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Extraction and Partial Purification of Exo-Polysaccharide from *Lactobacillus Rhamnosus* Isolated from Vaginal Specimens

Reyam M. Alshammery, Mouruj A. Alaubydi
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

Lactobacillus spp. is one of the most important strains used worldwide in different applications that range from medical to industrial uses. Therefore, this study aimed to determine the potential capability of the putative probiotic *L. rhamnosus* isolated from clinical vaginal specimens to produce exopolysaccharide (EPS). From a total of 100 clinical samples, only 13 (13%) samples were represented as Lactobacillus spp, as characterized by the use of the API 50CHL system. The results revealed that the number of *L. rhamnosus* isolates constituted 4/13 (30.8%), with a confident percentage of more than 80%. In addition, characterization by 16S rRNA sequencing showed 100% similarity to the characterized species of *L. rhamnosus*. Also, the results showed a strong capability of all Lactobacillus spp. isolates to produce biofilm. On the other hand, the antimicrobial susceptibility test revealed increases of antimicrobial resistance. The selected *L. rhamnosus* showed the capability to produce exopolysaccharide with carbohydrate and protein concentrations of 74.774 µg/ml and 0.0145µg/ml, respectively. This bacterial isolate demonstrated the ability to form a thin capsule with carbohydrate and protein concentrations of about1.314 µg/ml and 0.01µg/ml, respectively.

Keywords: ***Lactobacillus rhamnosus,*** **Exopolysaccharide, EPS**

ألاستخلاص والتنقية الجزئية لمتعدد السكريد الخارجي لبكتريا *Lactobacillus rhamnosus* المعزولة من عينات مهبلية

ريام محمد، مروج عبد الستار

قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

ألخلاصة

عصيات الحليب هي واحدة من العتر المهمه في العالم والتي لها تطبيقات مختلفة تراوحت بين الاستعمال الطبي الى الاستعمال الصناعي . لذلك هدفت هذه الدراسة على تحديد القدرة المحتملة لسلالة البروبيوتيك المفترضة *L.rhamnosus* المعزولة من العينات المهبلية السريرية لإنتاج عديد السكريد الخارجي (EPS). فمن مجموع 100 عينة سريرية ، مثلت فقط 13 (13 ٪) عينات تعود الى انواع العصيات اللبنية، عند توصيفها باستخدام API 50CHL system أظهرت النتائج أن *L. rhamnosus* شكلت 13/4 (30.8٪) مع نسبة ثقة تزيد عن 80٪ ، وكذلك التوصيف باستخدام تسلسل الرنا الريباسي 16s أظهر تشابه 100 ٪ مع الأنواع *L. rhamnosus*. كما أظهرت نتائج هذه الدراسة القدرة القويه لكل العصيات اللبنية على انتاج الغشاء الحيوي . كشف اختبار الحساسية المضادة للميكروبات زيادة في مقاومة العزلات اتجاه مضادات الميكروبات المستعملة. *L. rhamnosus* المختارة أظهرت القدرة على إنتاج متعدد السكريات الخارجي والذي

*Email: mourujrabea@gmail.com

بلغت فيه تراكيز السكريات والبروتين بنسب 74.774 ميكروغرام / مل و 0.0145 ميكروغرام / مل على التوالي . هذه العزلة تملك القدرة على تكوين طبقة خفيفة من غلاف المحفظة تكونت من السكريات والبروتين بتراكيز1.314ميكروغرام / مل و0.01 ميكروغرام / مل على التوالي.

Introductio:

