

Extraction and Characterization of *Listeria monocytogenes* Exopolysaccharide

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(Received 4/ 3 / 2014 ; Accepted 5/ 5 / 2014)

ABSTRACT

This study was aimed to extract exopolysaccharide (EPS) produced by *Listeria monocytogenes* and investigate the maximum production by using different culture media and various factors. The EPS was obtained from the culture supernatant by cold ethanol precipitation and yield maximum production (0.368mg /ml) in basal salt solution (BSS) as dry weight. The total carbohydrate content was determined by phenol sulfuric acid method. The functional groups were detected by Fourier transform infrared (FTIR) spectroscopy at frequency range of 400- 4000cm⁻¹. Different alcohols were checked for their efficiency on precipitating EPS, those other than ethanol, methanol and isopropanol were able to sediment the EPS, while isoamylalcohol show no effect on precipitation. The results also indicated that the medium volume play an important role in EPS production.

Keywords : *Listeria monocytogenes* , EPS, FTIR spectroscopy.

استخلاص و توصيف متعدد السكريات الخارجي لجراثيم *Listeria monocytogenes*

الملخص

تضمنت الدراسة استخلاص متعدد السكريات الخارجي (EPS) المنتج من قبل *Listeria monocytogenes* للحصول على أعلى إنتاجية باستخدام أوساط غذائية مختلفة وتأثير عوامل مختلفة. تم ترسيب متعدد السكريات الخارجي من رشح المزرعة باستخدام الايثانول المبرد وكانت أعلى إنتاجية (0.368) ملغم /مل كوزن جاف باستخدام وسط المحلول الملحي (BSS) وقدر محتوى الكربوهيدرات الكلي باستخدام طريقة الفينول-حامض الكبريتيك. تم تحديد المجاميع التركيبية باستخدام طيف الأشعة تحت الحمراء (FTIR) عند التردد 400-4000 سم⁻¹.

استخدمت أنواع مختلفة من الكحولات وقد أظهر الايثانول والميثانول والايثوبروبانول كفاءة في ترسيب EPS، في حين ان ايزواميل الكحول لم يظهر أي تأثير في الترسيب. أظهرت النتائج أيضا أن حجم الوسط الزراعي يلعب دورا مهما في إنتاج متعدد السكريات الخارجي.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacterium with optimal growth temperature range of 30 - 37°C. It is one of the major food-borne pathogen causing disease in both humans and animals and has affected the food industries due to contamination of equipments that lead to cross contamination on food products (Chen *et al.*, 2010; Pagadala *et al.*, 2012). Contamination of surfaces in the processing environment can be attributed to attachment of pathogenic bacteria. Consequently, attachment of pathogenic microbes leads to biofilm formation that will occurs when microcolony formed on the surface adheres with a conditioning film that traps the surrounding fluid and nutrients (Sauer *et al.*, 2007; Lee *et al.*, 2013).

Microbes release polysaccharides extracellularly as exopolysaccharides (EPSs) into the environment, the naturally occurring polysaccharides possess a unique combination of functional and environmental friendly features. they are renewable in nature, nontoxic, and biodegradable (Freitas *et al.*, 2011). EPS is located at the cell surface of, or outside and detached from bacteria and can fill the space between cells existing in a colony, substances associated with exopolymeric matrices have multiple functions. Some serve as signaling molecules or messengers and others are energy and nutrient reserves with an important role in polymer degradation and surface adhesion (Marvasi *et al.*, 2010).

EPS is often produced at a lower temperature than is required for optimum growth (Abdul Razak *et al.*, 2013). It also requires higher carbon content in the medium and decreased nitrogen quantity, factors that could influence the production of EPS are the composition of the medium, especially carbon and nitrogen sources, and parameters like pH, temperature, and incubation time (Morikawa., 2006). EPS may be produced through specific biosynthetic pathways and are then exported and actively translocated to the cell surface. It can also be produced by spontaneous liberation of integral cellular components and different mechanisms to produce EPS are adopted by different bacterial species (Flemming *et al.*, 2007; Marvasi *et al.*, 2010).

