Lactic Acid Bacteria Biosurfactant Role That Isolated from Human Breast Milk in Inhibit Eyes Pathogenic Bacteria

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Received in:3/December /2017, Accepted in:6/June/2017

Abstract

Biosurfactants have a wide-range of applications due to their unique properties like specificity, not toxicity (from LAB) and relative ease of preparation. These properties hold promise of biosurfactants to increase breast milk benefit were isolated and described into *Lactobacillus plantarum*, *Lactobacillus fermentum* ,*Lactococcuslactis*, and *Leuconostocmesenteroides*.The degree of microbial destruction of disease, which promotes the effective remediation of disease spreading.This paper presents a review of available research, methods and publications regarding Biosurfactant extraction from Lactic Acid bacteria isolated from human breast milk. 3 samples of human breast milk was provided, LAB were isolated and described, Biosurfactants recovery and surface activity were tested and extracted endo and extra cellular. In other side 26 samples from eye patients were ordered, diagnosed and their sensitivity to biosurfactant were studied. The results showed that 5 isolates of LAB from human breast milk were biosurfactant producer but *L. plantarum* was the more efficiency in surface activity. In other side, out of 26 eyes sample 18 were positive to pathogenic bacteria included *E.coli* (7), *Klebseilla* (5), *Staphylococcus aureus*(3)and *S.epidermides* (3).Extracellular Biosurfactanthad good effect against tested bacteria, but endocellular (extraction by normal method) had not any effect against any bacteria, whereas by solvents method were the more active against all tested bacteria. The results are promising enough to continue the quest for enhancement of inhibition growth of pathogenic bacteria with biosurfactant application (extracted extracellular by solvents) to look forward for biosurfactant as a solution of antibiotic resistance problem. In this study we concluded that*L. plantarum* was the more effectiveness in biosurfactant surface activity and the extracellular biosurfactant by solvent method for extraction were better than endocellular and normal method of extraction.

Keyword: Lactic Acid bacteria, Biosurfactant , eye pathogenic bacteria

Introduction

Human breast milk consists of high amounts of necessary nutrients for infants, including carbohydrates, essential fatty acids, proteins, vitamins and minerals [1]. It plays an important role in development of infants due to transfer of microflora originated in breast milk [2]. Human breast milk also contains indigestible nutrients that exert potent bioactive functions on establishment of infants [3][4].

In recent years, more than 200 different species of bacteria have been described in human milk, like probiotics [5][6][7], including bifidobacteria, *Streptococci* and lactic Acid Bacteria (LAB) [8].

Probioitics are "live microorganisms and when they administrated in adequate amounts they will confer a health benefit on the host" [9], LAB are a group of gram-positive bacteria including many of genera [10], within the group of LAB *Lactobacillus*, *Lactococcus* species due to their potential beneficiary properties as probiotics, the activity of these bacteria is known to inhibit a large number of pathogenic bacteria, the use of these bacteria products to control certain infections has started gaining acceptance, the alarming rise of inappropriate antibiotic use and antimicrobial resistance, along with renewed interest in ecological natural methods to prevent infections [11], within these products of LAB are CFS (Cell Free Supernatant) which have a very interesting field for researches [12][13][14] or Bacteriocin [15].

Biosurfactant is one of these products that produced by some species of LAB not all of them [16], and other microorganisms like *Pseudomonas* [17] ,and yeast [18] ,and can be defined as the surface-active biomolecules, with wide-range of applications, due to their unique properties like specificity, not toxicity and relative ease of preparation, these surface-active biomolecules have attached wide interest, due to their unique functional properties, biosurfactants were used in several industries including organic chemicals, mining, foods, cosmetics and many others [19].

Because of both the importance of human breast milk and its use traditionally by mothers for their babies infected eyes,and biosurfactant application, and no researches detect the effect of this product against eye bacterial contamination, for all these above reasons, this study was objected to determine the effectiveness of LAB biosurfactant that isolated from human breast milk against the growth of bacteria that isolated from eye patients. So this product (biosurfactant) if prove its benefit in therapy, would be able to use it as antibiotic alternative agent (natural agent).

