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Research Article

## Study the optimum conditions for biosurfactant and bacteriocin production from *Lactobacillus plantarum* isolated from different sources

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### Abstract

The goal of current research is to study the optimal conditions for the biosurfactant (lipid) and bacteriocin production from local isolate *Lactobacillus* sp. A total of 120 *Lactobacillus* sp. isolates were obtained from several sources, recognized according to biochemical tests, and screened utilizing primary and secondary screen processes for bacteriocin and lipid production. According to the observed results, the isolate *Lactobacillus* sp. ZMJ20 obtained from the clinical sample of healthy Iraqi females revealed to the highest lipids emulsifying activity  $E_{24\%}$  (60%) and decrease in surface tension to 29.2 mN/m and higher bacteriocin inhibitory impact against pathogenic isolate *Candida albicans* with a diameter of (22 mm). Therefore, the isolate *Lactobacillus* sp. ZMJ20 was selected for identification and optimization studies. The isolate was recognized as *Lactobacillus plantarum* by VITEK 2 system and ensured via molecular identification by PCR technique. Modified MRS medium was used in the fermentation processes for bacteriocin and lipid production by of *L. plantarum*. The findings of optimum conditions showed that fructose was the best source of carbon and energy instead of dextrose (glucose) and the best nitrogenous sources were meat and yeast extract at ratio (1: 1) with a percentage of (2.5%) instead of peptone, meat, and yeast extract together. The ideal pH was (6.5), the optimum temperature was (37°C) and the best incubation period for bacteriocin and biosurfactant (lipid) production was after 48 and 72 hours respectively. All the production processes are adopted under anaerobic conditions.

**Keywords:** Bacteriocin, Biosurfactant, Emulsification, Antifungal, Inhibition

### Introduction

Humans have a normal microbiota that includes the species *Lactobacillus*, which has essential roles in the health of the GIT and the vagina (Duar et al., 2017). It includes several species that are both generally recognized as safe (GRAS) and included in the qualitative presumption of safety list, as well as numerous strains that are often employed in the production of a wide range of foods and probiotics (Salveti et al., 2017; Koutsoumanis et al., 2019). Bacteriocins are produced by many different *Lactobacillus* strains, and these bacteriocins have a wide spectrum of antibacterial and antifungal activities (Mokoena, 2017).

Antibiotic resistance rate is troubling, as the discovery of novel antibiotics with diverse action mechanisms has slowed

considerably. The recent increase in antibiotic resistance cases has prompted scientists to re-evaluate alternate therapeutic options (Holmes et al., 2016). One choice to compensate lack of innovative antibiotics is to incorporate several beneficial compounds, such as bacteriocins, and biosurfactants as therapeutic strategies in clinical sites for the best treatment and control of fungal diseases (Seddighi et al., 2017).

Bacteriocins of Lactic acid bacteria (LAB) have significant applications in biotechnology since they are susceptible to proteases, non-toxic to human, constant at low pH, and simple to produce (Todorov, 2009). Most bacteriocins have a low molecular weight (3-10kDa) with hydrophobic and hydrophilic areas, have neutral electricity, and are GRAS (Savadogo et al., 2004). Biosurfactants are microbial-origin surface-active agents (bacteria and fungi) with hydrophobic

and hydrophilic areas (Okoliegbe et al., 2012). Biosurfactant controls consistency, slow staling, and solubilizes flavor oils in ice-cream and baking formulations, while medicinal and biomedical biosurfactant applications include antibacterial, anticancer, and astringent properties drugs for respiratory failure (Gautam et al., 2006).

Fungal infections are among the tough manageable diseases in human, particularly in the immunocompromised patients of AIDS, organ transplants, blood cancer and in neonates, with around 1.7 billion people suffering from these infections worldwide (Denning et al., 2015). *Candida* species are considered the most common reasons of mycotic invasive diseases in developed countries (Bassetti et al., 2016). The most important species regarded to be human pathogenic are *C. viswanathii*, *C. lusitaniae*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. albicans* (Chander, 2017). Recently, the emergence of strains resistant to antifungals drugs and failures of treatment after a high or long-term use of these drugs by patients has increased (Zahabi et al., 2020).

## Materials and Methods

### Collection and preparation of Samples for isolation of *Lactobacillus* spp.

One hundred and forty-two samples were gathered from various sources include (food and clinical) samples, with twenty-two samples coming from food (yogurt, cheese, liquid milk, Sourdough, and fermented vegetables) and one hundred clinical samples (sixty vaginal samples from healthy women). Besides these 20 ready isolates obtained from the environment laboratory in the Biotechnology Department at Science College, University of Baghdad. Another samples also were collected from the mouths of 30 infants and 10 adults. These samples included both stool and vomit as well as the contents of the infants' digestive systems.

### Isolation of *Lactobacillus* spp.

*Lactobacillus* isolates was isolated according to the procedure mention by Oyewole et al., (1990). the isolates subjected to biochemical identification tests (Karami et al., 2017) and then all bacterial isolates were kept in glycerol at 4°C.

### Identification of *Lactobacillus*

#### Biochemical identification

The selected *Lactobacillus* isolate was subjected to biochemical tests (GP card consist of 47 substrates) to determinate species of isolate. To perform this test, specific steps were carried out based on the manufacturer's instructions.