The aim of this study was to evaluate the production of exopolysaccharide by *Listeria monocytogenes* and study of some factors that affect its production.

MATERIALS AND METHODS

Bacterial isolate and culture medium

The organism *Listeria monocytogenes* was isolated from a clinical sample, and identified by using API- 20 techniques, obtained from Bacterial Strains Bank Unit / Biology department / College of Science in Mosul University. *Listeria monocytogenes* was maintained in tryptic soy broth (TSB) with 20% glycerol at -80°C. Cells were subcultured by inoculating brain heart infusion (BHI) and incubating at 37°C for 24h . The purified culture was stored on nutrient agar slant at 4°C as stock for further study.

Production of EPS

To find the optimal culture medium to produce EPS by *L. monocytogenes*, the following media were used: basal salt solution (BSS) (Bragadeeswaran *et al.*, 2011), nitrogen- free medium (NFM), and chemically defined medium (CDM). For the primary EPS production, 100 ml of each culture medium was prepared and inoculated with *L. monocytogenes* and then incubated at 37° C for 4 days (Borgio *et al.*, 2009).

Extraction of EPS

Bacterial culture was centrifuged at 5000 rpm for 15 min(REMI motors LTD (India)), the supernatant was collected and mixed with an equal volume of cold ethanol and incubated at 4°C for 24 hr. The precipitated EPS was collected by centrifugation at 5000 rpm for 20 min, then dried at 70°C until it was completely dried, then labeled as crude EPS. The EPS pellet was weighted before and after drying and considered as wet and dry weight respectively (Savadojo *et al.*, 2004).

Characterization of EPS

Estimation of total carbohydrate and protein contents of EPS

Colorimetric analysis was used for determination of total carbohydrate content in the culture supernatant by phenol-sulfuric acid method using glucose as a standard monitoring. The absorbance was measured at 488 nm (CEC1L, CE7200 (U.K.)), Protein content was determined by Lowery method with bovine serum albumin as standard (Dubois *et al.*, 1956 ; Lowry *et al.*, 1951).

Infra-Red spectroscopy analysis of EPS

The crude EPS was analyzed by the Fourier Transform infrared (FTIR) spectrophotometer (Biotech engineering management CO. LTD. (U.K.)). One part of the EPS pellet was mixed with nine part of potassium bromide powder (KBr) and then compressed into a thin disk. The disk was

then used for FTIR spectroscopy with the wave lengths ranging from 400 to 4000 cm^{-1} , to detect the C=O and – OH bonds in the sample (Vidhyalakshmi and Nachiyar, 2011).

Effect of different alcohols on EPS precipitation.

Firstly, to determine the efficiency of alcohols on EPS extraction, we compared between the effect of ethanol and a three chosen types of alcohols (methanol, isoamylalcohol, isopropanol) to precipitate and yield the highest EPS. An equal volume of tested alcohols were added to four flasks each which contains 25 ml of the supernatant obtained from the culture medium. The precipitated EPS was observed after incubation over night at 4°C, the precipitate was collected after centrifugation at 5000 rpm at room temperature for 20 min., and then dried at 70°C and weighted (Abdul Razack *et al.*, 2013). Secondly, dependent on the obtained results and to find the best ratio of the best tested alcohol through extraction, different volumes (ratio) of the best alcohol was added to 20ml of supernatant as follows: (1:1 , 1:2 ,1:3 , 1:4 , 1:5) the result was observed after overnight incubation at 4°C (Xu,2007).

Effect of culture medium volume on EPS production

To examine the effect of medium volume to yield maximum EPS, various culture volumes of BSS medium (20, 40, 60, 80, ml) were added in 100 ml flask then inoculated with *L. monocytogenes* and incubated aerobically at 37° C for 4 days (Wang *et a .*, 2011).