Materials and Methods

1. Milk Specimens Collection:

Milk specimens were taken from 3 milk nursing mothers (healthy women), milk were put in sterilized tube, and under sterilized conditions to bring them to Biology Laboratory/University of Al-Mustansiriayh.

1ml from each of the 3 specimens were put in tubes containing MRS (Man-Rogosa-Shape) broth, then, incubated in 37C˚ at 48h. in an aerobic condition, after that serial dilution were done for each tube to obtain single colonies, from the last dilution (1 ml) was taken to on petri dishes with MRS Agar and incubated at 37ºC for 24 hr [13].

2. Isolation and Identification the LAB:

Smear slide was prepared for each colony and staining, finally examined under a microscope, biochemical tests (fermentation medium) were prepared to diagnose the isolates finally even species. Fermentation medium prepared from each of (Trehalose, xylose and sorbitol) which were sterilized by Millipore filter (0.22µm) and added as 1% from each solution, change in color indicator of sugar fermentation [20].

3. Lactic Acid Bacteria Biosurfactant Production Ability Test:

The LAB isolates were tested for their ability to biosurfactant production by growing in MRS broth at 37C˚ for 24h., then 0.1ml from broth was transferred to blood agar medium and left to dry, after that the petri dishes were incubated in 37˚C for 24-72hr. , presence of heamolysis around the colonies is the positive result[21][22].

4.Biosurfactant Surface Activity Test:

The surface activity of biosurfactant was tested by culturing the isolates (biosurfactant producing) in MRS broth with 2% inoculation, tubes were incubatedin 37˚ for 24h, centrifuged at 6000 xg for 20 min, filtered by Millipore filter,sediment was suspended in 500 ml Normal Saline after

washing three times, to ensure presence biosurfactant binding to cells.Then, 0.2 ml from each of Supernatant and sedimentation separately were put in sterilized Petri containing 20ml Distal water and 20ml motor oil to being oil membrane in the center, after (30) sec. the more activity isolate was measured by measuring diameter push the oil from the center [23].

5.Biosurfactant Extraction (Extracellular):

The higher activity isolate was chosen to biosurfactant extraction., 10ml of overnight culture isolate cultured was inoculated to 500 ml of MRS broth and incubated for 24 hr,centrifuged at 10 000 xg for 5 min at 4ºC, Filtrated by Millipore filter.the filtrated supernatant represented the crude extracellular biosurfactant, [24].

6.Biosurfactant Extraction (Endocellular):

The same procedure followed above and after centrifugation, sediment cells were washed twice with distal water, then superintend with 100 ml of PBS (pH=7), moved by magnetic stirrer in room temperature for 2hr., centrifuged, sedimentation was concealed, supernatant was taken and filtered with Millipore filters [25] .

7.Partial Purification of Biosurfactant (Endocellular):

Extraction of the biosurfactant by using the solvents was done as another extraction method, chloroform : methanol with volume 1:2, added to inoculated MRS (10 ml ml of isolate and incubated at 37ºC for 24 hr , centrifuged at 6000 xgat 4ºC, mixed well (supernatant+solvents) and left to dry ,the weight was measured by following calculation [26]:

$$\text{Biosurfactant dry weight} = \text{Petridish weight containing drying biosurfactant} - \text{Empty petri dish weight}$$

8.Eye Samples:

- Methodology: Each of 26 samples were taken from eyes patient (from Al-Kindy hospital) put in swabs containing (2ml) normal saline separately , (1 ml) of each swabs was taken and cultured on brain heart infusion, or nutrient broth. (for activation the bacteria)., incubated in 37C˚ for 24hr. , serial dilution were made, the last dilution cultured on nutrient, mannitol salt and MacConkey agar to separate and purificate the bacterial isolates [27].
- Media: In the study, 26 samples, to bring them to the Laboratory, MacConkey, brain heart infusion, mannitol salt and nutrient agar were used to for isolation of bacteria.
- Identification: All bacterial isolates were identified based on their gram stain and biochemical reactions as described in Atlas *et al.*, 1995. [28]

