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PARTIAL PURIFICATION AND CHARACTERIZATION OF PLANTARICIN WZD3, A BACTERIOCIN PRODUCED BY *LACTOBACILLUS PLANTARUM* WZD3

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ABSTRACT

For partial purification of plantaricin WZD3, a bacteriocin produced by *Lactobacillus plantarum* WZD3 which has antifungal activity against *Candida albicans*, three procedures were performed; adsorption-desorption method, cold-acetone extraction method and n-butanol extraction method. The results showed that n-butanol extraction method was the best since the activity of bacteriocin increased to 320 AU/ml. Characterization of plantaricin WZD3were also studied and the results showed that this bacteriocin have high thermostability at different temperatures, it remained active without losing its activity after being treated with (20- 80) °C for (10, 30 and 60) minutes, but it retained only 50% of its activity after treatment at 100°C at the same periods of exposure and at 121°C for 15 minutes. Also, the activity of bacteriocin was stable at pH values (4-7) while at the pH values (8-11), the antifungal activity was decreased to the half, on the other hand, complete loss of activity was observed at pH (2, 3 and 12). Antifungal activity of plantaricin WZD3 was disappeared when it treated with proteolytic enzymes (pepsin and trypsin), whereas it retained whole activity when treated with lysozyme and α-amylase, indicating pure proteinaceous nature of it. The results of effect of storage period revealed that the activity of plantaricin WZD3 was gradually decreased during storage. In conclusion, plantaricin WZD3 could be a good candidate as antifungal agent and its properties are promising to use it as biopreservative instead of chemicals preservatives.

Keywords: n-butanol extraction, plantaricin purification, characterization of plantaricin.

Introduction

Bacteriocins are multi-functional proteinaceous substances ribosomally-produced from bacteria have antimicrobial activity at certain concentrations (Chikindas *et al*., 2018). There are large variations in their producers which are divided essentially into three groups: Archaea, Gram-negative and Gram-positive bacteria (Riley and Chavan, 2007). Various strategies for the purification of bacteriocins from complex cultivation broths have exploited their cationic and hydrophobic characteristics (Cheigh *et al*., 2004). Usual methods for bacteriocins extraction are based on their affinity to organic solvents, their variation in solubility in concentrated salt solutions and at a given pH value. Bacteriocins are usually secreted in the growth medium, so when producer cells are grown in broth culture, bacteriocins can be harvested in the supernatant after centrifugation of the culture. Subsequent precipitation with ammonium sulphate is the favoured method of concentration from supernatants.

Solvent extraction has also been reported (Piva and Headon, 1994; Messi *et al*., 2001). Researchers such as ten Brink *et al*. (1994) showed that butanol extraction yields almost pure (≥ 90%) bacteriocin, and that this simple procedure could be adopted for partial purification of bacteriocins. Upon obtaining an active partially purified bacteriocin, Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC) is most commonly performed to purify the bacteriocin. Other techniques, such as ultrafiltration or filter assisted size exclusion protein fractionation, can be used for purification (Kaškonienė *et al*., 2017). A few protocols use dialysis to purify bacteriocins (Rather *et al*., 2016). Purification usually results in great increase in the specific activity, but with only a partial recovery (≈ 10-20%) of the initial activity (Kanatani *et al*., 1995). For this reason, antagonistic assays are usually conducted using crude extracts from the test isolates. Upon obtaining a purified bacteriocin, SDS-PAGE can be used to determine an approximate molar mass, and the bacteriocin can be sent to sequencing to obtain the amino acid sequence, or it can be detected using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Zhu *et al*., 2016). This lab-based bacteriocin discovery approach is time consuming, as well as labour intensive, requiring qualified technicians to use the equipment needed (Kaškonienė *et al*., 2017).