Lactobacillus is one of lactic acid bacteria (LAB) which constitutes a group of Gram-positive, non-spore forming, rod-shaped, catalase-negative, and fastidious organisms, with a tolerance for low pH [1-3]. It is usually non-motile, aero-tolerant and its division occurs in one plane. LAB also ferments carbohydrates for energy demand, using endogenous carbon sources as a final electron acceptor instead of oxygen. The DNA of LAB has a low G + C content [4]. Some strains of LAB can be considered as probiotic bacteria [5], such as Lactobacillus genus with more than 100 species, for example *L. acidophilus, L. rhamnosus, L. casei, L. plantarum, L. bulgaricus, L. L.reuteri, delbrueckii* and *L. helveticus* [6, 7]. Lactobacilli are a broadly defined group characterized by lactic acid formation as a main end product of carbohydrate metabolism. It is fastidious bacteria (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, vitamins) [8]. Lactobacilli are part of the microbiota in the mouth, gastrointestinal tracts, and genital tracts of humans and many animals [9]. They are characterized by protecting the host from urogenital infections by decreasing the environmental pH due to lactic acid production, by producing various bacteriostatic and bactericidal materials such as hydrogen peroxide (H2O2), and through competitive exclusion [10,11]. *L. rhamnosus* GG is one of the best clinically studied probiotic microorganisms. This bacterium is capable of adhering to the human intestinal mucosa and colonizing there for more than seven days after being orally ingested by healthy adults [12]. It is also able to form biofilms *in vitro* on an abiotic surface such as polystyrene [13]. A biofilm is defined by Donlan and Costerton (2002) as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or/ and interface, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" [14]. It is also established that the exopolysaccharide produced by some biofilm forming strains is able to suppress the formation of biofilms by certain pathogens [15].

Exopolysaccharides (EPSs) are composed of monosaccharide residues of sugar and sugar derivatives. They are produced by plants, fungi, algae, and bacteria [16]. Among the various EPSs producing bacteria, LAB gained a special consideration. LAB is "generally recognized as safe (GRAS)" microorganisms and their capabilities to produce EPSs have a wide variety of structures without health risks [17]. EPSs produced by LAB have been receiving an increasing amount of attention because of their health benefits to the consumers. These advantageous effects include cholesterol lowering, antitumor, antioxidant, anti-ulcer, and immune-stimulating activities [18]. Bio flocculants, bio-absorbents, drug delivery agents, heavy metal removal agents, and others are among the new applications of EPSs [19]. Different species of lactobacillus produce EPSs, especially *L. plantarum* [20], *L. lactis*subsp. *cremoris (*25 to 132 mg/L) [21], *Lactobacillus casei*CG11(130 to 250 mg/L) [22], *L. delbrueckii* subsp. *bulgaricus*NCFB 2772 [23], and *L. casei* [24]. Microbial EPSs are generally found in two forms, depending on their sites as cell-bound EPSs, into those which closely adhere to the bacterial surface as capsular (cEPSs), and those released into the surrounding medium as free EPSs (fEPSs) [25, 26]. Thus, this study was designed with the aim of finding the ability of clinical Lactobacillus spp. to form EPSs that are to be used for further applications in the future.

Materials and Method:

Samples collection

Samples of 100 vaginal specimens were obtained from female patients aged 19-46 years. All samples were collected from AL-Yarmouk hospital/ women's competence and obstetrics department, during the period from November 2018 to February 2019. From a vaginal fluid, samples were collected with sterile cotton swabs inserted into the vagina. A selective medium, namely de Man, Rogosa, and Sharpe (MRS) agar (Difco, Detroit, MI, USA) was used for primary bacterial isolation, and then single colonies were stored at –20°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% (v/v) glycerol. The isolates were subsequently grown on MRS agar or broth media and incubated for 48 hrs at 37°C under anaerobic conditions (BBL® GasPak Anaerobic System; Becton Dickinson, Cockeysville,MD, USA).

Isolates Identification

The initial identification of the achieved pure cultures was based on Gram staining, the ability to grow on a selective MRS agar, and catalase test phenotype [27]. Also, the biochemical test, including the use of API 50 CHL kit (bio merieux / France), was used to facilitate the interpretation of fermentation patterns.

Molecular Diagnosis of Lactobacillus spp

For more cinfirmation, 16S Ribosomal RNA (16S-rRNA) sequencing was performed.

Extraction of DNA

The procedure was further extended using Wizard genomic purification kit (Promega, USA). The extracted DNA was estimated using Quantus (Promega) with the Quantiflour dye, where the estimated concentration was 20ng/1µl.

