

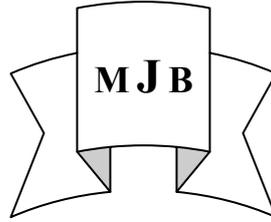
## Screening for Bacteriocins Production in Enteric *Bifidobacterium* Isolates and Study of Some Production Affecting Factors

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### Abstract

Enteric *Bifidobacterium* isolates were screened for bacteriocins production against food – born *Listeria monocytogenes* by agar – well diffusion assay. *Bifidobacterium longum B1* and *Bifidobacterium bifidum B2* was potent producer of antilisterial bacteriocins. Maximum bacteriocins production was achieved using MRS broth medium. The antilisterial activity appeared to be pronounced in mid logarithmic growth phase after 6h, 9h in *B1* and *B2* respectively. The optimum temperature and initial pH for bacteriocins production was 35°C and 6.5 respectively. Bacteriocin production decreased by the addition of NaCl to MRS broth. Glucose as a carbon source stimulated bacteriocin production in isolate *B1* (12.8 mm inhibition zone), while supplementation of MRS with fructose doubled bacteriocin production in isolate *B2* (13 mm inhibition zone). Inclusion of peptone to MRS broth clearly enhanced bacteriocins production in the two tested isolates, comparison to other tested nitrogen sources.

التحري عن إنتاج البكتريوسينات في بكتريا *Bifidobacterium* المعويه، ودراسة بعض

العوامل المؤثرة في الإنتاجية

### الخلاصة

اختبرت قدرة عزلات من بكتريا *Bifidobacterium* المعويه على إنتاج البكتريوسينات ضد بكتريا الاختبار *Listeria monocytogenes* ، بتقنية الانتشار بحفر الأغار، كانت العزلتين *B1 longum B.* و *B2 bifidum B.* الأغزر أنتاجاً للبكتريوسينات . وكان أعلى إنتاج للبكتريوسينات عند تنمية العزلات في وسط مرق MRS ، أبدأ إنتاج البكتريوسينات في منتصف طور النمو المتسارع بعد مرور 6 و 9 ساعات في العزلتين *B1* و *B2* على التوالي. وعند رقم هيدروجيني أبتدائي 6,5 و بدرجة حرارة 37 °م بوسط MRS. ينخفض إنتاج البكتريوسينات عند إضافة ملح الطعام NaCl الى وسط النمو . يعزز الكلوكوز تحفيز إنتاج البكتريوسين باعتباره مصدر كربوني في العزله *B1* (12.8 ملم قطر تثبيط) ، في حين تضاعف إنتاج البكتريوسين في العزله *B2* (13 ملم قطر تثبيط) عند إضافة الفركتوز لوسط MRS يعزز إضافة البيبتون من إنتاج البكتريوسينات باعتباره كمصدر نيتروجيني لوسط MRS مقارنة مع المصادر النيتروجينية الأخرى.

### Introduction

**B***ifidobacterium* members are the prominent group of microorganisms in the human intestinal flora of healthy breast - fed newborns, whereas they constitute more than 95% of total microbial population [1]. *Bifidobacterium* have

been shown several beneficial effects on host health, including, and protect the gastrointestinal tract from microbial infections [2]. Several mechanisms have been proposed to explain the efficacy of *Bifidobacterium* in preventing enteric infections, Bacteriocins are defined as

ribosomally synthesized proteins or protein complexes, usually antagonistic to genetically closely related organisms. Bacteriocins are produced by many bacterial species including lactic acid bacteria LAB (*Lactobacillus* and *Bifidobacterium*), many strains of these bacteria produce bacteriocins that have bactericidal effects particularly on other species or genera which live along in same microenvironments [3]. diverse bacteriocins have been identified from various species of LAB, including strains of *Bifidobacterium*, which their Inhibition of enteric pathogens is well documented, but isolation of bacteriocins by *Bifidobacterium* strains is still not well documented like *Lactobacillus*, very few studies exist in this issue [4]. To date some bacteriocins such as, bifidiocin I, bifidiocin B, and bacteriocin – like inhibitory substances (BLIS) have been purified and characterized, and found to inhibit growth of species of food – borne pathogenic bacteria like invasive *Listeria*, *E.coli*, *Salmonella*, and *Bacillus cereus*[5].