RESULTS AND DISCUSSION

Production of EPS on different media and its functional group analysis by FTIR spectroscopy

With a concern to find out the ability of *L. monocytogenes* to produce EPS in different chosen media, the quantity of EPS produced was determined and the results are shown in (Fig. 1), large amount of EPS production (0.368mg/ml) was obtained in BSS medium flowed by CDM medium, the lowest EPS production was recorded with NFM medium (0.102mg / ml) as dry weight.

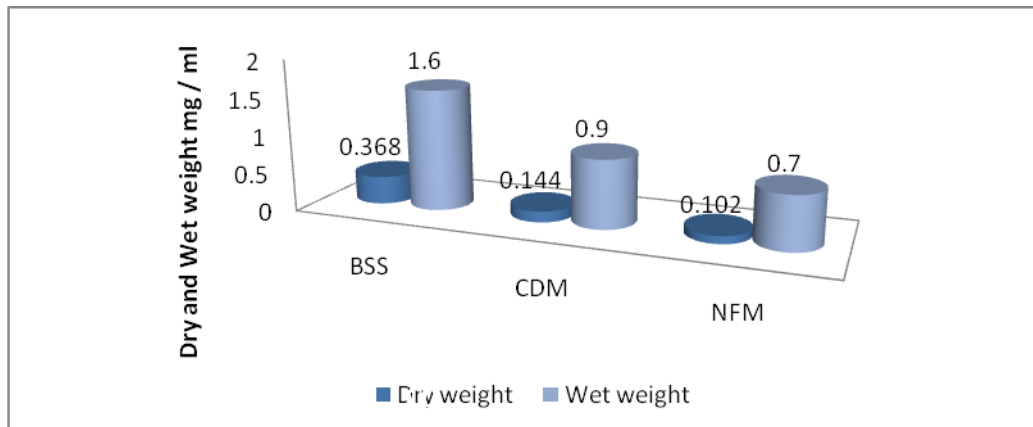


Fig 1: Quantity of EPS (mg / ml) on different media

The weight of EPS, carbohydrate concentration obtained using BSS medium (1790 $\mu\text{g/ml}$) was greater than that of CDM (960 $\mu\text{g/ml}$) and NFM (780 $\mu\text{g/ml}$) as shown in (Fig. 2).

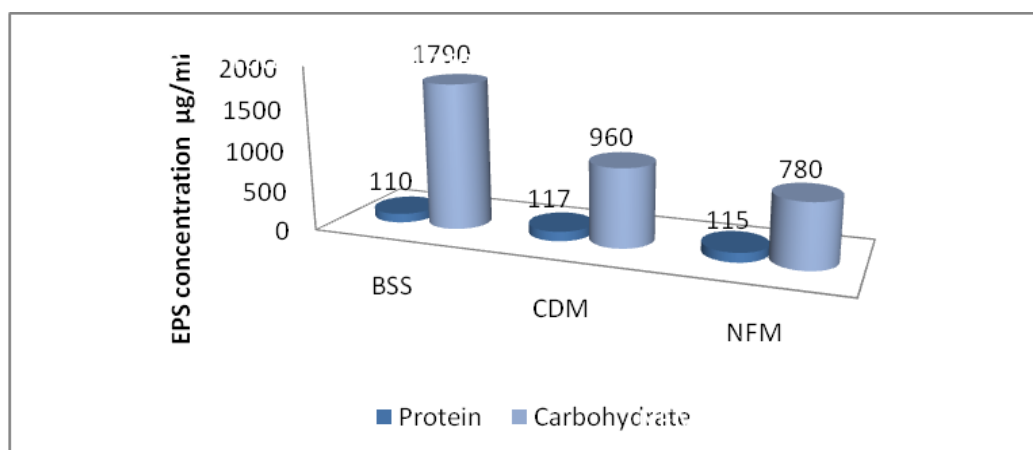


Fig. 2: Total carbohydrate and protein concentrations of EPS extracted by different media