9. Determine The Antibacterial Effects Of Biosurfactant:

The isolate that had the higher biosurfactant surface activity by wells diffusion method were used to detect the antibacterial effect of biosurfactant [29]. Mueller Hinton agar medium (MHA, Hi-media,India) were inoculated by 0.1 ml (0.5 MacFarland standard) of pathogenic

bacteria that isolated from eyes, added 0.1ml of Exo and EndoBiosurfactant and Supernatant (external Biosurfactant) and MRS broth as a control in each well prepared by cock pore. The plates were incubated at 37C˚ for 18-24hr., The antibacterial effect was measured by diameter the inhibition zone around the wells.

Results

In our study, Five strains of LAB were isolated from human milk, as shown in Table (1)and (2), according to results depicted in these tables, *Lactobacillusplantarum (Lb. p.)* and *Lactobacillus fermentum (Lb. f.)* were found and (1) strain of *Leuconostoc* (Le.), *Lactococcuscoccus* was found in two species *Lactococcuslactis* 1 (Lc. l_1) and *Lactococcuslactis* 2 (Lc. 1_2.) as shown in (Figure 1).

The five LAB isolates produced the biosurfactant at biosurfactan ability test, this productivity was different according the diameter around heamolysin area, *Lactobacillus* was the best among other LAB in Biosurfactant production Figure(2).

Biosurfactant surface activity test was estimated by oil spreading method, resulting higher biosurfactant surface activity in *Lb. p.*,whereas other isolates did not have any surface activity. *Lb. p.*biosurfactant was extracted by two types: extracellular and endocellullar, the first called EX.B and second, EN.B. and the EN.B. was extracted by two method: normal and solvent extraction assay., as remembered in above., EX.B and EN.B. were used in antibacterial activity against eyes pathogens.

In other side of our study, 18 out from 26 samples that collected from eyes patients, were observed for bacterial presence., *E.coli* and *Klebseilla* were found in these samples in large number, *Staphylococcus* also was found with two species *S. aureus*and *S. epidermidis* but in fewer number,

7 isolates were observed of *E. coli* and 5 isolates of *Klebseilla*, 3 isolates of *Staphyllcoccusaureus* and 3 isolates of *S. epidermides* , So we called them *E. coli* (1).to *E. coli* (7) ,*Klebseilla*(1)to*Klebseilla*(5), *S. aureus*(1)to *S. aureus*(3)and *S. epidermides*(1)to *S. epidermides* (3), to determine the activity of EX. B. and EN.B. against them as shown in(Table 3).

Also,our study appeared the antibacterial effect of *Lb. p.* Biosurfactant as shown in Table (4), showed all bacteria that submitted to EX.B. were sensitive and EX.B. gave higher effect against *E.coli* and lower effect against *S.epidermides* ,whereas *Klebsiella* was the medium between *E.coli* and *S.aureus*.

EN.B. extracted by normal method did not give any result against tested bacteria, perhaps because it was not concentrated, that is opposite of EN.B. extracted by solvent method which showed very high degree of effect against all tested bacteria, perhaps because it was the result of purification and concentration methods as shown in (Table 4) .

Discussion

Our results were agreed with Nino,(2016)& Martin , (2009) [30] [31]who found that human breast milk is rich with LAB and Bifidobacterium that considered as probiotic,it has been reported that breast milk is a good source to provide all the nutritional requirements for the rapidly and healthy growing of infants because it is a source of beneficial bacteria such as *Lactobacilli, Lactococci, Leuconostoc spp.*, which are the usual commensal bacteria present in breast milk and they play an important role in the defense system of the infant.

Also our results agreed with other researchers that said, the transparent areas on blood agar are referred to Biosurfactant produced by the microorganism and heamolysis area directly proportional to the amount of Biosurfactant produced by bacteria [32][33].

LAB are characterized within ability of Lysis blood and this is which gives probiotic recipe and Lysis blood is due to the ability of Biousrfactant to agglutinate, then analysis the blood [34].