*Listeria monocytogenes* is the causative agent of listeriosis [6], it's one of the most virulent food borne pathogens, with 20 to 30 % of clinical infections resulting in death worldwide. The ingestion of products contaminated with this organism may be a potential health threat to high-risk population such as immune-suppressed, children, pregnant women, and the elders [7]. The ubiquitous nature of *L. monocytogenes*, its ability to survive and grow in wide range of food products, its ability to survive adverse conditions such as vacuum, freezing, ultraviolet ray, and to resist conventional pasteurization, have heightened the awareness of this pathogen as a public health problem over the past decade [8]. Antilisterial bacteriocins production demonstrated

in human isolated *Bifidobacterium* [9], and because of most of *Bifidobacterium* members are reported as GRAS (generally regarded as safe), these microorganisms and their bacteriocins are novel and a good sources as biological preservative and also in human therapeutics [10].

The aim of this study was to present some data on bacteriocins production of local human enteric *Bifidobacterium* isolates, and study of some factors affecting their production .

## **Materials and Methods**

### **Bacteria and cultural conditions**

Twelve enteric *Bifidobacterium* isolates used in the study, were previously isolated from healthy breast – fed infant feces [11], activated in de Man, Rogosa and Sharpe broth medium (MRS) supplemented with 0.05% (w/v) L -cysteine HCl, incubated under anaerobic condition (anaerobic jar and gas pack) at 37°C for 48h. Cultures were streaked on MRS agar plates several times. The interesting isolates were identified to species level by sugars fermentation profile of human strains and compared with sugars fermentation scheme described in Bergey's manual of systematic bacteriology [12]. *Bifidobacterium* isolates maintained in MRS broth with 20% glycerol at – 18 °C as stock cultures, *Listeria monocytogenes* obtained from biology dept. Baghdad university, used as indicator bacteria, propagate in brain heart infusion medium (BHI)

### **Bacteriocins production screening**

*Bifidobacterium* isolates were analyzed for their antagonistic activities against indicator bacteria *Listeria monocytogenes* by agar – well diffusion assay [13]. Cell free solutions were prepared by, centrifugation of MRS broth grown cultures (6000 rpm for 30 min. at 4°C), obtained supernatants were neutralized to pH7

with 1N NaOH to exclude the antimicrobial effects of organic acids [14], filtered through 0.2 $\mu$ m pore-size filter, and 5 $\mu$ g/ml of catalase was added to eliminate the inhibitory activity of hydrogen peroxide[15], melted BHI agar seeded with overnight culture of *L. monocytogenes* at a final concentration 10<sup>6</sup> cell/ ml, poured into sterile Petri dishes and allowed to solidify at room temperature, wells 5mm were hollowed out in agar using a sterile cork borer, a volume of 50 $\mu$ L of tested supernatants were dropped separately in each well, and incubated at 4°C for 6h to facilitate diffusion into agar, plates finally were incubated at 37C for 48h, formed inhibition zones around the wells were measured and recorded in millimeter after subtraction 5mm (wells diameter)

#### **Effect of growth media on bacteriocins production**

Three culture media were used for bacteriocins production, MRS broth, yoghurt whey and date.

Yoghurt whey medium was prepared by adjusting the pH of yoghurt whey to 7 then autoclaved at 121°C for 15 min, the denatured precipitated proteins removed by centrifugation (6000 rpm 10 min. at 4°C), and the obtained supernatants used as a culture medium[16].

Date medium prepared from Iraqi Al-Zahdi dates, 100 gm of seedless date soaked in 300 ml of warm water for 2h, after the mixture filtered through several layers of gauze, autoclaved at 110°C for 15 min .and used as date medium.[17]

Fresh culture of the two bacteriocin producer isolates were inoculated 1% (v/v) into MRS broth, Yoghurt whey, and date medium, respectively, all cultures incubated at 37C for 48h. Antilisterial bacteriocins production was evaluated for each tested culture by mean of inhibition zones diameter (mm)

#### **Bacterial growth and bacteriocins production**

Bacteriocin producer isolates were grown in MRS broth medium for 48 h., samples were withdrawn at intervals during incubation period (0,3,6,9,12,15,18,21,24,36, and 48 h), growth monitored by measuring the optical density at 600 nm (OD<sub>600</sub>), and antilisterial bacteriocins production detected by measuring inhibition zones diameters(mm).

#### **Optimization of culture conditions**

*Bifidobacterium* selected isolates were subjected to different conditions to derive the optimum conditions for bacteriocins production. Temperature, pH, and sodium chloride (NaCl).