The nontoxic properties of Gram-positive bacteriocins, and much wider inhibitory ranges make them a unique beneficial tool for many medical and industrial applications; the most commonly known of these antimicrobials producing Gram-positive bacteria are lactic acid bacteria (LAB) (Zacharof and Lovitt, 2012). Bacteriocins produced by LAB are characterized by their biochemical, genetic, structural and metabolic activity. Most of them have limited molecular weight nearly from 3 to 10 kDa, are electrically neutral, have hydrophobic and hydrophilic regions, generally recognized as safe (GRAS) and have received a significant attention as an original approach to controlling pathogens in food products (Savadogo *et al*., 2004). The genus *Lactobacillus* is

part of the normal microbiota of human and contributes to the status of health of a various sites of the body, specifically the gastrointestinal tract (GIT) and vagina (Duar *et al*., 2017). It consists a great number of species GRAS and/or encompassed in the qualitative presumption of safety list, with many other strains broadly used in manufacturing of many foods or used as probiotics (Khandare and Patil, 2016; Salvetti and O'Toole, 2017; Koutsoumanis *et al*., 2019). Members of *Lactobacillus* produce a wide variety of bacteriocins that exhibit broader range of antibacterial or antifungal activity (Mokoena, 2017). *Lactobacillus* spp. are considered earnest of an alternative biological line to the combat of bacterial and fungal pathogens in the oral cavity, the GIT, and the urogenital system (Hu *et al*., 2013; Li *et al*., 2014). Some scientists in advanced countries have reported important *in vitro* inhibition of pathogenic vaginal *Candida* by certain lactobacilli species isolated from vaginal and non-vaginal sources (McLean and Rosenstein, 2000; Osset *et al*., 2001). In this study partial purification of plantaricin WZD3 and some characteristics were investigated.

Materials and Methods

Producer and indicator isolates

The bacteriocin producer was *Lactobacillus plantarum* WZD3, that isolated previously from yogurt and the indicator organisms were two isolates of *Candida albicans* (*C. albicans* CA and *C. albicans* CB) which obtained from Department of Biology, College of Science, Soran University, Erbil, Iraq.

Bacteriocin activity assay

To quantify the bacteriocin activity, Cell-free supernatant (CFS) was serially diluted two-fold with physiological saline solution. These dilutions were used to exam the inhibitory activity of bacteriocin against indicator yeast by agar well diffusion assay (Biyari and Fozouni, 2018). Bacteriocin activity was expressed as AU/ml and defined as the reciprocal of the highest dilution showing a distinct inhibition zone of the indicator yeast. AU was calculated as: (1000 / 100) × D, where 1000: constant, 100: volume of supernatant in a well (µl) and D: the dilution factor (Ołdak *et al*., 2017).

Partial Purification of bacteriocin

In order to choose the best method for concentration and partial purification of bacteriocin, three methods were used. All the methods were performed twice and the antimicrobial activity of bacteriocin in all of these methods was determined by the agar well diffusion method (Biyari and Fozouni, 2018):

1. Adsorption-desorption method

It was made according to Yang *et al.* (1992) with minor modification. Briefly, MRS broth was inoculated with the bacterial isolate and incubated at 37°C for 48 hours. Then, the culture broth was adjusted to pH 5.5 and heated to 70°C for 25 minutes to kill the cells which harvested by centrifugation at 15,000 x g for 15 minutes. Supernatant activity was determined by the agar well diffusion method. While the heat -killed cells were washed with sterile 5 mM sodium phosphate (pH 6) at a ratio (1:5) and resuspended in sterile 1.0 M NaCl solution (pH 2.0, adjusted with 5% phosphoric acid) at the same ratio (1:5), and mixed at 4°C for 1 hour. Then, cell suspension was centrifuged at 29,000 x g for 20 minutes, and the cells were re-suspended in 5 mM sodium phosphate (pH 6.5).

2. Cold-Acetone extraction method

MRS broth was inoculated with bacterial isolate and incubated at 37°C for 48 hours. Cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4°C. CSF was mixed with cold acetone at ratios (1:1, 1:2 and 1:3) separately and maintained at -20°C for 2 hours. The solvent and precipitate were separated by centrifugation at 6000 rpm for 15 minutes at 4°C, then was placed in glass petri dishes and allowed to evaporate in laminar cabinet at room temperature for 24 hours (Chung *et al*., 2011).