#### Molecular identification

PCR reactions were amplified utilizing a thermal cycler equipment (labnet, USA). Here's how the parts were put to use: A Master Mix (12.5 µl) containing buffer, deoxynucleosides (dNTP), MgCl<sub>2</sub>, *Taq* DNA polymerase and 1 µl (10 pmol) of each primer was used. One microliter of 100ng of DNA was utilized as a template, and 8.5 µl of deionized water were added to get the volume up to 25 µl. Oligonucleotide primers for polymerase chain reaction (PCR) were generated utilizing the NCBI reference sequence for *Lactobacillus*. The primer sequences were designed as follows:

**Table 1. The primers utilized in this study**

Primer	Seq. (5' – 3')	Annealing Temp. (C°)	Product size (bp)
F	AGAGTTTGATC CTGGCTCAG	60	1500
R	TACGGTTACCT TGTTACGACTT		

The *16s rRNA* gene amplification was performed as following program.

**Table 2. Program setup for amplification process (PCR Program)**

Steps	Temperature °C	Time	No. of Cycle
Initial Denaturation	95	Five minutes	One
Denaturation	95	Thirty seconds	Thirty
Annealing	60	One minute	
Extension	72	One minute	
Final extension	72	Seven minutes	One
Hold	10	Ten minutes	

Sanger sequencing was performed on PCR products utilizing an automated sequencer of DNA (ABI3730XL) from Macrogen Corporation in Korea. The finding was sent to us through email, and our brilliant program did the rest of the work.

### Screening program for biosurfactant (lipid) production

#### Primary (Qualitative) screening

Hundred-forty-two *Lactobacillus* sp. isolates were screened for production of biosurfactant utilizing plate assay on

medium of blood agar, phenol red agar plate and Cetyl trimethylammonium bromide CTAB/ methylene blue solid medium as described by [Anandaraj et al., \(2010\)](#).

### Secondary (Quantitative) screening for biosurfactant (lipid) production

#### Bacterial inoculum preparation

Isolates were cultured from MRS agar stocks by inoculating MRS broth with the bacteria and incubating it in an anaerobic Jar at 37°C for 48 hours. Following incubation, a spectrophotometer counted the cells in the suspension, and the resulting  $1 \times 10^8$  cell/ml (O.D 600 nm) cell concentration was achieved by diluting the cells in the same broth.

#### Biosurfactant production in liquid media

The most productive primary screen isolates were chosen for further cultivation in MRS broth and then subjected to secondary screening for biosurfactant production in liquid media. A flask (250 ml) containing fifty milliliters of MRS medium was added into 1.0 ml ( $1 \times 10^8$  CFU/ml, OD = 0.5) *Lactobacillus* inoculum and incubated at 37°C for 72 hours in a shaker (120 rpm) in anaerobic condition utilizing flashing system of N<sub>2</sub> gas, then the cultures were placed in the centrifuge at eight thousand rpm, for 20 mins, neglect supernatant and taking the pellet. Then, the cells were washed two times with D.W and resuspended in ten milliliters of phosphate buffer saline and were kept at 25°C for 24 hours then the cells were sonicated at 600Hz for 2 min with ice bath

#### Screening program for bacteriocin production

The well-diffusion assay was used as described by [Lewus et al., 1991](#) for estimate the best producing-bacteriocin isolate. *Candida albicans* was utilized as indicator. The activated yeast suspension, at a concentration of  $10^6$  cells per millilitre, was diluted to 0.1 millilitre and plated on SDA agar. Pour plates were drilled with a 6mm sterile cork borer to create wells for the agar wells diffusion procedure, and 100 µl of CFS was poured into each one. After incubating the plates aerobically at 30°C for 24 hours, they were kept at room temperature for 2 hours. The diameter of the inhibitory zone surrounding the paper discs or wells was calculated using a digital ruler and expressed in millimetres. the reaction between the organic acid and the H<sub>2</sub>O<sub>2</sub>, sodium hydroxide, and catalase solution.

#### Activity assay of bacteriocin

The bacteriocin activity assay was measured as described [\(Pilasombut et al., 2005\)](#), whereas Serial two-fold dilutions of

Thereafter, the bacterial suspension was centrifuged at 8000 rpm for 20 min. The supernatant was considered as intracellular biosurfactant (lipid) production. According to [Tahmourespour et al. \(2011\)](#), the supernatant was filtered through a sterile 0.22 mm pore-size filter (Millipore).

#### Measurement of Surface tension (S.T)

Using a Wilhelmy platinum plate and a QBZY-2 Tensiometer (China), we were able to make an educated guess as to the surface tension of an aqueous solution. The tensiometer was calibrated by placing 20 ml of supernatant in a 50 ml glass beaker. After placing the plate in the solution, the temperature was held constant at 25 °C while the surface tension of the supernatant was measured. The Wilhelmy plate was cleaned with acetone and then charred with an alcohol burner in between readings. According to [\(Sekhon et al., 2011\)](#), researchers utilized an average of three recordings to get a more reliable result.