The results from the FTIR spectroscopic analysis of EPS produced by the bacteria under study for the three tested media indicates the presence of different function groups such as C=O, COOH, and OH groups. The FTIR spectra showed the presence of the characteristic bands for OH group at 3350 cm^{-1} , 3327 cm^{-1} and 3384 cm^{-1} in BSS, CDM and NFM, respectively. The absorbance peak at 2924 cm^{-1} for BSS and NFM, and at 2925 cm^{-1} for CDM indicate the presence of C-H aliphatic group. The intensity of the bands at 1662 cm^{-1} in BSS and CDM, at 1655 cm^{-1} in NFM was due to C=O bond (Figs. 3, 4, 5). According to the obtained results of FTIR analysis and corresponding to the amount of yielded EPS, the BSS medium showed higher efficacy in supporting *L. monocytogenes* to produce large amount of EPS, so this medium was depended to estimate the EPS in the current study.

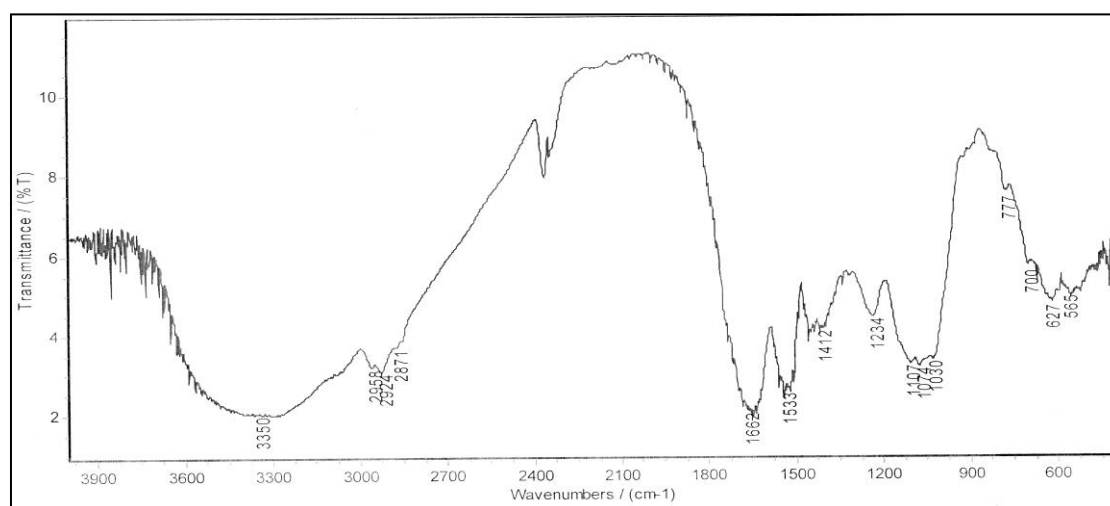


Fig. 3: FTIR analysis of *L.monocytogenes* EPS from BSS medium

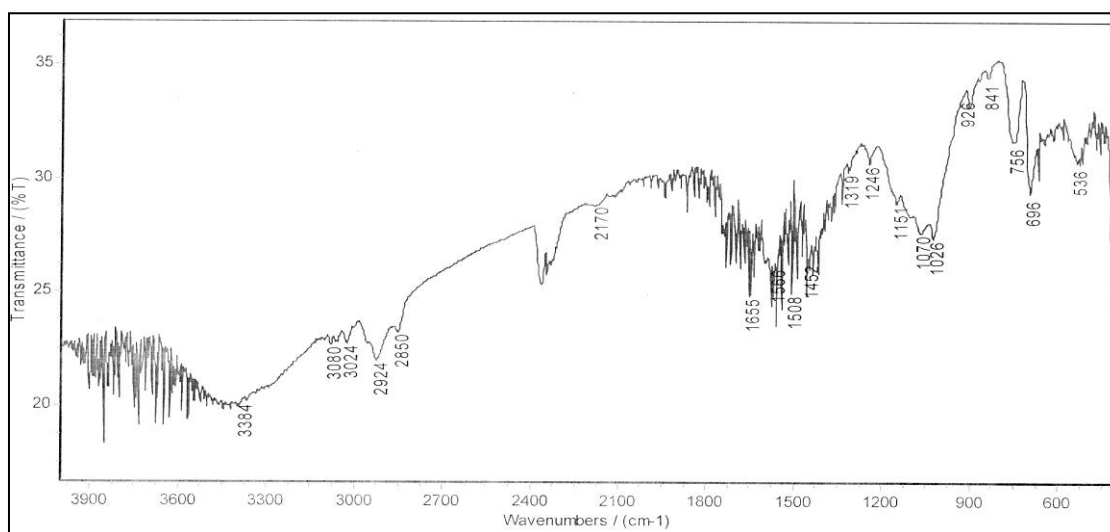


Fig. 4: FTIR analysis of *L.monocytogenes* EPS from CDM medium

The BSS medium was suitable for production of EPS which it contains carbon and nitrogen source greater than the other media. Many studies indicated that although the composition and amount of EPS produced by a bacterium are genetically determined traits, they are highly influenced by media components and cultivation conditions, and the extracellular biopolymers' synthesis by microbial cells depends on the carbon and nitrogen availability in the culture medium and EPS synthesis is generally favored by the presence of a carbon source in excess. Most exopolymer-producing microorganism utilize carbohydrates as their carbon and energy source and either ammonium salts and amino acids as their source of nitrogen (Freitas *et al.*, 2011; Czaczyk and Wojciechowska, 2003).