Detection of Gene Using PCR

The set of primers used in this study was 27F:AGAGTTTGATCCTGGCTCAG and 1492R: TACGGTTACCTTGTTACGACTT (Macrogen,Korea). The amplification program considered after the optimization procedure for the standard annealing point included the following steps; initial denaturation for 5min at 95ºC, denaturation for 30sec at 95C, annealing for 45sec at 60ºC, extension for 1min at 72ºC, and final extension for 7min at 72ºC. The resulted amplicons from the reactions were detected after electrophoresis using Ethiduim bromide dye. The resulted bands were documented using gel documentation system.

Sequence Analysis of 16srRNA

For final conformation and precise diagnosis of Lactobacillus isolates, the PCR forward and reverse primer products were sequenced using Sanger sequencing through ABI3730XL and automated DNA sequencer at Macrogen Corporation-Korea. After processing of sequencing raw data using Bioedit software, the results were aligned at NCBI database through BLAST software, and conformed by geneious software.

Detection of the Bacterial Isolates Capability of Biofilm Formation

This test was performed by using two procedures as in the following.

The first procedure was conducted according to a method describedby Freeman *et al* [28]**. An a**mount of MRS agar (5.2g) was dissolved in 50ml of distilled water, sterilized by autoclaving at 121^0C for 15 min, and then cooled to 55°C. Simultaneously, Congo red (BDH / England) and sucrose (BDH / England) were prepared by dissolving 0.2g and 5g in another 50ml distilled water, respectively, sterilized by autoclaving at 121°C for 10min, added to the medium and mixed well, and finally dispensed into sterile Petri dishes.

The second procedure was performed according to De Keersmaecker and Sigrid [13], by the activation of the isolates for 48hr. using MRS medium and glucose 1% under static microaerobic conditions at 37°C. The device used for biofilm formation is a microtiter plate 96 polystyrene peg, with a peg hanging into each microtiter plate well. For biofilm formation, the device was placed in its original sterile tray filled with 180µl of Trypticase soy broth (TSB) (Biosciences). Then 20µl ($1X10^8$) of the bacterial suspension were added and incubated for 72 hr. at 37°C. To determine the magnitude of biofilm formation, the pegs were washed in phosphate-buffered saline (PBS) three times. The residual attached bacteria were stained for 15 min with 200 µl of 0.1% (wt/vol) crystal violet. Excess stain was rinsed off by applying the pegs into a 96-well plate filled with 200µl PBS per well. The pegs were then left for 30 min until air dried and the dye bound to the adherent cells was extracted with 200µl of 96% ethanol. Additionally, sterile medium was always included (negative control) to verify that the influence on biofilm formation was not attributed to a nonspecific binding effect to crystal violet. The optical density (OD) of each well was measured at 630nm using an ELISA human reader/ Germany.

Antimicrobial Sensitivity Test

Antimicrobial sensitivity test was performed for each isolate characterized as *Lactobacillus spp*. by using 10 different types of antimicrobial agents disks (Erythromycin, Ciprofloxacin, Tobramycin, sulfamethoxazole, clindamycin, Imipenem, amoxillin, Trimethoprim, cefoxitin, and metroniodozol). The results of this experiment were recorded according to the standard guidelines recommended by the clinical and laboratory standard institute [29].

Extraction of Exopolysaccharides (EPS)

Different parameters were employed to obtain the best isolate which could produce EPS, such as morphology, biochemical activities, biofilm and capsular formation, and sensitivity to antimicrobial agents. There are two main methods which were applied and are considered as ideal procedures for extraction and partial purification of bacterial EPS.

Method 1: Cultivation of the selected *Lactobacillus rhamnosus* isolates was performed in 5 flasks, each one contained 250ml MRS medium with 100µl of activated *L. rhamnosus* broth culture ($1X10^8$). The mixture was incubated for 48h at 37°C. An overnight culture was heated to 100°C for10 minutes. After cooling, all samples were centrifuged at 13 200×*g* by a cooling centrifuge for 25 min, then the supernatants were collected and stored at –20 °C. Polysaccharides in approximately 50 ml supernatant were precipitated by the addition of three volumes of chilled 96% ethanol. The mixture was held at 4°C for 12 hr. Following re-centrifugation (13 200×*g* at 4°C for 15 min),the subsequent pellet was dissolved in distilled water and dialyzed for 48 h against distilled water using dialysis sac (MWCO 4000 to 6000 Dalton, Roth, Karlsruhe, Germany) with stirring. Residual polypeptides were then removed by precipitation with 15% (v/v) of trichloroacetic acid (TCA) (Fluka) and centrifugation at 14 000×*g*, after which the EPS-containing supernatant was re-dialyzed for 5 days. Produced EPS components were determined by measuring protein and carbohydrate concentrations by means of colorimetric methods [30].