#### Emulsification Index (E24%) measurement of biosurfactants

Two milliliters of cell-free supernatant were placed into two milliliters of toluene, vortexed over two minutes, and kept unattended for 24 hours. The height of the emulsifier layer was measured at at  $25 \pm 1$  °C. The emulsification index is calculated by multiplying the height of the emulsified layer (mm) by the entire height of the liquid column (mm) and multiplying by 100. measured according to [\(Entissar et al., 2013\)](#) and as the following equation:

$$\text{Emulsion Index (E24)} = \frac{\text{Height of emulsion layer}}{\text{Total height of broth}} \times 100$$

cell-free supernatant CFS (crude bacteriocin) were prepared by using physiological saline solution. These dilutions of bacteriocin were examined the inhibitory activity against indicator yeast (*Candida albicans*) by assay of well agar diffusion [\(Lewus et al., 1991\)](#) and the results were expressed as the arbitrary unit (AU) in the equation as follows:  $(1000 / 100) \times DF$ , where DF: the dilution factor, 100: volume of supernatant in a well (µl) and 1000: constant [\(Oǻdak et al., 2017\)](#).

#### Optimal conditions for the production of biosurfactant (lipids) and bacteriocins

The best conditions for production biosurfactant (lipids) and bacteriocins from a locally selected strain were discovered. Modified MRS fermentation media (PM), pH, temperature, type of carbon and nitrogen supplies, and incubation time were among the factors. Optimization tests were carried out in an Erlenmeyer flask containing 50 ml of selected fermentation media and 1% fresh bacterial culture. The flasks were kept at 37 °C celsius degrees in a shaker incubator (120 rpm). At the

end of each condition, the bacteriocins activity by Well-Diffusion assay and biosurfactant production by measurement of surface tension and E24 (%) were determined.

### Impact of carbon sources

Fifty ml of MRS broth without glucose was created in Erlenmeyer flasks (250 ml) and supplemented with (20 g/L) of several carbon sources (Arabinose, glucose, lactose, fructose, starch, mannose). Different carbon sources were employed after autoclaving for 10 minutes. The flasks were autoclaved for 10 minutes before being inoculated with 1% ( $1 \times 10^8$  CFU/ml, OD = 0.5 on McFarland) *Lactobacillus* isolated and incubated in a shaker incubator (120 rpm) in anaerobic circumstances with N<sub>2</sub> gas (flashing system) at 37°C for 48 and 72 hours for production of bacteriocins and biosurfactant respectively.

### Impact of nitrogen source

In each 250 ml Erlenmeyer flasks, fifty ml of the defined MRS broth (20 g/L) fructose as carbon sources were created and supplemented with four different nitrogen sources include (peptone, yeast extract, meat extract, urea) were utilized in this experiment to select the best nitrogen source which support the maximal bacteriocins and biosurfactant production and growth. The following method was used for this purpose: each source of nitrogen individually, 1/2 amount of 2 sources of nitrogen and equal amount of 3 sources of nitrogen at 24 g/L concentration were utilized, as in Table 3. Following autoclaving; flasks were inoculated with 1 % (v/v) *Lactobacillus* inoculum and incubated in anaerobic conditions utilizing N<sub>2</sub> gas (flashing system) at 37°C for 48 hours to synthesize bacteriocins and 72 h for biosurfactants production.

**Table 3. The influence of several sources of nitrogen and ratios on bacteriocins and lipid production**

Nitrogen source	Nitrogen source Weight (g/l)
Peptone	24
yeast extract	24
meat extract	24
UREA	24
Meat extract + Peptone	12 +12
Meat extract +yeast extract	12 +12
Peptone +yeast extract	12 +12
Peptone +meat extract +yeast extract	8 +8 +8

### Production Medium (PM)

Depending on results of experiments above, the MRS medium composed of 2% fructose as a carbohydrate source and 1.2% yeast extract + 1.2% Meat extract as a nitrogen sources, that prepared in this study was used in all subsequent experiments in this study and referred to as production medium (PM).

### Impact of pH

Fifty ml of PM media in 250 ml Erlenmeyer flasks were adjusted to pH 3 ,4, 5 ,6, 6.5 ,7,8 and 9 respectively, with HCl (0.1N) or NaOH (0.1N) and autoclaved. The medium of culture was added into one percent of an overnight culture of the choosed isolate  $1 \times 10^8$  cell/ml (O.D. 600 nm) at 120 rpm at 37°C for 48 hours to synthesize bacteriocins and 72 h to synthesize biosurfactants.

### Impact of temperature

The pH of PM media was set in to 6.5, sterilized and placed into one percent of ( $1 \times 10^8$  cell/ml) overnight culture at 120 rpm and incubated anaerobically at different temperatures

(5,15, 20, 25, 30, 37 and 40°C) °C for 48 hours to synthesize bacteriocins and 72 hours to synthesize biosurfactants.

### The impact of the incubation time

The pH of PM media was adjusted to 6.5 then sterilized and inoculated with one percent of  $1 \times 10^8$  cell/ml overnight culture (OD 600 nm) of the choosed isolate, which was incubated at 37 °C at 120 rpm for various periods (0, 18, 24, 48, 72, 96, and 120) hours. At the end of each incubation period biosurfactant synthesis and bacteriocin as well as the dry weight of biomass were determined as mentioned above.

### Biomass Measurements (dry weight)

Cell dry weight was calculated by filtering a specified volume of the growing culture via 0.2 m filter paper. After drying the filter paper at 60 degrees Celsius, it was weighed. The weight difference of filter paper with and without biomass is represent the biomass weight. Biomass values were recorded for all samples during the 24, 48,72,96 and 120 h (Bordoloi et al., 2008).