3. n-butanol extraction method

MRS broth was inoculated with bacterial isolate and incubated at 37°C for 48 hours. Cells were harvested by centrifugation at 6000 rpm for 15 minutes; CFS was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15 minutes (Powell *et al*., 2007). The supernatant was mixed thoroughly with n-butanol at a ratio 1:1.The mixture was centrifuged at 4000 rpm for 10 minutes to achieve phase separation .The organic phase was evaporated at 65°C by rotary evaporator, then the sediment was re-suspended in 1.0 mM sodium phosphate buffer (pH 6) and referred to as partially purified bacteriocin (PPB) (Abo-Amer, 2007).

Characterization of partially purified bacteriocin (PPB)

Effect of temperature

To evaluate thermal stability, PPB solution was heating to (20, 25,30,35,40, 60, 80, and 100) °C in a water bath for (10, 30 and 60) minutes respectively and 121°C for 15 minutes. All samples were cooled to room temperature and tested for remaining activity. It was calculated as follows:

$$\text{Residual activity}\,(\%) = \frac{\text{Remaining units}}{\text{Original units}} \times 100$$ (Ali, 2010)

Effect of pH

To evaluate pH stability, the pH of PPB solution was subjected to different pH values ranging from 2.0 to 12.0 (at increments of one pH unit) by mixed it with 1.0mM potassium phosphate buffer, After 30 minutes of incubation at 37°C, the pH of the sample was readjusted to the initial pH 5 with 1N NaOH or 1N HCl to evaluate the residual bacteriocin activity.

Effect of enzymes

To analyze sensitivity to various enzymes, PPB solution was incubated for 1 hour at 37°C in the presence of enzymes; α-amylase, lysozyme, pepsin and trypsin at a final concentration of 1mg/ml. After incubation, the enzymes were boiled for 3 minutes to denature it and cooled to room temperature and tested for remaining activity.

Effect of storage period

PPB solution was examined for stability during different storage temperature for different periods. About 5 ml of PPB solution was stored at different temperatures (37, 25, 4 and -20) °C for different times (one, two, three, four, eight and twelve) weeks. Then, the residual bacteriocin activity was assayed. All treated samples were tested for residual activity against *Candida* spp.by agar well diffusion method.

Results and Discussion

Partial purification of bacteriocin

In adsorption-desorption method, bacteriocin was extracted from CFS of *L. plantarum* WZD3 culture using the modified method described by Yang *et al.* (1992) based on the hydrophobicity and the charge of the compounds secreted by cells. The established method allowed extracting and partially purifying the bacteriocin molecules in a fraction called crude extract. Although, this method which developed in 1992 by Yang and his team to minimize steps used for bacteriocins purification which exploits their cationic nature, it is not suitable for all bacteriocins, for example; it was suitable for nisin, pediocin ACH and leuconocin Lcm1 which recover about 90% of bacteriocin but only 44% was recovered in case of sakacin A (Sharma *et al.*, 2019). In the present study, no recovery for bacteriocin was occurred and no antifungal activity against *C. albicans* CA and *C. albicans* CB by the agar wells diffusion method was observed. These results disagreement with those of Yang *et al.* (1992) and De Giani *et al.* (2019) that showed an increase in specific antimicrobial activity measured through the dimension of the inhibition halo, also they observed that the activity was not shown at neutral and alkaline pH, but it was recovered when the pH was lowered to 4, the lactobacilli physiological pH.

Cold-acetone extraction method is a method used for extraction of bacteriocin from bacterial culture broth by cold acetone (at −20°C). The results in figure 1 shows that the antifungal activity of bacteriocin was 20 and 40 AU/ml against *C. albicans* CA and *C. albicans* CB respectively at the ratio 1:1(CFS: cold acetone) , whereas at both ratios (1:2 and 1:3), the activity of bacteriocin was 20 AU/ml against both of indicator yeasts. Efficacy decreased due to the fact that bacteriocin from one bacteria to another has different effects when purifying with acetone. In the case of this bacteriocin, the efficacy decreased against *C. albicans* CA isolate, while efficacy against *C. albicans* CB isolate remained the same before and after purification with acetone. Similar results reported by (Daba *et al.*, 1994; Matikevičienė *et al.*, 2017).

