio therefore called Alminosilicat Zeolite. These results agreed with the study of Tekeşoğlu & Ergün (2021) and Senila *et al*. (2022).

Natural zeolite was used for the first time in this study as far as we know to support and improvement of bacteriocin production by *L. carnosum*. The results showed in figs. (1 & 2). The maximum specific activity 1093 $AU.mg^{-1}$ protein of bacteriocin when added1.5% zeolite. While minimum specific activity 978 $AU.mg^{-1}$ protein of it with 2.0% zeolite.

Senila *et al*. (2022) mention the using natural zeolite in his study as support for microbial community formation during wastewater treatment. Mühlbachová & Šimon (2003) revealed the effects of zeolites on microbial populations and their activities were completely unknown, but they found out an increase in microbial biomass. Silva *et al*. (2021) observed addition Zeolite leading to improve biohydrogen production from dark fermentation of C5/C6-sugars and *Sargassum* sp. Biomass. The zeolite was useful to raise the hydrogen titer and continuous high-rate feeding with C5 and C6-sugar. This impacts on the microbiota and fermentation products. Also, it adsorbed protons in solution by ion-exchange. It was assumed that these protons sink was reducing the NADH/NAD+ redox imbalance. This imbalance is usually associated to creation of more reduction products such as lactate.

Several studies explained the addition zeolites enhanced anaerobic digestion process (AD) under both thermophilic and mesophilic conditions. The zeolite was function as ion-exchangers for avoiding the inhibitory effect of ammonia towards the microbial community. By removing or adsorbing free ammonia, the overall process was improved (Kotsopoulos *et al*., 2008). Zeolites have porous structure; therefore it's able to improve hydrogen production. Also it was able to add of trace elements such as Ca, Na, K and Ba. These elements were vital to provide optimal conditions for the growth of microorganisms (Moghaddam *et al*., 2018; Montalvo *et al*., 2020). Recently, zeolite has been used in various industries due to its unique properties and the presence of water molecules within its structure. When water is removed, the resulting empty spaces can be occupied by other molecules. The occupation of these spaces by

molecules is called adsorption (Al-Mashhdany *et al*., 2023). In this study the role of zeolite based on previous studies was increase the bacterial biomass led to increase the bacteriocin production.

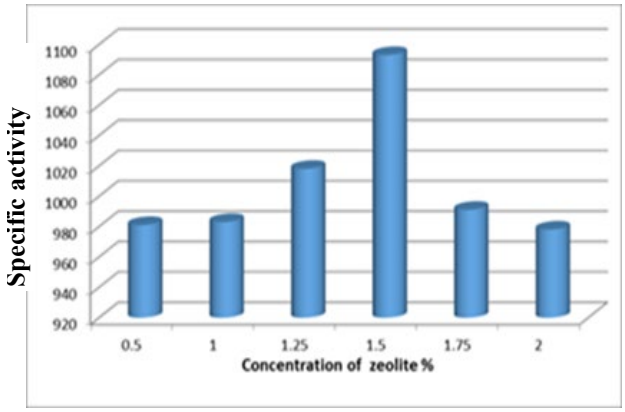

Fig. (1): Optimum concentration of zeolite for bacteriocin production by *L. carnosum*.

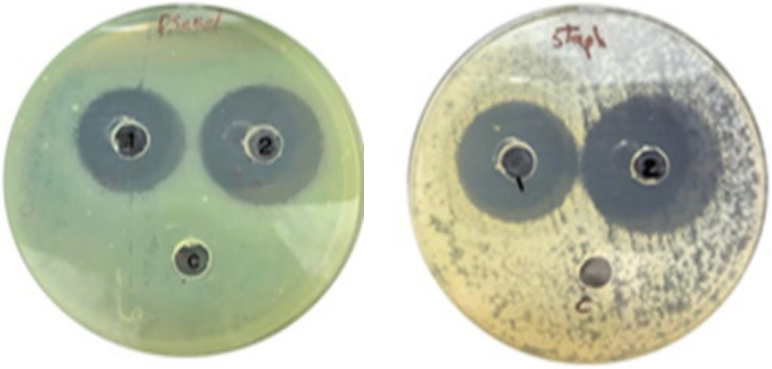

Fig. (2): Antibacterial activity of bacteriocin against indicator bacteria: *S. aureus* and *P.aeruginosa* 1: optimum conditions. 2: After using 1.5% zeolite. 3: control.