- **Optimization of incubation temperature:** MRS broth was inoculated with fresh culture 1% (v/v) of tested bacterial isolates, incubation was continued at 20, 25, 30, 35, 37, 40, and 45°C for 24h, and antilisterial activity of each bacteriocin was observed in term of inhibition zones diameter (mm).

- **Optimization of pH:** MRS broth were adjusted to different pHs value (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0), seeded individually with fresh cultures 1% (v/v) of tested bacteria isolates, incubated at 37°C for 24 h, and antilisterial activity detected in term of inhibition zones diameter(mm).

- **Effect of NaCl:** MRS broth was supplemented with different concentration of NaCl 0.5, 1.0, 1.5, 2.0, and 2.5 % (w/v), inoculated with 1% (v/v) fresh culture of tested bacterial isolates, incubated at 37°C for 24h, and antilisterial bacteriocins level detected.

#### **Effects of carbon and nitrogen sources on bacteriocins Production**

Various sugars, fructose, lactose, sucrose, and starch, 2 % (w/v) as a carbon sources supplemented into MRS broth, and their effects on bacteriocins production were

investigated in comparison to the classical MRS broth (glucose sole carbon source). The effect of two nitrogen sources, tryptone and peptone 2% (w/v) was studied in comparison to the classical MRS broth medium (yeast extract as nitrogen source). Fresh culture 1% (v/v) of bacteriocin producer isolates were inoculated individually to the MRS broth supplemented with previously mentioned ingredients, all cultures were incubated at 37C for 24h, and the antilisterial activity level are detected as described previously.

**Results and Discussion**

Twelve human infants fecal Bifidobacterium isolates were screened for bacteriocins production against indicator bacterium *L. monocytogenes* by agar – well diffusion assay, only two isolates (17%) were showed antilisterial activity detected by zones of inhibition (mm), they were selected as potential bacteriocin producers. The Two candidates were designated as **B1** and **B2**, identified to the species level according to sugars fermentation profile of human strains (Table.1). The two isolates were belonged to the following species:

*Bifidobacterium infantis* **B1**, and *Bifidobacterium . bifidum* **B2**.

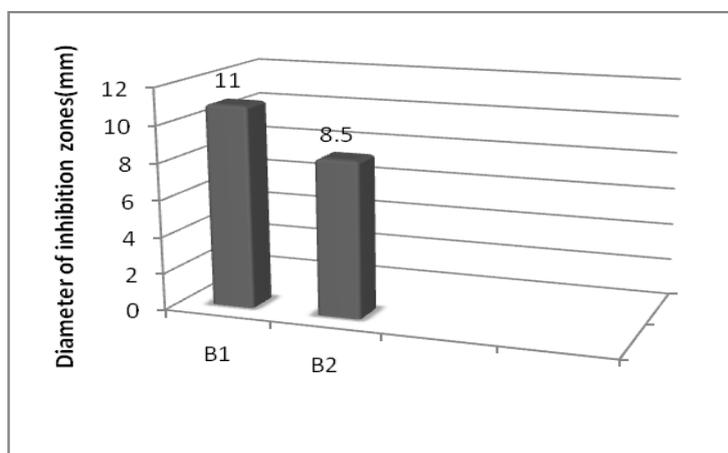
**Table 1** Sugars fermentation profile of Bifidobacterium isolates

Bacterial isolates	glucose	fructose	lactose	galactose	Maltose	trehalose	Xylose	raffinose	ribose	Suggested species
<b>B1</b>	-	+	-	-	-	-	-	-	-	<i>Bifidobacterium. infantis</i>
<b>B2</b>	+	+	+	+	+	-	-	-	-	<i>Bifidobacterium . bifidum</i>

(+Positive reaction , - Negative reaction)

A maximum antilisterial activity was shown by the isolate **B1**, it exhibited a larger inhibition zone with 11 mm

diameter, while the isolate **B2** showed 8.5mm diameter inhibition zone (Fig -1).