Method 2: After the culture incubation period, the cultures were heated at 100 °C for 15 min and the cells were eliminated by centrifugation at 4000×*g* for 30 min at 25º C. By using vacuum rotary evaporator (Büchi R210/ Germany) with 0.093 MPa at 40 °C, the volume of the supernatant was concentrated to approximately 50 ml. The EPS in the solution was precipitated by adding three volumes of chilled 96% ethanol for 24 h at 4 °C with stirring. The precipitant was collected by centrifugation (20 000×*g* for 30 min at 4°C) and dissolved in distilled water. The next step was dialysis for 48 h at 4 °C against water which was changed after 24 h (4000 to 6000 molecular weight cutoff for the dialysis membranes, Roth,Karlsruhe, Germany). After dialysis, the crude EPS was purified by suspending in 15% TCA and centrifugation at (20 000×*g*). Next, an exhaustive dialysis was performed for 5 days with one water change per day [31].

Extraction of capsular polysaccharides (CPS)

CPS extraction was achieved according to the method described by Polak *et al*., [31]; the bacterial cells were washed twice with 0.9% NaCl and then treated with 0.5% phenol for 4 h at 25ºC. After centrifugation (12 000×*g* for 15 min, at 4 °C), the pellets were excluded and the solubilized CPS present in the supernatant fraction was extracted using the same procedure as for EPS.

Chemical analysis of Exopolysaccharides (EPS) extracts

Determination of protein concentration: Protein concentration was determined using Bradford method [32].

Determination of the carbohydrates concentration: Depending on Dubois *et al.* [33], the phenol-sulfuric acid method was used to determine the amount of carbohydrates in the prepared extracts.

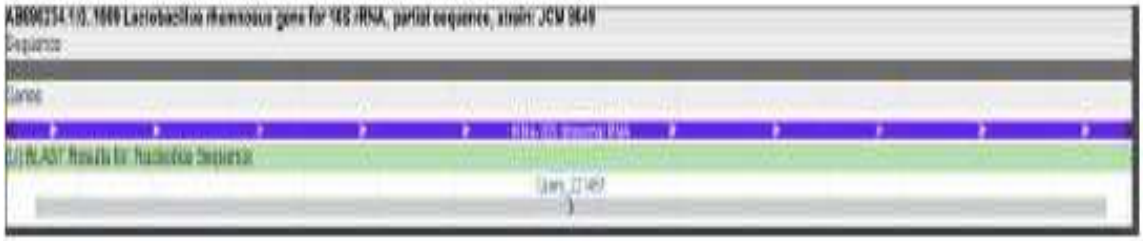
Results and discussion

Identification of bacterial isolates

Several characteristic parameters (microscopic morphology, colony morphology and biochemical tests) were followed to identify the bacterial isolates. The results revealed that 13 (13%) of the bacterial isolates were *lactobacillus spp.,* out of the 100 total number of vaginal specimens. Analysis with the API 50CHL kit showed a positive result for 4/13 (30.8%) isolates as *Lactobacillus rhamnosus.*

Furthermore, primary characterization based on the biochemical results facilitated the choice of appropriate molecular methods for further species identification. Also, according to16S rRNA analysis, the results showed 100% similarity to the species *L.rhamnosus* with the band of PCR product 1534 bp (Table-1, Figure-1), the partial 16S rRNA sequence (GenBank accession number, EU402395)

Table 1-Data analysis



Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactobacillus rhamnosus gene for 16S rRNA, partial sequence, strain: JCM 8649	2645	2645	100%	0	100%	AB690234.1