The BSS also contain 3.5% sodium chloride (NaCl) and this salt will enhance the EPS production, Pan *et al.*, (2010) found that when the colonies of *L. monocytogenes* were incubated with tryptic soy broth as nutrient and NaCl as additional nutrients, the formation of biofilm was enhanced as a synergistic effect on the combination of glucose, NaCl and TSB. On the other hand Lee and his colleagues (2013) in their studies on the effect of sodium chloride on *L.monocytogenes* ATCC 19112 (serogroup 1/2c), the organism was placed on the cellular stress by NaCl instead of NaCl as additional nutrient to the cells, they showed that this microorganism was able to form biofilm with minimal nutrient requirements, for example, in presence of NaCl only.

Wong (2012) showed that when *Listeria* grown in a minimal medium (D10 and MCDB202) it produced more capsule-like material than those grown in a rich media (BHI) when imaged using SEM. From this image it can be seen that *Listeria* cells grown in BHI appears as separate, single rods showing little evidence of EPS formation, whereas the cells grown in minimal media were surrounded by visible coating of EPS, and also were surrounded by strings-like structure spanning between cells.

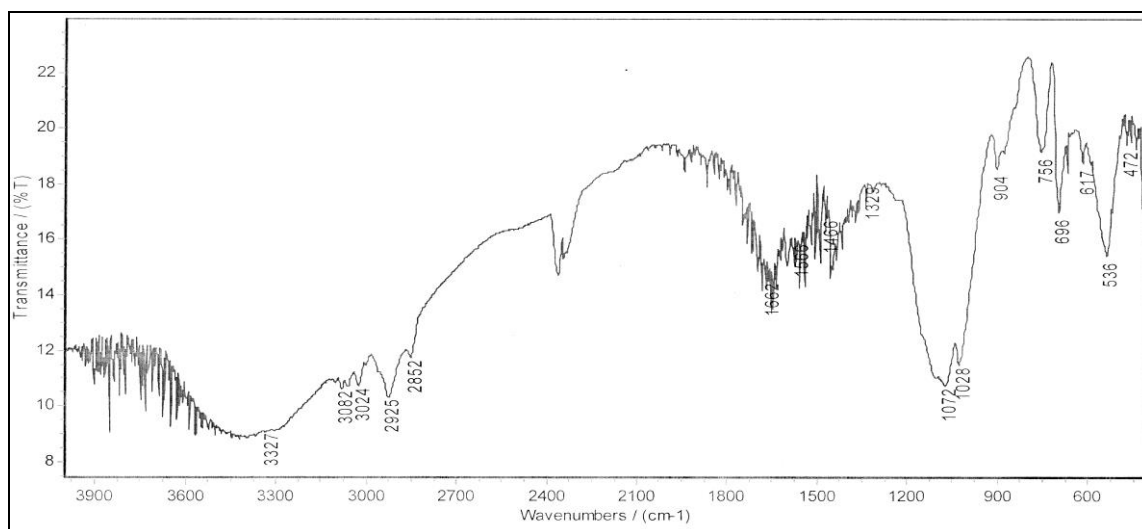


Fig. 5: FTIR analysis of *L.monocytogenes* EPS from NFM medium

Effect of different alcohols on EPS precipitation.