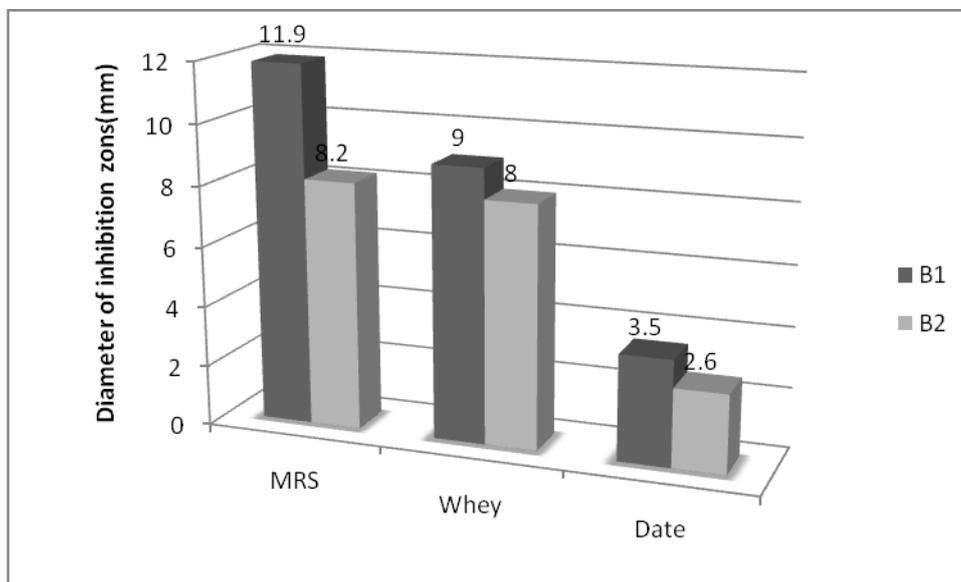
**Figure1** Inhibition zones of *Bifidobacterium* isolates (**B1**,**B2**) against *L. monocytogenes*

The antagonistic effects of the isolates **B1** and **B2** against *L. monocytogenes* appeared to be pH independent and seems to be related to the production of bacteriocins, whereas, the bacterial supernatants pHs adjusted to 7 prior to detection to eliminate the antimicrobial effects of organic acids (acetic acid and lactic acid), which are the major metabolites of *Bifidobacterium* that drop pH, which may be sufficient to antagonize many pathogens [18], and the inhibitory activity of hydrogen peroxides also was eliminated by the addition of catalase[26], so the inhibitory activity attributed to the bacteriocins production, in that several previous studies have reported the ability of intestinal strains of *Bifidobacterium* to produce antibacterial compounds with potent antilisterial activity [4,19]

In this study just two of tested *Bifidobacterium* isolates exhibited antagonistic activities against target bacterium, although both bacterium life along with same environment, human gastrointestinal tract which induce the production of inhibitory substances by

*Bifidobacterium*, as was reported previously in that 99% of enteric *Bifidobacterium* strains make at least one bacteriocin[18]. The failure of other tested strains to inhibit *L. monocytogenes* might due to a lack of cell – cell contact between the indicator bacterium and producer bacteria by detection procedure agar – well diffusion assay [13].

Bacteriocin production was carried out in three culture media, MRS broth medium as synthetic complex commercial recommended medium for bacteriocins production, and two row food wastes, yoghurt whey, and date medium. Bacteriocins production was varied clearly among these media ,the two former media supported bacteriocins production, a maximum bacteriocins level were appeared in MRS medium for the two producer isolates, and the isolate **B2** revealed no significant difference between MRS medium and Yoghurt whey medium in bacteriocins yield. Both bacteria showed poor bacteriocins level in date medium(Fig.2 )



**Figure 2** Influence of different culture media on bacteriocins production by *Bifidobacterium* isolates **B1** and **B2**