Table (1): Components of natural Zeolite

SiO_2	Fe_2O_3	Al_2O_3	TiO_2	CaO	Na_2O	K_2O	MgO
39.82%	0.71%	27.88%	0.10%	0.25%	13.20%	0.25 %	0.04 %

Purification of bacteriocin

Four different saturation rates of ammonium sulfate (20-40, 40-60, 60-80 and 80-100%) were used to detect the optimal range for bacteriocin precipitation. The results appeared that the optimal saturation range for bacteriocin precipitation was (40-60 %). The bacteriocin activity was 640 $AU.ml^{-1}$ and specific activity was 3926.38 $AU.mg^{-1}$ protein table (2). This result agreed with the study of Budde *et al*. (2003), while Lahiri *et al*. (2020) observed the precipitation of bacteriocin produced by *Leuconostoc lactis* was at saturation range between 40%-80 %. Maurya & Thakur (2012) reported the bacteriocin from the culture filtrate of *Leuconostoc* NT-1 was precipitated by the addition of 21–60% (NH4)2SO4. The ammonium sulfate salts (salting out) were used for partial purification by precipitating enzymes or proteins. It was the first step of purification to reduce the volume and concentrate of enzymes. Ammonium sulfate is chosen for its low-cost, high solubility, lack of toxicity to most enzymes and stabilizing effect on some enzymes (Fathi *et al*., 2021). Ion exchange chromatography showed one peak in the wash step but had no bacteriocin activity thus it was neglected. The two peaks appeared in the elution step. The peak with 0.25-0.5M of NaCl

at fractions numbered 35 to 40 showed the highest bacteriocin activity as appeared in fig. (3). The fractions were pooled and tested for bacteriocin activity with 1280 AU.ml^{-1}. The specific activity of 13195.8 AU.mg^{-1}, fold purification of 12 and protein yield of 19.2 % are shown in table (2). Budde *et al*. (2003) used SP-sepharose in a second step to purify leucocins 4010 from *L. carnosum* 4010. Its activity was 4,915,200 AU.ml^{-1}, specific activity 1,117,091 AU.mg^{-1}, fold purification 320 and protein yield 16%. Maurya & Thakur (2012) efficiently purified *Leuconostoc* NT-1 bacteriocin using DEAE cellulose column, with a bacteriocin yield of 10.2%. After the ion exchange purification step, fractions representing bacteriocin activity were collected, pooled and concentr-

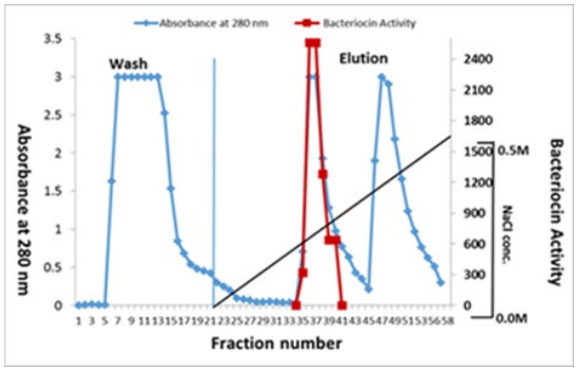

Fig. (3): Ionic exchange chromatography for purification of bacteriocin.

ated. Then, it was applied in Sephacryl S-200 previously equilibrated with 0.05M phosphate buffer pH 7.0. There is only one absorption peak which represents bacteriocin with maximum activity after elution with phosphate buffer. These results are illustrated in figs. (4 and 5). Protein concentration, activity and specific activity of bacteriocin were measured. Results in table (2) showed that the specific activity of the purified bacteriocin was (25600 AU.mg^{-1}). Also, the purification fold was 23.3 and the overall yield was 12.8%. Maurya and Thakur (2012) purified *Leuconostoc* NT-1 bacteriocin using gel filtration, with bacteriocin yield of 4.9 %. In a study done by Rasheed *et al.,* (2020) used gel filtration in the purification of *Lactobacillus acidophilus* HT1 bacteriocin. Where the specific activity was 12800AU.mg^{-1}, 10 purification fold and 20% yield.