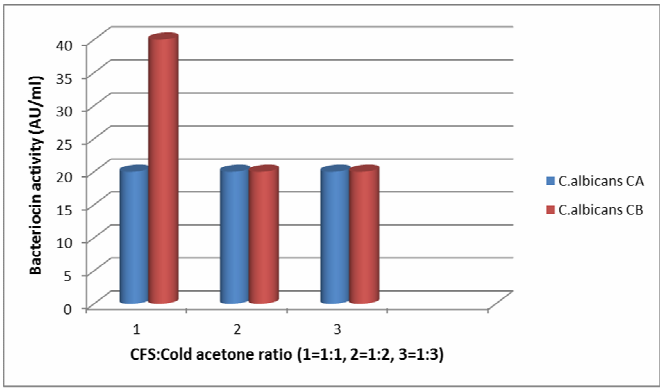

Fig. 1: Antifungal activity of bacteriocin extracted by cold acetone method against indicator yeast

In n-butanol extraction method, crude bacteriocin extract was heated at 80°C for 10 min before starting purification to denaturant proteases and any heat-labil proteins as investigated by Powell *et al.* (2007). Heating step did not affecting on bacteriocin activity, since the activity remaining 80 AU/ml against *C. albicans* CA and 40 AU/ml against *C. albicans* CB, respectively. Bacteriocin was partially purified by extraction with n-butanol in a ratio 1:1, it was removed from the aqueous phase and could be recovered from the organic phase. By using this method, the antifungal activity of bacteriocin reached to 320 AU/ml against both of *C. albicans* CA and *C. albicans* CB compared with antifungal activity of crude bacteriocin extract which was only 80 AU/ml against *C. albicans* CA and 40 AU/ml against *C. albicans* CB, respectively before partial purification with n-butanol (figure 2).

Butanol extraction exhibited complete recovery of bacteriocin activity, suggesting that at least part of the bacteriocin molecule has a hydrophobic character and shares this property with other bacteriocins (Daba *et al.*, 1991; Noonpakdee *et al.*, 2009). Extraction of bacteriocins using n-butanol in a 1:1 ratio was reported for plantaricin 35d (Messi *et al.*, 2001), plantaricin AA135 (Abo-Amer, 2007), plantaricin VGW8 (Ali, 2010) and plantaricin JY22 (Lv *et al.*, 2018).

It was found that the antifungal activity of the crude extract prepared by extraction with n-butanol was significantly higher than that of the other extraction methods used in this study. Therefore, n-butanol extraction method was selected as the best method for the partial purification of bacteriocin produced by *L. plantarum* WZD3. Okpara *et al.* (2014) showed that the partially purified inhibitory compounds obtained from *L. plantarum* have antagonistic activity against the growth of *C. albicans*.

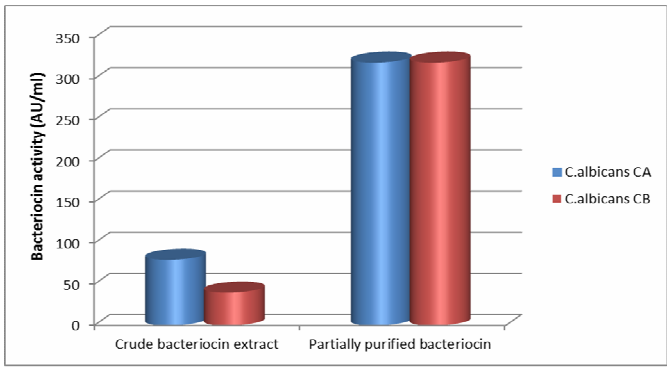

Fig. 2: Antifungal activity of partially purified bacteriocin produced from *Lactobacillus plantarum* WZD3 by n-butanol extraction method in comparison with crude bacteriocin extract against indicator yeast

Characterization of partially purified bacteriocin (PPB)

Figure 3 illustrates the effect of temperature on the antimicrobial activity of PPB produced by *L. plantarum* WZD3 against *C. albicans* CA and *C. albicans* CB. The PPB showed high thermostability at different temperatures (20, 25, 30, 35, 40, 60 and 80) °C for (10, 30 and 60) minutes, it remained active without losing its activity after being treated with (20- 80) °C for all three periods, but it retained only 50% of its activity after treatment at 100°C at the same periods of exposure to temperature and also it lost 50% of its activity after autoclaving treatment (121°C for 15 minutes). Heat stability of bacteriocin could be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions and stable cross-linkage. Temperatures stability is important if the bacteriocins are to be used as a food preservative, because many procedures of food preparation involve a heating step. Similar results were reported by (Navarro *et al.*, 2000; Messi *et al.*, 2001; Goh and Philip, 2015).