## Results and Discussion

### Collection, Isolation, and Identification of *Lactobacillus*

A total of 142 samples were obtained from several sources, included fermented vegetables (10/142, 7.04%), mouth of healthy adults and infants (20/142, 14.1 %), infant feces and vomits (20/142, 14.1 %), vagina of healthy women (60/142, 42.3 %), yogurt, cheese and liquid milk (10/142, 7.04%), sourdough (2/142, 1.4%) and ready isolates (20/142, 14.1 %). Among of the 142 collected samples, only 120 (84.5 %) isolates were belonging to the genus *Lactobacillus* spp. after subjecting of these samples to different microscopic, morphological, biochemical and VITEK system. The results of *Lactobacillus* were shown as the in the (Table 4) and (Fig. 1).

**Table 4. The results of microscopic, morphological, biochemical of *Lactobacillus* samples**

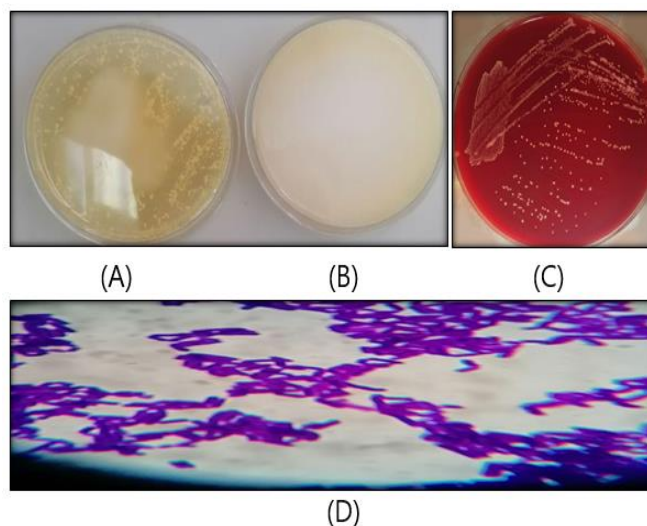
Test	Result
<b>Microscopic Field</b>	Gram positive reaction purple rods that existed singly, in pairs, or in short chains
<b>Cultural Characteristics</b>	
Calcium carbonate-MRS agar	The disappearance of the white color deposited in the dish as a result of decomposition by the acid secreted by the bacteria
Shape of colonies	smooth, creamy, small, large, soft, white color or convex colonies, surrounded by clear zone with circular shape and entire edge and
Blood agar	gray colonies with alpha hemolysis
<b>Biochemical examinations</b>	
Catalase	Negative
Oxidase	Negative

### Primary Screening

In this study 120 bacterial isolates were selected for estimation of their capability to form biosurfactant. The isolates of bacteria were cultured on three different media, include blood agar, phenol red agar plate and Cetyl trimethylammonium bromide CTAB/ methylene blue solid medium. The results were shown as in the (Fig. 2).

The hemolytic activity assay is the gold standard for determining the quality of a biosurfactant manufacturer. Sixty-nine of the 120 isolates tested positive for hemolytic activity, as evidenced by the appearance of a clear zone surrounding the colonies with a diameter ranging from fourteen to twenty-nine millimetres. The results also showed that the isolates (ZMJ2, 3, 5, 20, 30, 31, 33 and 34) revealed the highest hemolysis clear zone (27, 26, 27, 30, 28, 26, 26 and 25 mm)

respectively. Based on these findings, it was determined that 75.3% of *Lactobacillus* isolates have biosurfactant production capabilities. Although *Lactobacillus* species do not generate hemolysin, it has been shown that certain species agglutinate blood cells, hence the blood agar technique is commonly employed to screen for biosurfactant synthesis. Cultures of *Lactobacillus* species responsible for beta hemolysis can generate biosurfactants, as demonstrated by Rodrigues et al., (2006b) and Anandaraj et al., (2010).



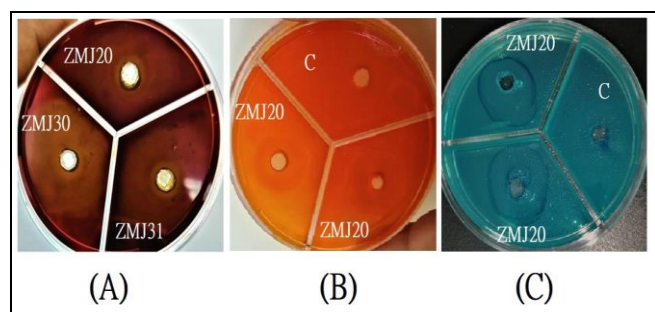
**Fig. 1 Morphology and microscopically examinations of bacterial isolates of the *Lactobacillus* sp. on various plates agar and under large objective lens (A): *Lactobacillus* sp. on MRS agar containing calcium carbonate (B): Control: MRS agar containing calcium carbonate only (C): *Lactobacillus* sp. on blood agar and (D): Microscope field (100x).**

### Screening of isolates of *Lactobacillus* for production of bacteriocin and biosurfactant

Only (44/120) (70 %) isolates of *Lactobacillus* spp. were form different clearance yellow zone ranged from (10 - 25 mm) on phenol red agar. The isolates (ZMJ2, 3, 5, 20, 30, 31, 33 and 34) showed the highest clear yellow zone (18, 19, 18, 23, 22, 21, 18 and 19 mm) respectively. The pH indicator phenol red, also known as phenol sulfonphthalein, is widely employed in the field of cell biology. Most culture media, Hardy CHROM A agar, include salt (sodium salt), XLT-4 agar, modified oxford agar (MOX) and mannitol salt agar (MSA), which inhibits most bacteria dependent on salt (Cangliang et al., 2017).

