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The Effect of some Variables on the Quantity of Exopolysaccharide Produced by *Klebsiella pneumoniae*

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ABSTRACT

The current study attempted to compare the production of exopolysaccharide (EPS) by a clinical isolate *Klebsiella pneumonia*e under different selected conditions using the colorimetric method, phenol-sulfuric acid. The results revealed that the chemically defined medium was the most efficient for the production of free bacterial exopolysaccharide. The type and concentration of carbon source were of critical role in determining the amount of the yield EPS. The elevated fructose concentration furnished the highest level while lactose promoted the lowest yield. The pH ranges between 6- 8 of the medium promote the maximum production; lower and higher than this range the production will decrease. In the batch culture vessel used in the current study, a bacterium inoculum of 3-5% (v/v) produced a valuable amount of EPS after three days of incubation.

Keywords: Exopolysaccharide, *Klebsiella pneumoniae*, Biofilm.

تأثير بعض المتغيرات على كمية متعدد السكريات الخارجي المنتج من *Klebsiella pneumoniae*

الملخص

حاولت الدراسة الحالية مقارنة انتاج متعدد السكريات الخارجي من قبل العزلة الـسريرية *Klebsiella pneumoniae* تحت ظروف مختلفة باستخدام طريقة التقدير اللونية phenol-sulfuric acid. بينت النتائج ان الوسط الزرعي chemically defined medium كان الاكثر كفاءة لإنتاج متعدد السكريات الخارجي الحر. وان نوع وتركيز مصدر الكربون كـان ذا دور أساسي في تحديد كمية منتج متعدد السكريات الخارجي. التراكيز المتزايدة من الفركتوز أعطت التركيز الأعلى بينما اللاكتـوز اعطى التركيز الاقل من المنتج. مديات pH بين 6-8 للوسط الزرعي مهدت للإنتاج الأمثل، بينما عند المديات الاقل او الاعلى فان الانتاج قل. في وعاء الزرع المغلق المستخدم في الدراسة الحالية فان لقاح البكتريا 3-5 % (حجم / حجم) انتج كمية وفيرة من متعدد السكريات الخارجي بعد ثلاثة ايام من التحضين.

INTRODUCTION

In their natural environments, most bacteria occur in aggregates whose structural and functional integrity is based on the presence of extracellular polymeric matrix, exopolysaccharide (EPS); some of which devote more than 70% of their energy in its production (Poli *et al*., 2010; Qiang *et al*., 2013). Microbial EPSs are high molecular weight carbohydrate polymers that make up a substantial component surrounding the cells where additional free bacteria can be entrapped, tightly packed and attached to tissue or surface (Sutherland, 2001; Bendaoud *et al*., 2011). Bacterial EPS either being covalently bound to the cell surface as a capsular polysaccharide, loosely attached to the cell surface as a slime polysaccharides or glycocalyx, or found in the extracellular medium as amorphous matrix (Poli *et al*., 2010).

EPS is proved to serve as a structural role, promote adherence to surface, help nutrient acquisition and cellular recognition, play a role in the protection of microbial cell against adverse

environmental conditions, predation by protozoans, and phage attack. It is also involved in the pathogenisity of bacteria and protection from antimicrobial agents and host defenses (Jiang *et al*., 2011; Savadogo *et al*., 2004). It is a common component of the biofilm and an important feature for its maturation. Bacterial cells initiate the adhesion to the surface by using EPS glycocalyx polymers and develop microcolonies, the architecture of biofilm, which may be composed of 10-25% cells and 75-90% extracellular matrix consisting mainly of polysaccharides, adhesions, and proteins (Jiang *et al*., 2011; Zubair *et al*., 2011).

In clinical settings, biofilm is of particular importance as a cause of chronic infections; for instance, its role in the ability of *Kingella kingae* to colonize the pharynx and oral cavity and start sever localized infections was established (Neil *et al*., 2009; Parsek and Singh, 2003). Similarly, its role in the pathogenisity of *E. coli*, *Pseudomonas aeruginosa* was proved (Bendaoud *et al*., 2011). Compared with the planktonic ones, bacteria within the biofilm are 1000-times more resistant to conventional antibiotics and host immune response due to the diffusion barrier; this leads to suggest that biofilms are accounting for over 80% of microbial infections in the body (Jiang *et al*., 2011). For this reason our effort aimed to screen for the favorable conditions that promote the best extraction of EPS produced by the pneumonic pathogen, Klebsiella as a substantive agent for its pathogenicity and establishment of the infection, and try to account the preferred *in vitro* conditions that encourage maximum production to achieve valuable amounts of this compound used in the experimental study particularly in respect to search about the antichemicals and control reagents.

MATERIALS AND METHODS

Bacterial isolate and maintenance: The study depended on the *Klebsiella pneumoniae* isolated from pneumonia infection and diagnosed using an API 20E System strip by Bacterial Strains Bank Unit in Biology department/ college of Science/ Mosul university. It was refreshed in nutrient broth at 37ºC for 24 hr. and maintained in nutrient agar slant at 4ºC over the experimental periods.

Basal medium and primary conditions for EPS production: Three media were tested for their ability to induce *Klebsiella pneumoniae* to produce EPS: the chemically defined medium (CDM), basal salt solution (BSS), and nitrogen- free medium (NFM). They were prepared according to Borgio *et al*., (2009). The pH value was adjusted to 7.2±0.1. For the primary EPS production, 0.5 ml of freshly growing *Klebsiella pneumoniae* was inoculated in 100 ml of each basal medium and incubated at 37ºC for three days (Qiang *et al*., 2013).