When we compared between ethanol and other alcohols on the ability to precipitate *L.monocytogenes* EPS, we found that cold ethanol gave higher yield of EPS (0.64 mg / ml) after 24 hours considered as dry weight (Fig. 6).

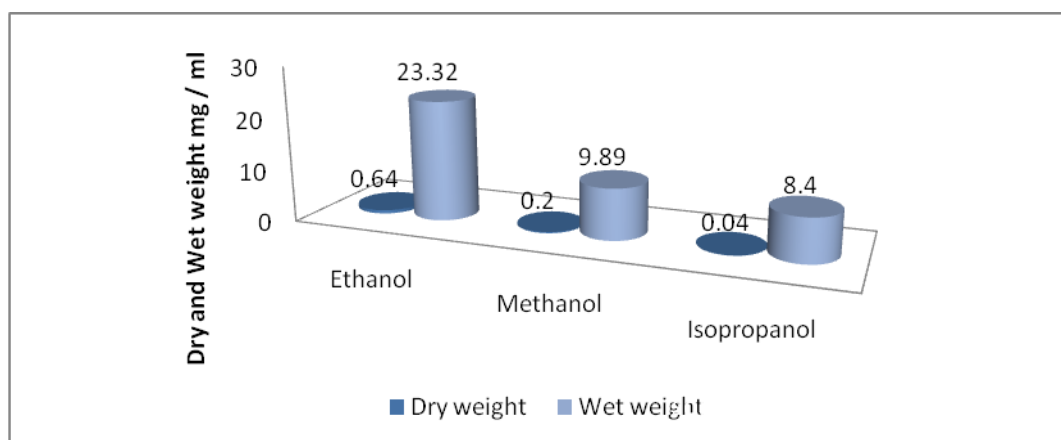


Fig 6: Effect of different alcohols on *L.monocytogenes* EPS precipitation

The amount of total carbohydrate in BSS medium was (313µg/ml) and the amount of crude EPS after precipitation also indicates ethanol was the best precipitate for EPS that yield (588µg/ml) while isopropanol led to the lowest yield (94µg/ml) (Fig .7).

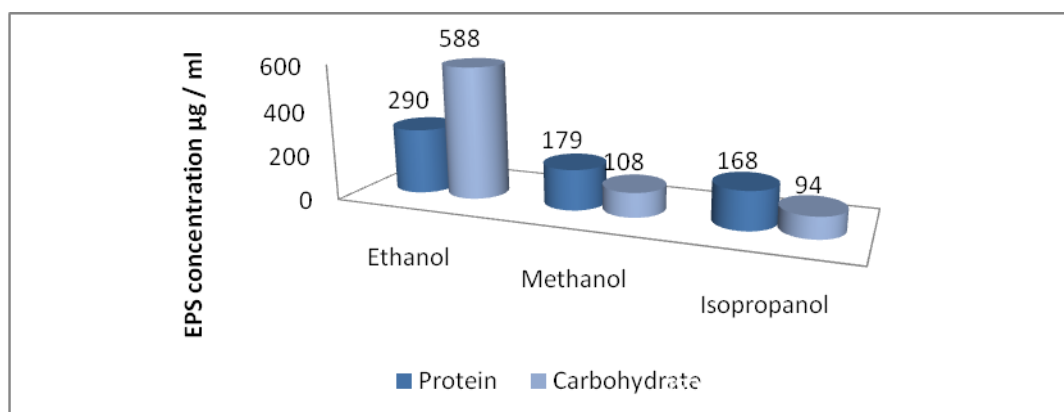


Fig. 7: Total carbohydrate and protein concentrations of EPS extracted by different alcohols