All isolates (44/120) were formed dark blue halos on CTAB agar, that indicating of biosurfactant production with a diameter ranged from (7-25 mm). The isolates (ZMJ2, 3, 5, 20, 30, 31, 33 and 34) showed the dark blue halos (25, 25, 20, 25, 18, 20, 25 and 20 mm) respectively. Whereas 9.38% of isolates were found to be positive for the CTAB agar plate test

in the prior investigation, 52.8% of isolates were found to be CTAB positive in the study by Shoeb et al., 2015. According to the results of primary screening eight isolates named ZMJ2, 3, 5, 20, 30, 31, 33 and 34 that exhibited higher lipid biosurfactant production selected for secondary screening.



**Fig. 2** Culturing of *Lactobacillus* isolates (ZMJ20, ZMJ30 and ZMJ31) on (A): Blood agar, (B): Phenol red agar, (C): CTAB agar.

### Secondary Screening (Quantitative Screening)

Eight *Lactobacillus* spp. were chosen from methods of primary screening and re-screened for biosurfactant and bacteriocin formation by cultivating the isolates in MRS medium.

### Screening of *Lactobacillus* isolates for Biosurfactant Production in Liquid Media

According to the results of surface tension and emulsification activity, the results indicated that all 8 isolates have ability for production of biosurfactant. The surface tension ranged from (29.22) to (37.22) mN/m, with emulsification activities ranging from 37 to 60% indicating a significant biosurfactant activity (Table 5). The isolate ZMJ20 showed a greater reduce in tension of surface to (29.22 mN/M) and higher E24 (%) reached 60%. As noted in previous study by Georgiou et al., (1992), significant surface activity may be detected in biosurfactants recovered either from culture supernatant (excreted biosurfactant) or from cells (cell-bound biosurfactant). Although it has been reported that lactobacilli produce more cell-bound than excreted biosurfactants, other microorganisms like (*Pseudomonas* and *Bacillus* species) form mostly excreted biosurfactants, which are known to have different surface-active features depending on the composition of the homologue and the producing-strain, carbon source, time, and culture conditions (Rikalovic et al., 2013).

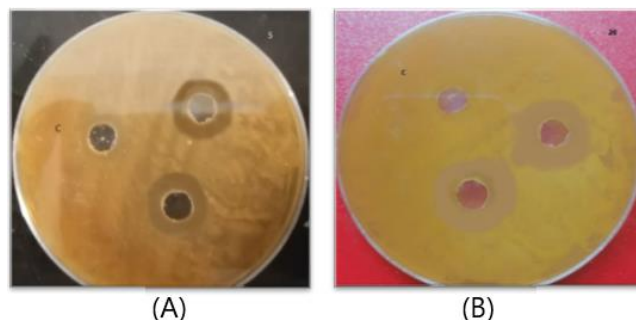
### Screening of *Lactobacillus* isolates for Bacteriocin Production

Eight high-yield samples of bacteriocin were taken based on the primary screening according to the antimicrobial activity. The results were achieved by using the well diffusion method in SDA agar, the yeast (*Candida albicans*) isolate was selected to be used as indicator.

Among eight isolates that were screened, two *Lactobacillus* isolates (ZMJ20, and ZMJ5) exhibited the highest bacteriocin production; the maximal antifungal activity based on clear zones was (22 mm) observed by the isolates *Lactobacillus* ZMJ20, as shown in (Fig. 3) certainly reflecting the activity of the antifungal compound. Furthermore, this method was utilized to determine the antifungal activity due to its sensitivity in determination. (Table 5) shows the bacteriocin activity formed by *Lactobacillus* isolates, representing the screening result after two days. According to results obtained from screened isolates for biosurfactant (lipid) and bacteriocin production, the isolate *Lactobacillus* ZMJ20 was selected for the remaining studies in the current work.

**Table 5.** Secondary screening of *Lactobacillus* isolates for biosurfactant and bacteriocin production.

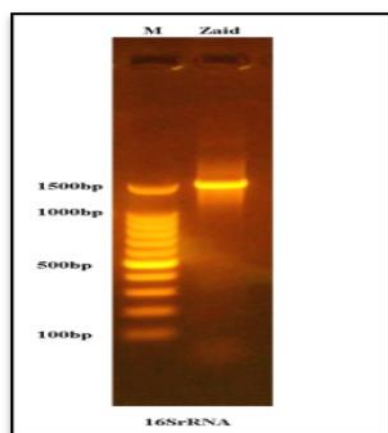
No. isolate	S.T (mN/m)	E24 (%)	Bacteriocin activity (mm)
ZMJ2	32.2	37	15
ZMJ 3	37.2	40	14
ZMJ 5	34.2	57	19
ZMJ 20	30.2	58	22
ZMJ 30	29.2	60	16
ZMJ 31	30.4	40	15
ZMJ 33	34.5	50	16



**Fig. 3** shows the Antifungal action of isolates of *Lactobacillus* against indicator *Candida albicans* by method of agar-wells diffusion in the secondary screening (A: the isolate *Lactobacillus* ZMJ5 ,B: the isolate *Lactobacillus* ZMJ20 )

### Molecular identification

Single isolate which isolated from vagina was chosen for molecular identification for confirming the diagnosis. The amplification and sequencing of 16S rRNA of the total genomic DNA from selected *Lactobacillus* isolate was performed for further identification to species level. Fig. 4 displays a PCR product of 1500bp size that was amplified using a universal primer. The investigated microorganism was finally identified by aligning the sequences of the chosen isolate with those of 16S rRNA molecules available in the Gen-Bank database.