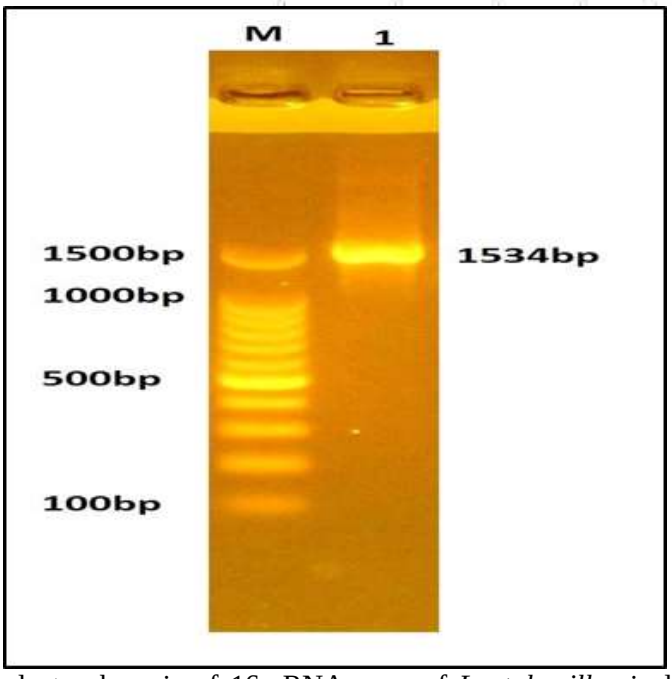


Figure 1-Agarose gel electrophoresis of 16s RNA gene of *Lactobacillus* isolate using PCR, which were fractionated on 1% agarose gel (100 V/75min) and stained with Eth.Br. Lane1:100bp DNA marker .Lane 2: *L.rahmnosus.*

This result was confirmed to that documented by Ronald *et al.*, [34] about *L. rhamnosus* that could be recommended as one of vaginal microbiota.

Detection of the biofilm formation ability of the bacterial isolates

The test of bacterial isolates' ability of biofilm formation on Congo red showed a positive results, as shown in Figure-2.

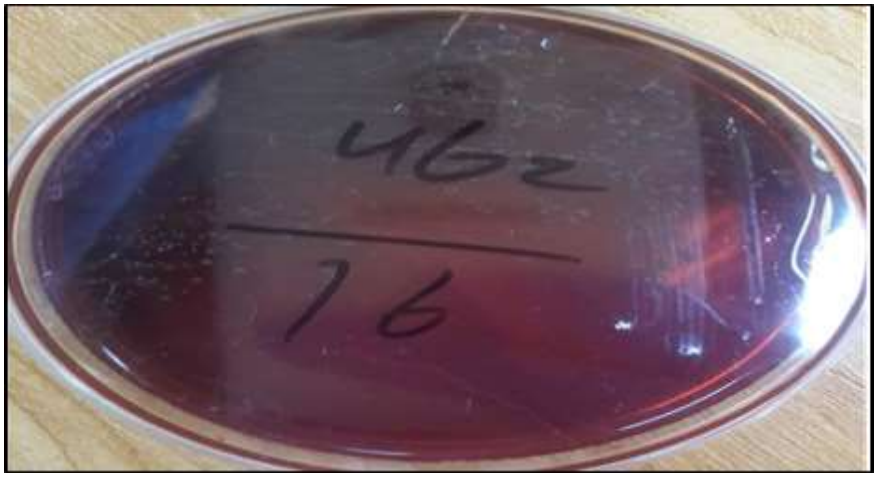


Figure 2-Positive result of *Lactobacillus rhamnosus* biofilm formation on Congo red agar plate.

This result was confirmed by microtiterplate format assay, which was used to detect the strongest one. The results in Table-2 revealed that all bacterial isolates showed high ability to form biofilm. The selection of bacterial isolates was achieved depending on biofilm formation. This result is in agreement with that reported by Lepargneur and Rousseau [35], who described the advantageous ability to form biofilms, which is a distinctive feature enabling the bacteria to resist environmental conditions, leading to the effective colonization and preservation of their population. In addition, Costerton *et al*. [36] elucidated that the probiotics bacterium form complex populations, known as biofilms, possess several characteristics useful for the development of a microbial population tolerating different abiotic or biotic factors. In the present study, this test resulted in the selection of the isolate L.10 as the strongest biofilm forming isolate.