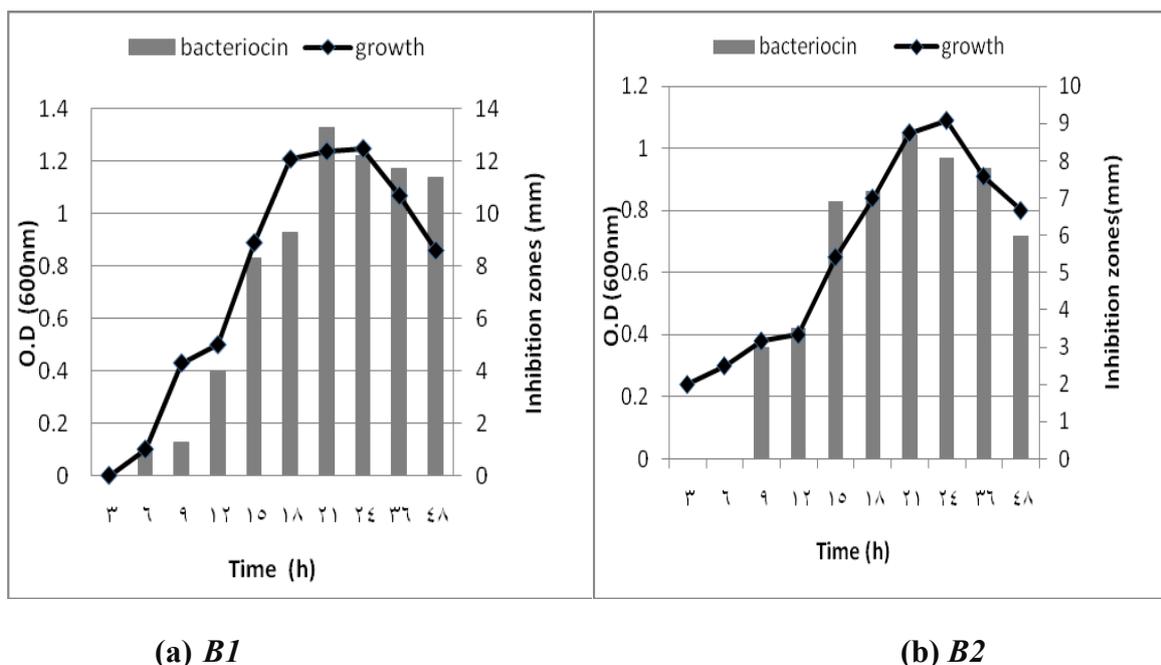
Production of bacteriocins depended on type of culture medium, and influenced by media composition , MRS medium seemed to be more suitable medium than other tested media, similar results were observed in

previous studies [20], because it favoring bacterial growth and high cell densities are frequently beneficial for bacteriocins production . Although bacteriocins production is often performed in complex media mostly

MRS, which promote abundant growth and relatively high level of bacteriocins, but in recent years it seems more economical to use some of west of food as the raw materials, such as; soy been milk, soy been residues , dairy why, and molasses, In this study yoghurt whey medium appeared to be the best raw material medium for Production, due to it supplementation of some prebiotic substances or hydrolyzed milk that enhance the growth and survival of *Bifidobacterium* which corresponds to bacteriocins production [21]. As it was seen date medium doesn't support bacteriocin production, this was in agreement with the finding of Al-zahrani and Al-Zahrani [16], which

may be related to the chemical composition of the date that proved to have not only the highest percentage of monosaccharide such as glucose and fructose, but also more complex sugars that are so difficult to assimilate by bacteria.

The effect of incubation period on bacteriocin production was studied using MRS broth medium. It was observed that the production in the two tested isolates were found in the middle of logarithmic growth phase, it detected after 6h in isolate *B1* and 9h in *B2*, the production peaked at the mid of stationary phase and remained relatively stable long after growth had ceased (Fig. 3 - a,b ).



**Figure (3 a,b)** Growth and bacteriocins production by *Bifidobacterium* Isolates *B1* and *B2*

Bacteriocins production occurs during the active growth phase, as the production was observed with onset of logarithmic phase and early of stationary phase, which related to the highest densities of bacterial cells, this was also confirmed in production of plantaricin KW30 and bacteriocins of *Lactobacillus delbrueckii* in MRS medium [22]. Detection of the two bacteriocins in

logarithmic growth phase, indicating that the bacteriocins are primary metabolites, which is in agreement with the bacteriocins production data from LAB [23].

The Effects of physical factors on bacteriocins production including temperature, pH, and different concentrations of NaCl, were estimated using MRS broth (Table-2).

Bacteriocin production detected at all tested temperatures(20, 25, 30, 35, 37, 40, and 49°C), the rate of production was increased with an increase of temperature, the highest activity was observed at 37°C, (12.4 and 9 mm inhibition zone for *B1* and *B2* respectively), after 40°C the incubation temperature had a significant adverse effect on production. Regarding pH, optimal pH for bacteriocins production was 6.5 in the both producer isolates, they revealed maximum bacteriocins

production level, as 10.3 mm inhibition zone for *B1* and 8.7 mm for *B2*. Regarding the effect of various concentration of NaCl, the antilisterial activity in both tested bacterial isolates was significantly lower, but didn't destroyed completely by the addition of different concentration, the highest level of bacteriocins were recorded in MRS broth with the lowest NaCl concentration 0.5%.