**Fig. 4** PCR product (1500 bp) of 16s RNA gene for *Lactobacillus plantarum* ZMJ20 visualized under UV light after staining with ethidium bromide. The electrophoresis was on 1% agarose gel at 5 volts/cm for 2 hours. DNA ladder=100 bp.

Depending on the morphological, microscopic and biochemical tests as well as molecular identification, the isolate (ZMJ20) was identified as *L. plantarum* with identity value (89%) utilizing the basic local alignment search tool (BLAST) of the Gen bank.

#### Optimization of medium compositions and culture condition

Bacteriocin and biosurfactant productions from *Lactobacillus* ZMJ20 were affected by medium composition and cultivation conditions (Leal-Sánchez et al., 2002) and according to literature recorded by Rodrigues et al., (2006). Fermentation process parameters in this study, such as carbon source,

nitrogen source, pH, temperature and impact of the incubation period, were evaluated.

#### Impacts of the carbon sources

The bacteriocin production and bio-surfactants were tested in the presence of various carbon sources used at a concentration of 2% (w/v) in the production medium. Results indicated in (Fig. 5), that antifungal activity of bacteriocin reached (23.5 mm) clear inhibition zones and emulsification activity E24% (62.3 %) with lowering the tension of surface of the culture to (28.03 mN/m) were achieved when fructose was utilized as energy and carbon source.

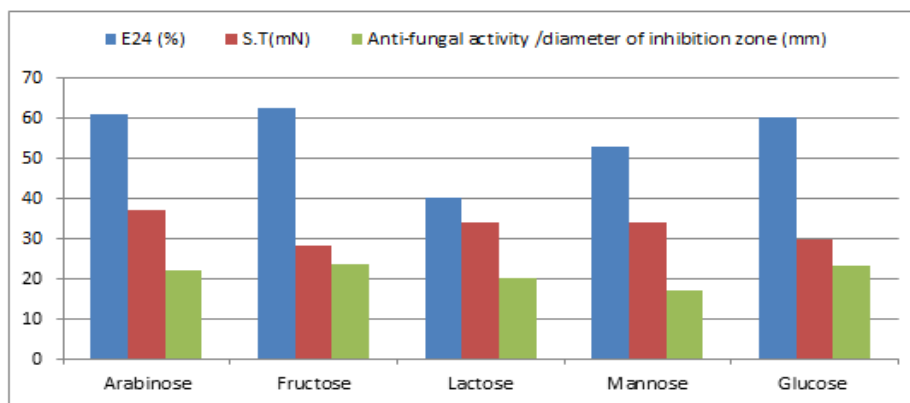
**Table 6. Identification of *Lactobacillus* isolate (ZMJ20) using 16S rRNA gene Sequences**

Description	Ident	Accession	Max score	Total Score	Query cover	E Value
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> strain HBUAS56167 16S ribosomal RNA gene, partial sequence	89.21%	MT231789.1	1182	1182	95%	0.0

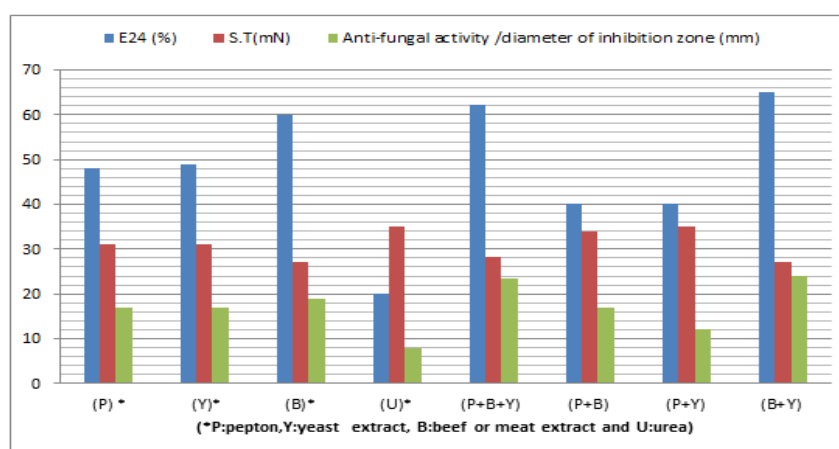
At the same time, the lowest production of bacteriocin and biosurfactant observed with starch as carbon sources, the antifungal activity (7 mm), E24% (20%) and surface tension (38 mN/m) were recorded, respectively. These results were disagreed with (Kadhun et al., 2020 and Dyae et al., 2019) who glucose or lactose was the best carbon source that supports the production of bacteriocin, While these results agree Jameel and Haider (2021) in their study about biosurfactant production from *Lactobacillus plantarum*. When lactose was the only carbon and energy source, the emulsification activity of the biosurfactant (E24%) increased to 63%, and the surface tension decreased to 25.41 mN/m. The surface tension of fructose was 28.38 mN/m, and its E24 percentage was 57%.