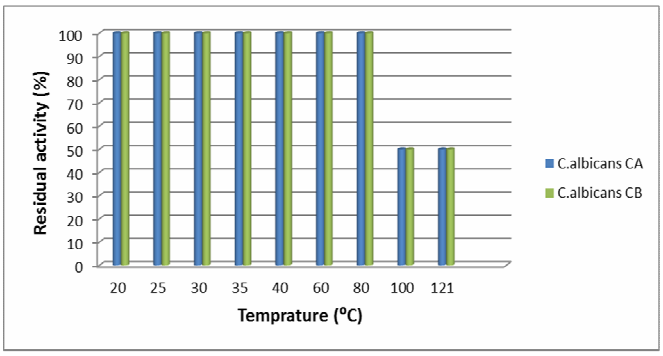

Fig. 3: Residual activity of partially purified bacteriocin (PPB) at different temperatures for 60 minutes (except 121°C for 15 minutes).

Results in figure 4 show that the activity of PPB was stable at pH values (4-7) while at the pH values (8-11), the antifungal activity was decreased to the half against both of indicator yeasts. On the other hand, complete loss of activity was observed at pH (2, 3 and 12). LAB bacteriocins are generally stable at acid or neutral pH, indicating that the substances are well adapted to the environment of the bacteria producing them. Similar results were reported by (Messi *et al*., 2001; Dhewa, 2012; Goh and Philip, 2015).

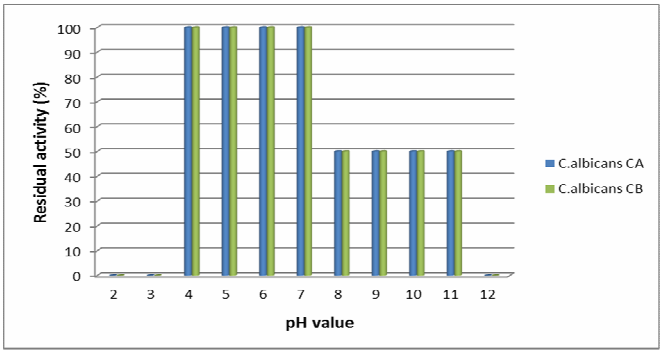

Fig. 4: Stability of partially purified bacteriocin (PPB) produced by *Lactobacillus plantarum* WZD3 at different pH values.

The effect of enzymes on the antimicrobial activity of PPB was investigated and the results showed that the antifungal activity of it was disappeared when it treated with proteolytic enzymes (pepsin and trypsin), whereas it retained whole activity when treated with lysozyme and α-amylase, indicating pure proteinaceous nature of PPB (figure 5). Similar results were reported by (Navarro *et al*., 2000; Chen and Hoover, 2003; Goh and Philip, 2015).

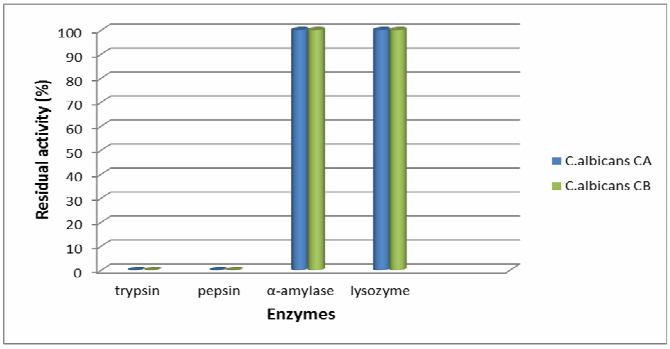

Fig. 5: Sensitivity of partially purified bacteriocin (PPB) produced by *Lactobacillus plantarum* WZD3 towards some enzymes