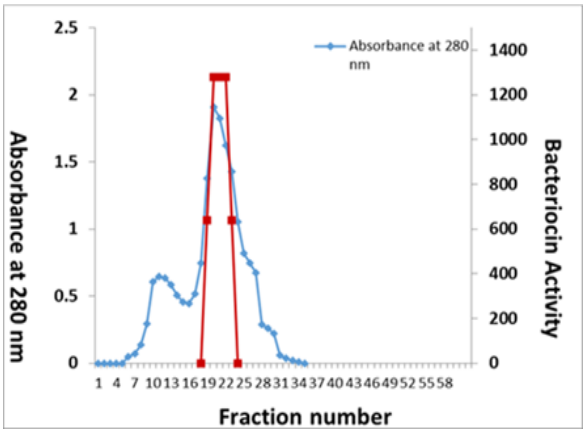

Fig. (4): Gel filtration chromatography for purification of bacteriocin.

Fig. (5): Fractions (20, 21 and 22) of purified carnosin with the highest activity against indicator *S. aureus*.

Table (2): Purification steps of carnocin produced from *L. carnosum.*

Purification steps	Volume (ml)	Bacteriocin activity	Protein concentration	Specific activity	Total activity	Purification (folds)	Yield (%)
Crude Bacteriocin	250	320	0.292	1095.89	80000	1	100
Ammonium sulphate	50	640	0.163	3926.38	32000	3.5	40
DEAE-cellulose	12	1280	0.097	13195.8	15360	12	19.2
Sephacryl S-200	8	1280	0.05	25600	10240	23.3	12.8

Characterization purified bacteriocin

Molecular weight determination by SDS-PAGE

The molecular weight of carnosin was found to be 8,000 Dalton, as appeared in figs. (6 & 7). Budde *et al*. (2003) reported the peptides produced by *L. carnosum* were designated leucocin B-4010 with molecular sizes of 4.6kDa. Afrin *et al*. (2021) found the molecular weights of bacteriocins produced from *lactobacillus* ssp. was around 7.0 to 10.5kDa.

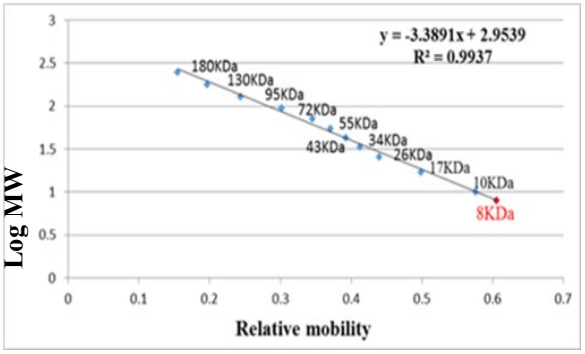

Fig. (6): Determination of molecular weight of purified carnosin.

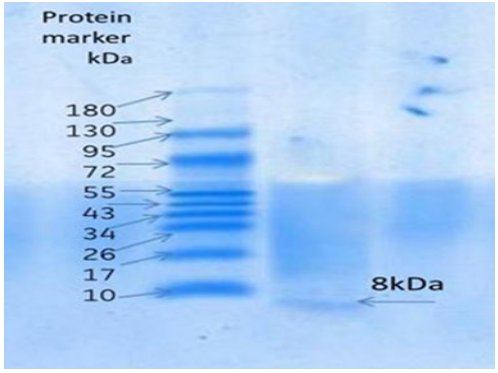

Fig. (7): (SDS-PAGE) of the purified carnosin.

Effect of pH on carnosin

That purified carnosin was steady at pH values ranging from 2.0 to 11.0. At these values, carnosin retained its activity. It was lost 15 % of their entire activity at pH values 12.0 as appeared in fig (8). These results were indicating bacteriocin is stable with a wide range of pH. These results agreed with Stiles (1994) which reported the bacteriocin of *L. camosum* is active over a vast range of pH (from 2 to 9). Also Dündar (2006) revealed the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* retained their activities without any loss at pH values 2.0 to 10.0 at room temperature.

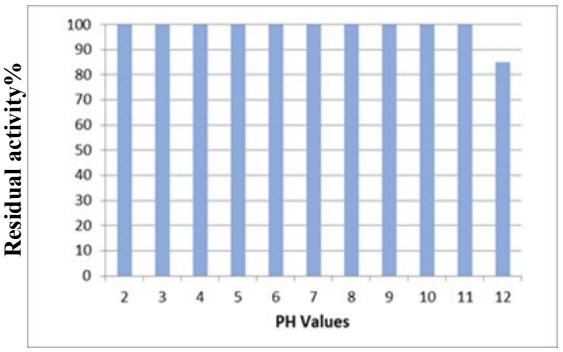

Fig. (8): Stability of purified carnosin at different pH values.