#### Impact of nitrogen sources ratio

The impact of variable nitrogen sources ratio on the formation of bacteriocin and biosurfactant by *Lactobacillus plantarum* ZMJ20 was examined. Different nitrogen sources were examined. According to the results in (Fig. 6), bacteriocin production and biosurfactant formation were variable with several nitrogen sources. Hence, The highest antifungal activity (24 mm) clear inhibition zones for the bacteriocin and E24% (65%) and lowering the surface tension (27mN/m) were achieved when (beef (meat) extract: yeast extract) ratio was (1: 1), while the lowest activity and production were obtained when (Urea) with lowest inhibition zones (8 mm) and E24% (20%) and surface tension (35mN/m).



**Fig. 5** Impact of carbon sources on biosurfactant and bacteriocin production from *Lactobacillus plantarum* (ZMJ20) grown in modified MRS medium pH 6.5, 37 °C in shaker incubator (120 rpm) under anaerobic condition by flushing with N<sub>2</sub> gas.



**Fig. 6** Impact of several sources of nitrogen on biosurfactant and bacteriocin production from *Lactobacillus plantarum* (ZMJ20) grown in modified MRS medium pH 6.5, 37 °C in incubator 120rpm under anaerobic condition by flushing with N<sub>2</sub> gas.

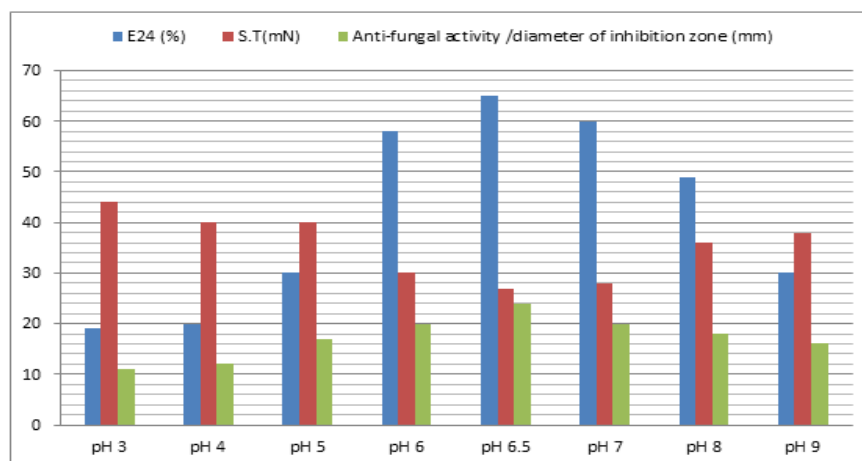
These results were agreed with Okoliegbe et al., (2012); Zainab and Nadhim, (2022) and Nouralhuda and Luti, (2022) mentioned that bacterial cells need nitrogen sources to complete their metabolic pathways and their role in protein and enzyme synthesis for cell growth. It was reported that using yeast extract as a nitrogen source was usually accompanied by enhanced bacteriocin production because of growth factors and free amino (3 or 4 amino acids) as a short peptide (Aasen et al., 2000).

### Impact of pH value

Modified PM media were selected in the current study to determine the initial pH effect in media on the formation of bacteriocin and biosurfactant by *Lactobacillus plantarum* ZMJ20. The media were adjusted to variable values of pH. Based on the findings in (Fig. 7) indicate that the highest antifungal activity (24mm) clear inhibition zones for the bacteriocin and E24% (65) and surface tension (27 mN/m)

were achieved at pH 6.5, while the lowest activity was obtained at pH 3 showed (11mm) inhibition zones and drooping in the value of E24% to 19 % and surface tension (44 mN/m). Other researchers also reported similar results (Todorov et al., 2005; Todorov, 2008). The optimum pH for *Lactobacillus* growth is 5.5 – 6.2; their growth usually occurs at pH 5.0 or less. Their growth rate mainly decreased in an alkaline media or neutral PH (Kandler et al., 1986). According to the mentioned result, the pH was considered a critical factor that can affect the production of that affects bacteriocin by lactic acid bacteria, which attributed to the pH having a regulatory effect driven by ions in media or to its effect on the envelope of cells in medium (Bruno et al., 1998; Guerra et al., 1986) reported that the optimal pH ranges for maximum biosurfactant production were 6-6.8. The biosurfactant synthesis decreased without pH control, meaning it is essential to maintain its value during fermentation (Bednarski et al., 2004).





**Fig. 7** The effect of different pH on bacteriocin and biosurfactant production by *L. plantarum* (ZMJ20) grown in PM in shaker incubator 120rpm, under anaerobic conditions utilizing N<sub>2</sub> gas.