The differences between isolates activity related to the produced biosurfactant concentration from these isolates [35][36], they found that high concentration of biosurfactant lead to movement the oil particle from their places, due to biosurfactant ability to change the surface tension The good production of *Lb. p.* biosurfactant made this isolate the best for extraction and determination the antibacterial inhibitory effect. [23][37].

Eyes bacterial contamination may be due to use not clean hands (workers during working) or cosmetics tools like eye lashes , face sponge, brushers and mascara (during using them from person to another), and bacteria were observed in eyes area were considered true opportunistic pathogenic bacterium, matching, there were evident that the most frequently
found bacteria were approximately the same in kind of cosmetic tools or currency contamination [11][38].

The strong effect of Biosurfactant rented to its properties like: biodegradability, emulsifying, pH tolerance and surface activity which helps in reducing surface tension and the interfacial tension, leading to reduction adhesion of microbes over the surface, causing slow down the colonization of other strains which are responsible for fouling [16].

These results corresponded with Salleh*et al*., 2011 [40] who refer to normal method of extraction is not efficient way of extraction because of the lipid nature of biosurfactant, this is confirmed by the results of solvents method of extraction,which was independent and favorite method especially the mixture of Chloroform: Methanol by (1:2)vol:vol because of solvents mixture lead to easier polarity organization between the solvent as extraction material and Biosurfactantwhich want to extract it [40].

Other authors divided the physiological role of Biosurfactant activity to three major pathways. First, Lipophilic cell wall filled with hydrophobic substance of polysaccharide nature and have high affinity for hydrocarbons formation. Second, Hydrocarbons emulsifying and solubilizing compounds synthesis.Third, Hydrophobic cell wall on the lipophilic, compound basis formation provides direct contact with the hydrocarbon molecules. These ways are typical for the most microorganisms[23][26][41].

Conclusions

*L. plantarum*isolated from human breast milk was the most effective in biosurfactant surface activity. *L. plantarum* extracellular biosurfactant had the inhibitory effect on growth all test bacteria isolated from infected eye.*L. plantarum* endocelullar biosurfactant by normal method had not effect on growth any isolate in opposite of biosurfactant extracts by solvents method which had the higher. Biosurfactant extraction solvents method confirmed that it is the best method and give the best results.

Recommendations

Purification the Extra and Endobiosurfactant, study its effect on pathogenic bacteria from another sources like urinary tract, intestine, wound and others,study the synergistic effect of biosurfactant and other agent from LAB like Bacteriocin to increase the efficiency of effect*in vitro* to use it as antibiotic alternative agent.

Acknowledgments: The authors would like to thank AL-mustansiriyahUniversity (**www.uomustansiriyah.edu.iq**.) Baghdad –Iraq for its support in the present work.

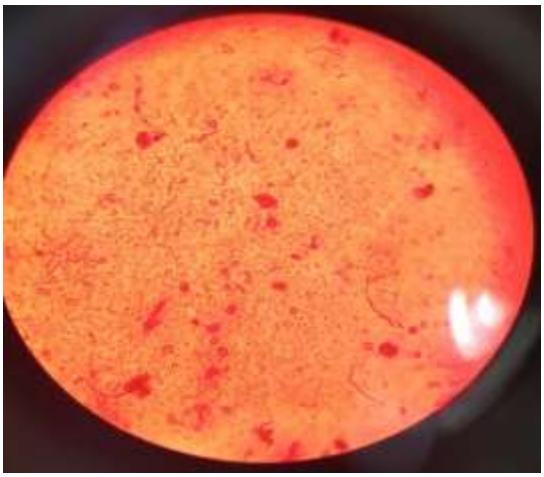

Figure (1): *Lactobacillus* in microscope

Table (1): Morphology, gram stain and biochemical tests of isolated strains of human breast milk