Effect of temperature on carnosin

The thermal stability of purified carnosin at various temperatures where it was active at 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C for fifteen minutes. However, 13% of its activity was missing after autoclaving (121°C /15min/ 15psi), as showed in fig. (9). The bacteriocin produced by *Leuconostoc* spp. such as

leucocin A and carnosin were stable at 100ºC whereas leucocin S was active at 60ºC (Stiles, 1994).Choeisoongnern *et al*. (2020) reported the bacteriocin produced by *Pediococcus pentosaceus* was stable at temperature 100 °C for two hours or 121°C for 20 min.

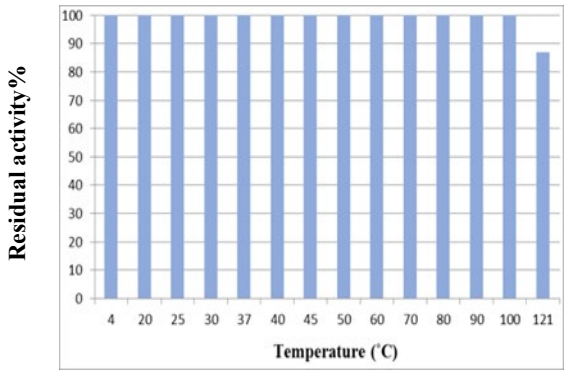

Fig. (9): Effect of different temperatures on the activity of purified carnosin.

Effect of Enzymes on carnosin

The activity of carnosin after being treated with different enzymes was recorded in fig. (10). The bacteriocin remained unaffected after treatments with lipase, α-amylase and lysozyme. It lost all activity after treatment with Proteinase-K, trypsin and pepsin. This indicated that the bacteriocin was protein in nature. The bacteriocin was missing its native structure as well as activity in the presence of the mentioned enzymes. These results agreed with the research of Qiao *et al*. (2020). Ivanova *et al*. (2000) reported that *L. lactis* bacteriocin was stable after treatments with lipase, amylase, trypsin and α-amylase, while Proteinase-K inhibited the bacteriocin activity.

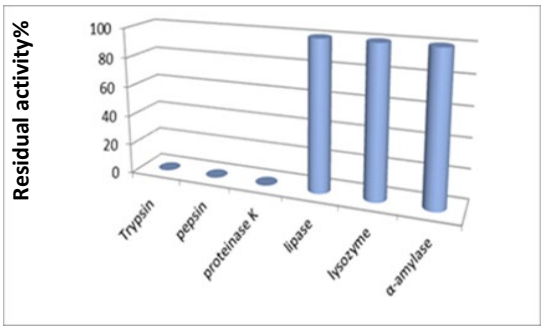

Fig. (10): Activity of purified carnosin after treated with enzymes.

Effect of organic Solvents on carnosin

The different organic solvents (acetonitrile, diethyl ether, ethanol, methanol and isopropanol) were not effect on the activity of the carnosin. These results are similar to the study of Otunba *et al*. (2022) who revealed the *Pediococcus pentosaceus* bacteriocin was stable on treatment with organic solvents. This indicated that there are no lipid constituents within the bacteriocin therefore it was stable after organic solvent treatments (Yildirim *et al*., 2014).

Effect of metal ions salts on carnosin

The various salts of metal ions on the activity of the carnosin were detected. The result showed stability of bacteriocin after mixing with the 5mM of NaCl, KCl, MgSO4, MnSO4, CuSO4 and Ca $(NO3)^2$. These results are similar with the study of Ibrahim *et al*. (2020). Who observed the *Lactobacillus plantarum* bacteriocin was stable with the presence of divalent ions (Mn^{2+}, Mg^{2+} and Ca^{2+}). But the bacterial growth was affected by the Hg^{2+} at low concentrations. It may be due to the blockage of the active sites of Bac-IB4 by these metal ions.