### Impact of temperature

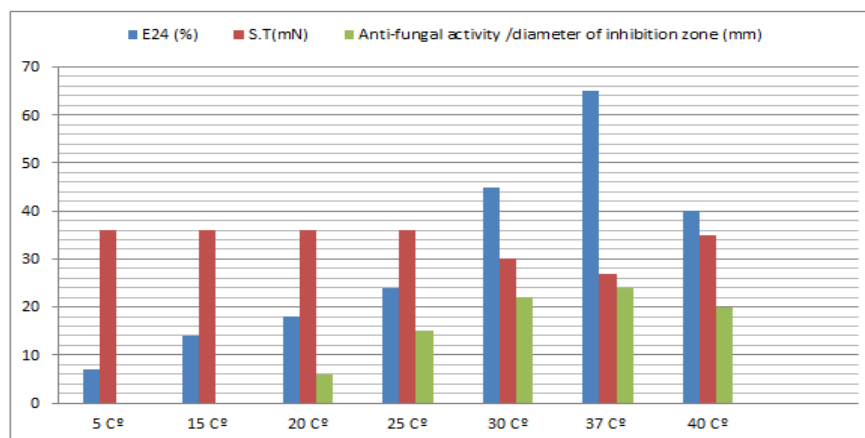
Since the generation of biosurfactant and bacteriocin by *Lactobacillus plantarum* ZMJ20 is strongly influenced by incubation temperature, a range of temperatures was tested. Results in (Fig. 8) indicate that the highest antifungal activity (24 mm) clear inhibition zones for the bacteriocin and E24% (65%) and surface tension (26.9mN/m) were achieved at incubation temperature 37 °C, while no inhibition zone at (5,15 °C) and the lowest activity was obtained at 20°C showed (6 mm) inhibition zones and drooping in the value of E24% to 18 % and surface tension (36 mN/m).

The same results were noted by Hiba et al., (2020) who found that the optimal temperature for bacteriocin production from

*Lactobacillus acidophilus* HT1 was 37 °C. However this result disagreed with Casla et al., (1996) who found that the maximum bacteriocin production from *L. curvatus* was obtained at 30 °C.

### Impact of the incubation period

Several periods of incubation (0 to 120 hrs.) were tested to reveal the optimum periods for growth of bacterial cells and production of bacteriocin and biosurfactant by *Lactobacillus plantarum* ZMJ20. The results in Fig. 9, show that the maximum antifungal activity (24 mm) clear inhibition zones for the bacteriocin were observed at 48 hrs, beyond 48 hrs no bacteriocin activity was observed.



**Fig. 8** The different incubation temperatures impact on *L. plantarum* (ZMJ20) grown in PM in shaker incubator 120rpm, under anaerobic conditions utilizing N<sub>2</sub> gas.

These results agreed with the result reported by Amortegui et al., (2014) for bacteriocin production from *L. plantarum*; the optimum period was found to be 48h. The maximal

biosurfactant production E24% (65%) and lowering the value of surface tension (26.8mN/m) was achieved at 72 hrs. with an increase in dry biomass up to 4.6 g/l, while the lowest were

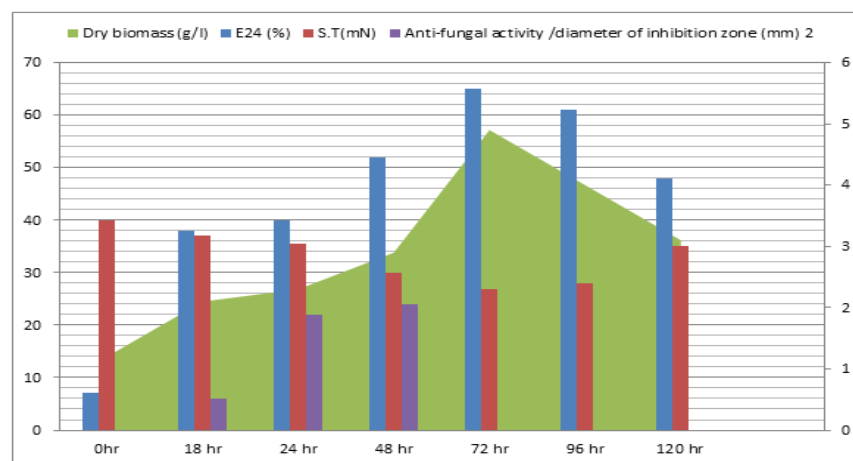
obtained during (0,18 and 24 hrs). However, the emulsification activity dropped and the surface tension values rose during prolonged incubation (more than 72 hours). During this time, the culture conditions may have changed, thereby reducing the availability of anaerobic conditions, nutrients, and toxic metabolites that restrict bacterial growth. The results of the current investigation indicated that the amount of biosurfactant generated by *Lactobacillus plantarum* increased with incubation time, beginning at the early stationary phase (48h) and peaking at 72h.

Biosurfactant biosynthesis seemed to be most active during the exponential growth phase, indicating that it was

synthesised as a main metabolite alongside the development of cellular biomass (growth-associated kinetics).

Bonilla et al. (2005) found that as the creation of secondary metabolites advanced, biosurfactant production ceased; this might cause issues with emulsion formation and the adsorption of surfactant molecules at the water/oil interface.

The synthesis of biosurfactant (emulsan) by *Acinetobacter calcoaceticus* RAG-1 is highest while the organism is in its stationary growth phase (Rodrigues et al., 2006).



**Fig. 9 Effect of Different incubation times on bacteriocin and biosurfactant production by *L. plantarum* (ZMJ20) grown in PM in shaker incubator 120rpm, under anaerobic conditions utilizing N<sub>2</sub> gas.**

### Conflict of Interest

The author hereby declares no conflict of interest.

### Consent for publication

The author declares that the work has consent for publication.

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