Table 2- Biofilm formation ability of Lactobacillus isolates using microtiter plate format assay

***Lactobacillus* isolate**	**Result of the mean of biofilm triplicate**	**Control**	**The result of divided mean of Triplicate sample to Control**	**Biofilm Production**
L.1	0.93333	0.09567	9.7557228	Strongly adherent
L.2	0.95867	0.09567	10.0205916	Strongly adherent
L.3	0.85567	0.09567	8.94397408	Strongly adherent
L.4	0.674	0.09567	7.0450507	Strongly adherent
L.5	0.81467	0.09567	8.51541758	Strongly adherent
L.6	0.96567	0.09567	10.0937598	Strongly adherent
L.7	0.99133	0.09567	10.3619735	Strongly adherent
L.8	0.82367	0.09567	8.60949096	Strongly adherent
L.9	0.767	0.09567	8.01714226	Strongly adherent
L.10	0.993	0.09567	**10.3794293**	Strongly adherent
L.11	0.80267	0.09567	8.38998641	Strongly adherent
L.12	0.90267	0.09567	9.43524616	Strongly adherent
L.13	0.908	0.09567	**9.4909585**	Strongly adherent

*** Non –adherent: C≤D ---OD < 0.120*weakly adherent: D<C≤2*D ---OD >0.120 but ≤ 0.240*moderately adherent: 2*D<C≤4*D*strongly adherent: 4*D<C --- OD > 0.240**

Antimicrobial susceptibility test

Antimicrobial susceptibility test was done for different *Lactobacillus* isolates. Several researchers documented that some species of lactobacilli are intrinsically resistant to vancomycin and aminoglycosides [37, 38], whereas other revealed that glycopeptides have variable activities against different species and strains [39]. The results of the present study showed increasing resistant of the bacterial isolates against common antimicrobial agents (Table-3). A standardized method to study the antimicrobial susceptibility of *Lactobacillus* has not been completely published, probably because they have been considered as "GRAS" by the Food and Drug Administration, USA [40]. Many researchers developed alternatives for the semi-quantitative disc assay for lactobacilli [41, 42]. As previously described by other researchers [43], the growth of lactobacilli in Muller Hinton was poor and irregular and it was not possible to measure the diameter of the inhibition halos. MRS appropriate for all *lactobacilli.*

Table 3-Antimicrobial susceptibility test for vaginal *Lactobacillus rhamnosus* isolate

Antibiotic	Resistant %	Intermediate %	Sensitive %	L2	L6	L7	L10
				Antimicrobial inhibition zone diameter (mm)			
Erythromycin	**≤ 0.5**	**1 – 4**	**≥ 8**	**23**	**17**	**12**	**22**
Ciprofloxacin	**/**	**/**	**/**	**12**	**R**	**13**	**12**
Tobramycin	**/**	**/**	**/**	**R**	**R**	**R**	**R**
Sulfamethoxazole	**/**	**/**	**/**	**R**	**25**	**R**	**15**
Clindamycin	**≤ 0.5**	**1**	**≥ 2**	**16**	**12**	**R**	**24**
Imipenem	**≤ 0.5**	**1**	**≥ 2**	**28**	**35**	**28**	**24**
Amoxillin	**/**	**/**	**/**	**29**	**30**	**34**	**23**
Trimethoprim	**/**	**/**	**/**	**10**	**17**	**10**	**16**
Cefoxitin	**/**	**/**	**/**	**10**	**10**	**R**	**R**
Metroniodozol	**/**	**/**	**/**	**R**	**10**	**R**	**R**

On the other hand, this test revealed a higher resistant of Lactobacillus spp. against some broad spectrum antimicrobial agents such as Tobramycin, Cefoxitin, and Metronidazole. The results disagree with those reported by Gueimonde *et al*. [38].