**Table 2** Effect of environmental factors (temperature, pH, and NaCl) on bacteriocins production

Diameters of Inhibition zones(mm)		
	<i>B. infantis B1</i>	<i>B. bifidum B2</i>
<b>Temperature(°C )</b>		
20	1.6	1.0
25	1.8	1.8
30	5.4	4.0
35	9.3	7.4
37	12.4	9.0
40	4.3	5.8
45	2.2	1.3
<b>Initial pH</b>		
5	0	0
5.5	4.8	2.5
6	6.8	4.6
6.5	10.3	8.7
7	7.2	6.6
7.5	5.4	5.1
8	2.3	2.0
<b>NaCl (%)</b>		
0.5	3.2	3.0
1	3.0	2.4
1.5	1.5	1.5
2	1.2	0.8
2.5	0.8	0.5

Understanding the influence of environmental factors on the induction of bacteriocins is essential for the effective commercial application of bacteriocin – producing LAB in biopreservation and therapeutic application, It seems that bacteriocins production is influenced strongly by several environmental factors, such as pH, temperature, and NaCl [24]. These environmental factors may influence growth negatively and thereby the

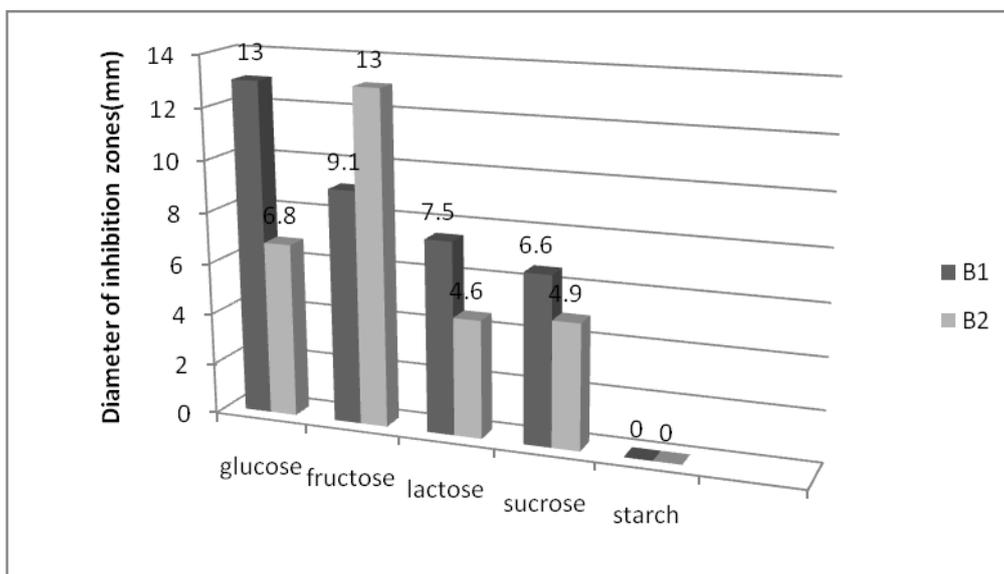
secretion of bacteriocins, Further, it has been suggested that some of environmental factors reduce the binding of bacteriocins to its receptors on target microorganisms [25].

Most of *Bifidobacterium* members are mesophilic, which prefers growth in a thermal ranges 35 – 40° C, after growth are ceased, and all metabolic activities, including bacteriocins production occurs within these temperatures[26], and this was also

confirmed previously in production of different human bifidobacterial bacteriocins [27]. pH is known to be important for biomass as well as bacteriocin production, because , aggregation, adsorption of bacteriocin to the cells and /or proteolytic degradation depend on pH and can affect bacteriocin activity [28]. The negative effect of high salt concentration on bacteriocin production has been previously reported for *Bifidobacterium* [29], however low concentration of sodium chloride (1 to 2 %, w/v) can sometimes enhance bacteriocin production, as it was proved in previous researches with curvacin *A* production [30]. The protective effect of sodium chloride presence on *L. monocytogenes* may be due to the presence of Cl<sup>-</sup> anions that inhibit the binding of bacteriocin molecules to the surface of the cell

membrane as proposed by *Bhunia et al* [31].