In this study, various solvents were tested for their efficiency in extracting the EPS from the culture. The results showed that the standard solvent was ethanol, it precipitated EPS within 24 h and overnight incubation, whereas methanol sedimented EPS instantly upon addition to the culture supernatant. Ethanol precipitates polysaccharides preferentially and it represents the standard alcohol used to extract exopolysaccharide from microorganisms in most other studies (Qiang *et al.*, 2013). In the extraction method of EPS from *Bacillus subtilis* (Abdul Razack *et al.*, 2013) recorded that methanol sedimented EPS instantaneously upon addition to the culture supernatant, whereas when they used ethanol it required 24 h for incubation to precipitate EPS.

Our results revealed that isopropanol showed effective action but less than ethanol, other study also used cold isopropanol in EPS purification from *Staphylococcus aureus* (Kolodkin-Gal, *et al.*, 2012), but Xu, (2007) demonstrated that isopropanol precipitated protein more than polysaccharides. According to our results, isoamylalcohol showed no effect on extraction of EPS because after the addition of isoamylalcohol to the culture supernatant for 24 h, there was no obtained EPS pellet and the solution stayed as two separated layers even after 48 h, this is also reported by Abdul Razack and his colleagues (2013) when they found the same result as they showed a form of white interface at the junction between solvent and supernatant which apparently consists of lipids and proteins.

According to previous results, ethanol was depended as the best precipitating agent to produce EPS by *L.monocytogenes*, and to find the best ratio for precipitating, we found that when we added (100)ml ethanol to 20ml supernatant it gave higher yield of EPS (6.90mg / ml) as dry weight. The amount of total carbohydrate in BSS medium was (8800 µg/ml) and the amount of crude EPS after precipitation was yield (6300 µg/ml) (Table 1). The same conclusion was observed in *Pseudomonas fluorescens Biovar II*, where Xu (2007) recorded that using 75% ethanol volume yield higher total carbohydrate.

Table 1: Effect of different ethanol volume on *L.monocytogenes* EPS precipitation

Ethanol ratio	Estimation of EPS			
	Wet weight (mg/ml)	Dry weight (mg/ml)	Carbohydrate (µg/ml)	Protein (µg/ml)
1:1	4.8	1.6	3500	9200
1:2	6.1	2.3	5900	9500
1:3	10.25	2.75	6100	9600
1:4	12.15	4.95	6200	9700
1:5	23.8	6.90	6300	9800

Effect of medium volume on EPS production

The correlation between the resulted EPS with the different culture medium volume was pointed up (Table 2). The higher EPS production was obtained when the culture medium volume was (40) ml in 100ml flask, so the medium liquid volume may play an important role in EPS production, the same result of that reported by Wang and his colleagues (2011) which also found that 25ml of culture medium volume in 200ml flask give maximum EPS produced by *Bacillus thuringiensis* 27. Several studies have reported that medium volume was vital in order to increase aeration during incubation process, which had a great effect on the cell growth and EPS production and conclude that the aeration stimulate extracellular polysaccharide production (Czaczyk and Myszka, 2007; Vu *et al.*, 2009; Poli *et al.*, 2010). The production of most bacterial EPS occurs under aerobic conditions, for optimal production of some EPSs maximal aeration was required (e.g. xanthan gum) where as for others, synthesis is maximized under microaerophilic conditions (e.g. bacterial alginate) (Freitas *et al.*, 2011). Also Qiang and his colleagues (2013) observed that EPS produced from *Klebsiella sp.H-207* was slightly affected under a medium volume range of 20 to 60ml and then it decline sharply when medium volumes were more than 60ml.

Table 2: Effect of medium volume on EPS production

medium volume (ml)	Estimation of EPS	
	Carbohydrate ($\mu\text{g/ml}$)	Protein ($\mu\text{g/ml}$)
20	77	194
40	84	152
60	82	150
80	74	128

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