Determination of EPS production

Many bacteria that belong to the LAB have been described to produce polysaccharide layers on their surface, which, together with glycoproteins, form a structure commonly referred to as the 'glycocalyx'. These exo-cellular polymers include capsular polysaccharides (CPS), which form a cohesive layer that is covalently linked to the cell surface, as well as EPSs which are loosely attached to the cell surface and either form a slime layer or are secreted into the environment [44]. Hassan (2008) documented that only some LAB bacterium can simultaneously produce both CPS and EPS [45]. While, Pham and coworkers (2000) mentioned that EPS from LAB should be considered as minor products diverted away from glycolysis [46]. The results of the present study revealed that the selected *L. rhamnosus* has an ability to form CPS (Figure-3) and EPS. These results were improved through the determination of the amount of carbohydrates in the partial purified extract.

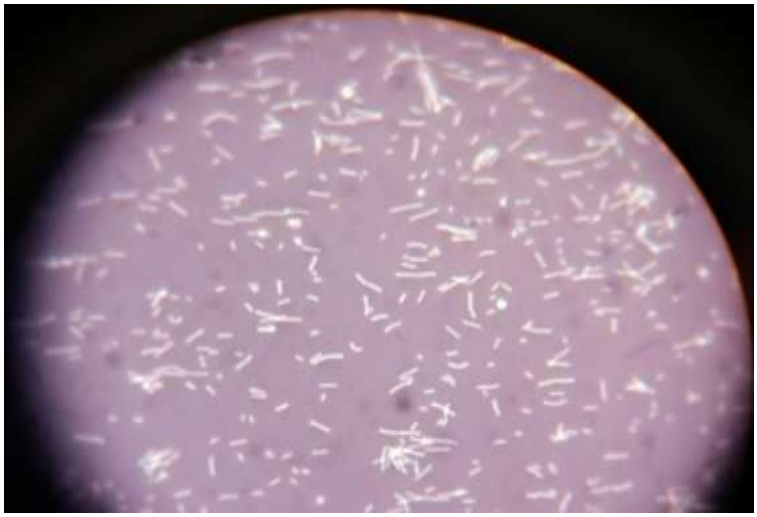


Figure 3-Light microscope view of *Lactobacillus rhamnosus* negatively stained with Nigrosin stain (100x).

Determination of the carbohydrate and protein concentration

The results in Table- 4 show the efficiency of the first method for the extraction of EPS as well as with less protein residues. This difference between both methods may be due to the heat treatment step, because treatment of the sample by heating as a first step in the polysaccharide isolation procedure could act as a critical point for complete recovery of EPS. This point could be ascribed to the separation and dissolution of the polysaccharide that is adhered to the cell wall and the inactivation of the enzymes which are potentially capable of degrading the polymer [31]. These results are consistent with those of Adekemi *et al.,* (2018) [47], who documented that the chemical composition of the EPS from *L. plantarum* showed the presence of carbohydrates and proteins. Moreover, the protein concentration in the partial purified extract was lower after applying the first method (0.0145mg/ml) as compared to that from the second one (0.026 mg/ml). On the other hand, the results of the determination of nucleic acids showed that the amount of nucleic acids in the extraction samples recorded a high concentration, especially using the first method which also showed lower purity as compared with the second method (Table-4). These results may reflect that the components of the extracted EPS were related to the biofilm layer form by the bacterial isolate. Therefore, it could be suggested that the first method is suitable to extract bacterial EPS. However, the detected amount of capsular carbohydrate reflects either the thin capsule which surrounded the bacterium or a low efficiency of the extraction method.

Table 4-Biochemical analysis of bacterial isolate exopolysaccharide and capsule obtained using different extraction methods.

Sample type	carbohydrate conc.(µg/ml)	Protein conc. (µg/ml)	Nucleic acid	
			conc. (ng/ml)	Purity (260/280)
Partial purified extract (method 1)	74.774	0.0145	776.2	1.48
Partial purified extract (method 2)	17.5625	0.026	121.2	1.54
Capsular polysaccharides	1.314	0.01	0.1	0.04

It can be concluded that all vaginal lactobacillus spp. isolates can produce biofilm and EPSs, which play important roles in bacterial adherence to vaginal epithelial cells. This important fact makes lactobacillus strains a good probiotic for vagina. Finally, the current study showed that lactobacillus isolates are resistant to most usable antimicrobial agents.

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