Effect of surfactants on carosin

The impact of some surfactants on the activity of carnosin was investigated. The surface active elements SDS and EDTA at 5 mM concentration did not effect on bacteriocin activity. Also triton X-100, tween 80 and tween 20 at concentrations of 5 % did not impact on the activity of bacteriocin. These results agreed with Cui *et al*. (2020), which displayed that the EDTA and Tween-80 at different concentrations don't effect on antibacterial activity of *E. faecalis* CG-9 bacteriocin. Another study was indicated the bacteriocin with high activity was raised after adding SDS since the antibacterial activity of SDS itself. It was resulted in a synergistic impact leading to SDS improvement in the bacteriocin's antibacterial activity. While

Tween-20, Tween80 and urea decreased their activity (Perumal & Venkatesan, 2016).

Antibacterial activity of crude and purified carnosin

The spectrum of antibacterial activity of bacteriocin against gram-positive and gram-negative bacteria is shown in table (3). It was an assay of crude and 16, 32 µg.ml^{-1} concentrations of purified carnosin. The findings showed that crude and purified carnosin have significant antibacterial activity against all tested bacterial isolates compared to the control (P>0.01). The results showed that 32 µg.ml^{-1} concentration of purified carnosin was more effective against all bacterial isolates than 16 µg.ml^{-1} and the crude. In the current study, the highest activity of crude and purified carnosin at 16 and 32 µg.ml^{-1} was against *S. aureus*. This activity was 32.83±0.28 mm and 35.16±0.28 mm, 26.5±0.5 mm respectively. Which presented significant differences in carnosin activity against tested bacterial isolates. This indicated the activity of carnosin had a wide spectrum against pathogens at different concentrations as appeared in fig. (11). Similar results by Al-Rawi *et al*. (2023) observed the antibacterial activity of bacteriocin from *Lactobacillus spp.* with the inhibition zone in different diameters. It was against various pathogenic bacteria (*E. coli, Pseudomonas aeruginosa, Staphylococcus epidermidis,* and *Streptococcus mutans*). Also, Taher (2016) showed that the inhibitory activity of *Pediococcus pentosaceus* bacteriocin was high against Gram-positive bacteria or Gram-negative bacteria and yeast. Cui *et al.* (2020) displayed that the antimicrobial activity of the purified bacteriocin. It was able to inhibit both gram-negative and gram-positive bacteria, even some fungi. Therefore it can be beneficial and valuable in the food industry. These bacteriocins were broad-spectrum antibacterial activity. It may be because the bacteriocin has a different mode of action from that of antibiotics. There are many therapeutic agents used for treating infectious diseases. But they are one of the main causes of morbidity-mortality in the world. The prolonged antibiotic therapy was stimulated bacterial-resistance. Some bacteria have developed ways to circumvent the effects of antibiotics. Therefore, new drugs have been developed for control of bacterial resistance (Khelkal, 2016).

Table (3): Antibacterial activity of carnosin.

Bacterial isolates	Inhibition zone (mm) of crude and purified carnocin at different concentrations (Mean ± SD)			
	crude	32µg.ml^{-1}	16µg.ml^{-1}	*P*-value
S. aureus	32.83 ± 0.28 B a	35.16 ± 0.28 A a	26.5 ± 0.5 C a	0.01sig
L. monocytogenes	25.33 ± 0.28 B c	29.17 ± 0.28 A c	21.33 ± 0.3 C d	0.01sig
S. typhimurium	24.00 ± 0.5 B d	28.33 ± 0.3 A c	25.17 ± 0.3 C b	0.01sig
P.aeruginosa	28.00 ± 0.5 B b	30.67 ± 1.04 A b	23.17 ± 0.28 C c	0.01sig
E. coli	28.17 ± 0.57 B b	28.83 ± 0.28 A c	22.67 ± 0.28 C c	0.01sig
P-value	0.01 sig	0.01 sig	0.01 sig	

(*) LSD test was used to calculate the significant differences between tested means, the capital letters represented the levels of significant between concentrations, while small letters for the levels of significant between pathogens, highly significant start from the letter (A)(a) and decreasing with the last one.(Sig: significantly)

Fig. (11): Spectrum activity of concentrations of purified carnosin against pathogens. A: 16µg.ml^{-1} B: 32µg.ml^{-1} C: control with D.W.