Effect of six periods of storage (one, two, three, four, eight and twelve) weeks on PPB activity against *C. albicans* CA and *C. albicans* CB was investigated at four different storage temperatures (-20, 4, 25 and 37) ºC. The results appeared that PPB maintained its full effectiveness at -20 ºC after the periods (one, two, three, four and eight) weeks while it retained only 25% of its activity after 12 weeks of storage (figure 6). At 4ºC, PPB maintained its full effectiveness after the periods (one, two, three and four) weeks while it retained only 25% of its effectiveness after (8 and 12) weeks of storage (figure 7). On the other hand, the PPB maintained its full activity after (one, two, three and four) weeks of storage periods, while it lost whole its activity after (8 and 12) weeks of storage at both of (25 and 37) ºC (figure 8). These results revealed that the activity of extracted PPB was gradually decreased during storage and indicating that the cold temperature may be the most appropriate preservation technique for bacteriocins (Ogunbanwo *et al*., 2003 ; Banerjee *et al*., 2013). The PPB in this study was called plantaricin WZD3 according to producer isolate *L. plantarum* WZD3. In conclusion, this bacteriocin might be a good candidate to use it as biopreservative and more studies are required about its antifungal activity against other types of fungi.

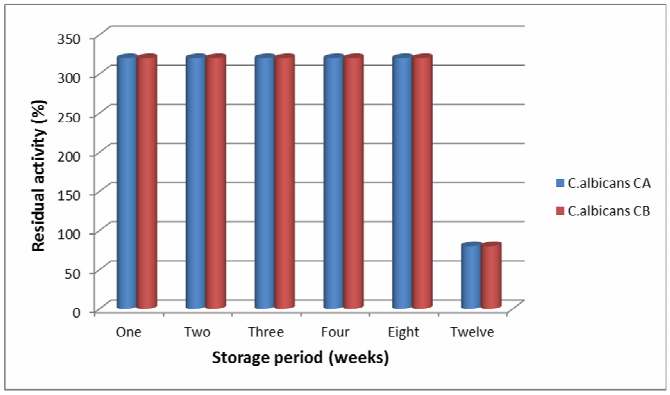

Fig. 6: Stability of partially purified bacteriocin (PPB) produced by *Lactobacillus plantarum* WZD3 at -20 ºC for different storage periods

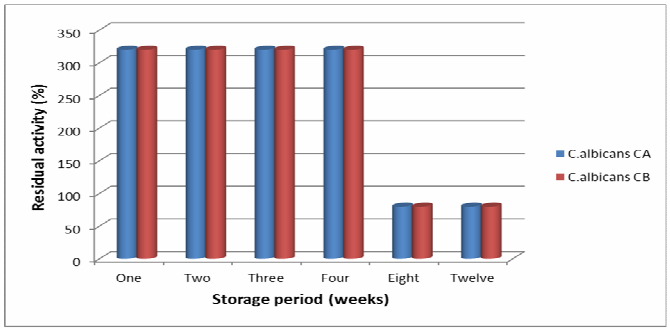

Fig. 7: Stability of partially purified bacteriocin (PPB) produced by *Lactobacillus plantarum* WZD3 at 4 ºC for different storage periods

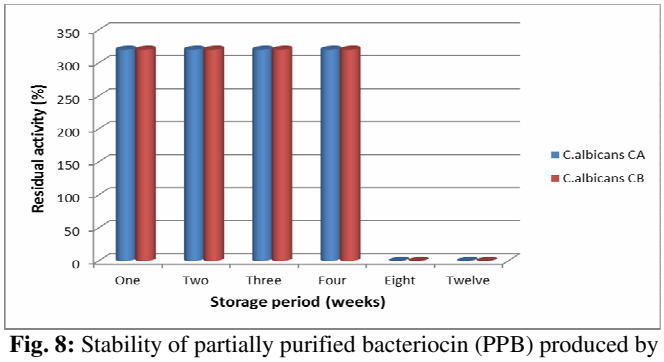

Fig. 8: Stability of partially purified bacteriocin (PPB) produced by *Lactobacillus plantarum* WZD3 at (25 and 37) ºC for different storage periods

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