Bacteria L.A.		Morphology in microscop	Gram stain	Catalase	Oxidase
Sample 1	*a.*	rod-shaped	+	-	-
	b.	cocci pairs	+	-	-
Sample 2	*c.*	cocci – short chain	+	-	-
	d.	ovoid cocci (chain) or double	+	-	-
Sample 3	*e.*	rod shaped	+	-	-

Table (2): Identification of LAB isolates according to different biochemical tests

Sugars fermentation test			Symbole of isolate	Result
Trehalose	Xylose	Sorbitol		
+	-	-	a	*Lactobacillus plantarum*
+	-	-	b	*Lactococcuslactis*
+	-	-	c	*Lactococcuslactis*
-	-	-	d	*Leuconostoc*
+	+	+	e	*Lactobacillus fermentum*

Figure (2): A: *Lb.plantarum* and B:*Lb. fermentum*

Table (3): Isolated bacteria species from eyes patients

Eye Sample symbol	Sample No.	Bacteria
2, 3, 6, 11, 14, 19, 23	7	*E.coli*
4, 9, 15, 18, 24	5	*Klebseilla*
5, 20, 25	3	*Staphylococcus epidermides*
10, 16, 21	3	*Staphylococcus aureus*
1, 7, 8, 12, 13, 17, 22, 26	8	Negative culture
	26	Total

Table (4): Antibacterial effect of *Lb. p.*biosurfactant against tested bacteria by agar well diffusion

Bacteria	Inhibition zones of Biosurfactant (mm)		
	External Biosurfactant	EnternalBiosurfactant	
		Normal method	Solvent Method
E. coli (1)	5	-	7
E. coli (2)	6	-	12
E. coli (3)	9	-	13
E. coli (4)	7	-	15
E. coli (5)	6	-	9
E. coli (6)	9	-	8
E. coli (7)	9	-	7
klebseilla (1)	6	-	12
klebseilla (2)	7	-	13
klebseilla (3)	3	-	20
klebseilla (4)	4	-	19
klebseilla (5)	5	-	12
S. aureus (1)	5	-	13
S. aureus (2)	6	-	12
S. aureus (3)	7	-	22
S. epidermides (1)	3	-	13
S. epidermides (2)	4	-	15
S. epidermides (3)	4	-	20

I.Z.: Inhibition zone

EXB: Extracellular Biosurfactant

ENB: EndocellularBiosurfactant

N.M: Normal Method

S.M: Solvent Method.

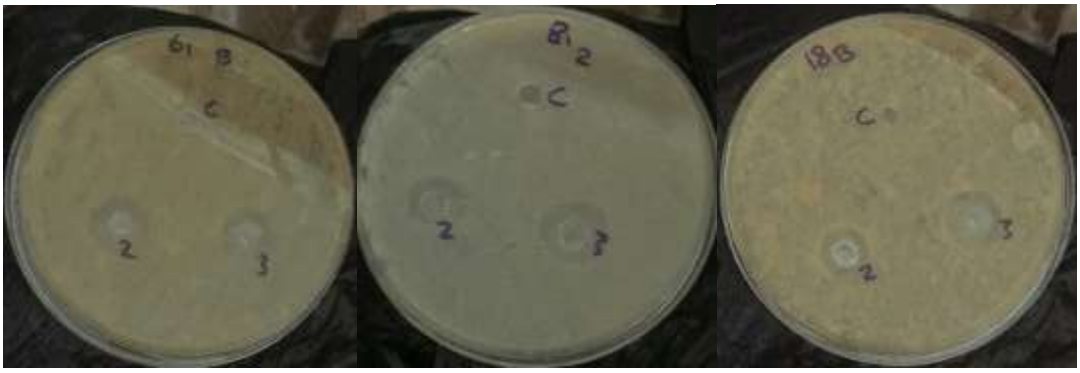

Figure (3): Antibacterial effect of *Lb. plantarum*biosurfactant against tested bacteria by agar well diffusion

6_1=*E. coli* (1) ,8_{12}=*Klebseilla*(3),18_B= *S. epidermides*(2), C: control 2: Extracellular Biosurfactant 3: EnternalBiosurfactant extracted by solvent method

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