Conclusion

This study concluded that LAB can be isolated from vegetables such as Cauliflower and Broccoli. Highest antibacterial activity of LAB isolated from Broccoli with a broad spectrum against Gram-positive and Gram negative pathogenic bacteria; Such as *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa*, *Salmonella typhi*. Natural zeolite can encourage the bacteriocin production by increasing the metabolic activities of the producer bacteria. Minerals that contain may be accelerate the enzymatic reactions and reduce the time of growth. The minerals then increase the production compared to free zeolite medium. The purified bacteriocin named carnocin. It was belonged to class II bacteriocin. It has some characters such as stable with wide range of pH and through different temperatures until 121˚C. It was no longer active with proteolysis enzymes but remained active with others. Finally no effect had been recorded of organic solvent, surfactant and metal ions salts on carnosin activity.

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Contributions of authors

I.N.K and **Y. M. B.** gave the idea of the research.

H.A.I: The steps of work such as collected the samples, diagnosed them, doing all the test about activity.

H.A.I wrote the manuscript with revisions, **I.N.K** and **Y. M. B.** diagnosed them and edited the manuscript with revisions and now take the care of publishing. All authors read and approved the final manuscript.

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Conflicts of interest

The authors declare that they have no conflict of interests.

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تحسين إنتاج الكارنوسين بواسطة الزيولايت الطبيعي والكشف عن النشاط المضاد للبكتيريا للكارنوسين المنقى من*Leuconostoc carnosum*

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المستخلص: كان الغرض من هذه الدراسة هو التحري عن تحسين إنتاج البكتريوسين باستخدام الزيولايت الطبيعي. الزيولايت هو معدن يتكون بشكل رئيسي من الالمينوسيليكات . تم الحصول على مائة وثمانون عزلة تعود الى بكتريا حمض اللاكتيك (LAB)من 120 عينة من اجزاء مختلفة من القرنابيط والبروكلي اخذت من اسواق مختلفة في مدينة بغداد خلال 3 اشهر. تم تشخيص عزلات LAB بواسطة طرق التشخيص القياسية . تم الكشف عن إنتاج البكتريوسين عن طريق قياس منطقة التثبيط تجاه العزلات البكتيرية الممرضة. تمت غربلة العزلات المنتجة لأختيار العزلة الأكفا اذ اختيرت العزلة *Leuconostoc carnosum* التي تسببت في أكبر منطقة تثبيط وتم تأكيد التشخيص بنظام.VITEK®2 حيث تم استخدام الزيولايت الطبيعي لأول مرة في هذه الدراسة لدعم وتحسين إنتاج البكتريوسين بواسطة*L. carnosum* ، وقد أظهرت النتائج أعلى إنتاج للبكتريوسين مع نشاط نوعي 1093 وحدة دولية/ملجم بروتين عند إضافة 1.5%.تمت تنقية الببتيد النشط من الراشح البكتيري الخاص ببكتريا *L. carnosum*الخالي من الخلايا في ثلاث خطوات: (1) الترسيب بنسبة 40-60٪ من كبريتات الأمونيوم المشبعة (2) كروماتوجرافيا التبادل الكاتيوني (3) كروماتوجرافيا ترشيح الهلام. تم تشخيص الكارنوسين المنقى بتحديد الوزن الجزيئي باستخدام SDS PAGE أذ كان حجمه (KDa8) كما وفقد الكارنوسين النشاط المضاد للميكروبات بعد معاملته بالأنزيمات المحللة للبروتينات المختلفة (الببسين والتربسين والبروتينيز .(K كان الكارنوسين مستقراً للحرارة عند 100 درجة مئوية لمدة 15 دقيقة وفقد 13% من نشاطه عند 121 درجة مئوية لمدة 15 دقيقة، ومقاوم للأس الهيدروجيني عند المدى 2.0-11.0 ويفقد 15% من نشاطه عند الرقم الهيدروجيني 12.0. لم يؤثر كلاً من حمض إيثيلين ثنائي أمين رباعي الأسيتيك، كبريتات دوديسيل الصوديوم. توين 80 واليورياعلى الفعالية . كما أن المذيبات العضوية واملاح الأيونات المعدنية ليس لهما أي تأثير على نشاطه .حيث أظهر الكارنوسين المنقى نطاقًا واسعًا من النشاط المضاد للبكتريا الممرضة والمسببة لتلف الأغذية مثل *Escherichia coli* ، *Listeria monocytogenes*، *Staphylococcus aureus*، *Pseudomonas aeruginosa*، *Salmonella typhi*

الكلمات المفتاحية: بكتريوسين، كارنوسين، بكتريا حمض اللاكتيك، *Leuconostoc carnosum*، زيولايت.