Different carbon sources were used for bacteriocins production, it was observed that glucose was the best carbon source for production in isolate **B1**, it exhibited a maximum production rate ( 12.5 mm inhibition zone) compared to the remaining sugars, while production relatively was doubled (13mm inhibition zone) with fructose supplementation in the isolate **B2**. Supplementation of MRS broth with lactose and sucrose had negative effects on production, whereas, the production was reduced by 57% , 68% in presence of lactose, and by 50%, 72% in presence of sucrose in **B1** and **B2** respectively, and no bacteriocins production was recorded in case of starch supplementation ( Fig.4).



**Figure 4** Effect of carbon sources on bacteriocins production in *Bifidobacterium* Isolates **B1** and **B2**

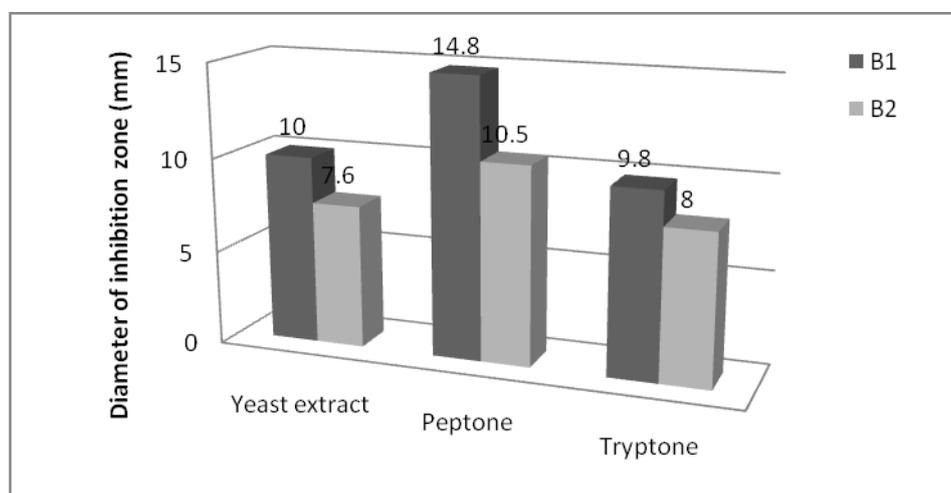
Different sugars has been successfully used in many studies for optimization of bacteriocin production in different bacterial strains, [32], however, glucose remained the best carbon source stimulated bacteriocins production, in that most workers demonstrated high

bacteriocins yield in association with the presence of glucose in growth media and not other monosaccharide's [33]. Because glucose is considered the main carbon source by most of microorganisms due to its rapid utilization and cellular energy conversion[14], however some

bacteriocins producer bacteria revealed high yield of bacteriocins production in association to inclusion of other sugars to growth media rather than glucose, may they have a complex enzymatic system that allow them to use other carbohydrates, for example *Enterococcus faecium* showed a variable sugar utilization rather than glucose for bacteriocins production [34], that suggest specific nutrients are required some times for production of bacteriocins. The similar effect of sucrose and lactose was also confirmed in nisin production by *Lactococcus lactis* subsp. *Lactis*. The negative effect of starch on bacteriocins, perhaps because the bacteria were

adsorbed to the surface of starch molecules that could ceased their good utilization[35]

(Fig.5) Shows the dependence of bacteriocin production on type of nitrogen source, replacement of yeast extract with peptone improved the yield of production by 1.4 relative to the using of classical MRS, whereas the two isolate **B1** and **B2** were displayed optimal yield of bacteriocins, 14.8mm and 10.5mm inhibition zone respectively, and the presence of tryptone led to slight increase of production in isolate **B2** with 8mm inhibition zone, but had no effect on isolate **B1**.



**Figure 5** Effect of nitrogen sources on bacteriocins production by *Bifidobacterium* isolates B2 and B2

Most of human indigenous bacteriocins producers microorganisms could not grow well and produce bacteriocins only with organic nitrogen sources, possibly for the absence in vitamins and DNA precursors, which was rich in yeast extract [36]. Substituted yeast extract with other nitrogen sources in classical MRS medium have variable effects on bacteriocins yields, but the stimulation of bacteriocin production by addition of peptone not agreed with the finding of researchers in respect with this subject, which found the enhancement of bacteriocin with presence of yeast extract and in some extent tryptone [37], maybe this is the first indication that the peptone is the key nitrogen source needed to production of bacteriocins by members of *Bifidobacterium*.

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