Extraction of free and cell-bounded EPS: After incubation, the bacterial cells were harvested by cooling centrifugation at 5000xg for 10 min. and relied to obtain the cell- bounded (conjugated) EPS while the supernatant was relied as a free EPS which was labeled as total EPS. To precipitate the free EPS, an equal volume of absolute ethanol was added to the supernatant and stored at 4ºC overnight. The precipitated EPS was collected by cold centrifugation and the sediment was placed at 75ºC until it dried and labeled as crude EPS (Poli *et al*., 2010). To extract the conjugated EPS, 1.5 ml of 30% NaOH was added to the cell sediment and placed in a boiling water bath for 15 min.; then it was centrifuged to obtain the supernatant and the EPS was precipitated and dried as mentioned(Myszka and Czaczyk, 2011).

Chemical estimation of EPS: EPS production in each media was estimated as follows:

(1) Fourier Transform Infrared (FTIR) spectroscopy: a thin film of the crude EPS disc was prepared by mixing one volume of the crude EPS with nine volumes of dried potassium bromide and pressing the mixture into a thin film. The disc was analyzed with FTIR- 600 spectroscopy (Biotech engineering management CO.LTD. (UK)) to screen the chemical bonds and groups.

(2) Quantification of the EPS concentration: the concentration of the total and crude EPS was estimated by spectrophotometery using phenol- sulfuric acid method for carbohydrate concentration and by Lowry- Folin method for protein concentration. The weight of the total and crude EPS was pointed in mg / 100 ml by a term of wet weight and dry weight respectively.

Investigation of the factors affecting the amount of EPS: CDM was relied as the basal medium to analyze the effect of the following variables on EPS, the principle of the procedures was based on (Bragadeeswaran *et al.*, 2011; Czaczyk and Myszka, 2007; Qiang *et al*., 2013) and the variables were assigned and their values were modified by the current study designation, the concentration of EPS was pointed in term of carbohydrate concentration:

(1) The effect of carbon source type and its different concentrations: to assess the efficacy of different carbon sources to induce EPS production, sugar- free basal CDM was prepared and supplemented with 0.5% (w/v) of each one of five different carbon sources; glucose, sucrose, xylose, fructose, or lactose. In addition to assess the production with different carbon source concentrations ranging from 0.5 to 6g %(w/v).

(2) The effect of pH value of the medium: CDM with different pH values ranging from 5-9 were estimated for the amount of EPS production.

(3) The effect of the bacterial inoculums size: CDM was inoculated with different *Klebsiella pneumoniae* inoculums in percentages between 1 to 6% (v/v) and the concentrations of the yield EPS was assessed.

(4) Evaluating the concentration of EPS in correlation with different incubation periods: the amount of EPS was evaluated after 1, 2, 3, 4, 5, 6, and 7 days of incubation in CDM with optimized conditions of carbon source concentration, pH values of the medium and the inoculum size.

RESULTS AND DISCUSSION

1. Chemical studies on the yield EPS in different basal medium: On comparing the three basal medium (CDM, BSS, NFM) for their ability to induce the primary EPS production by the clinical *K. pneumoniae* under study, it was found that the free EPS substance could be obtained in CDM and NFM. This was concluded from the results of the analysis of the bands and their peaks by the FTIR spectroscopy that were depended to inference the presence of the characteristic peaks of O-H, C-H and C-O group in the components of EPS. The FTIR spectra showed the presence of the characteristic bands of carbohydrate groups in the free EPS extracted from the CDM and NFM which was at 3356 cm^{-1} in CDM and 3421 cm^{-1} in NFM for the alcoholic O-H, at 2974 cm^{-1} in CDM and 2927 cm^{-1} in NFM for aliphatic C-H, and at 1662 cm^{-1}in CDM and 1637 cm^{-1} in NFM for C=O bonds in aldehyde or keton sugars (Fig.1 and 2).

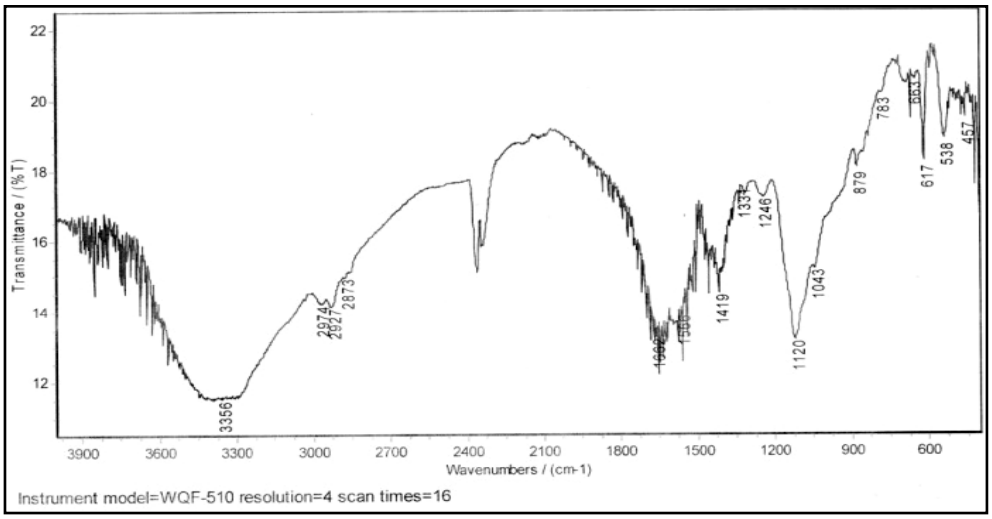

Fig. 1: FTIR spectra of the free EPS from CDM

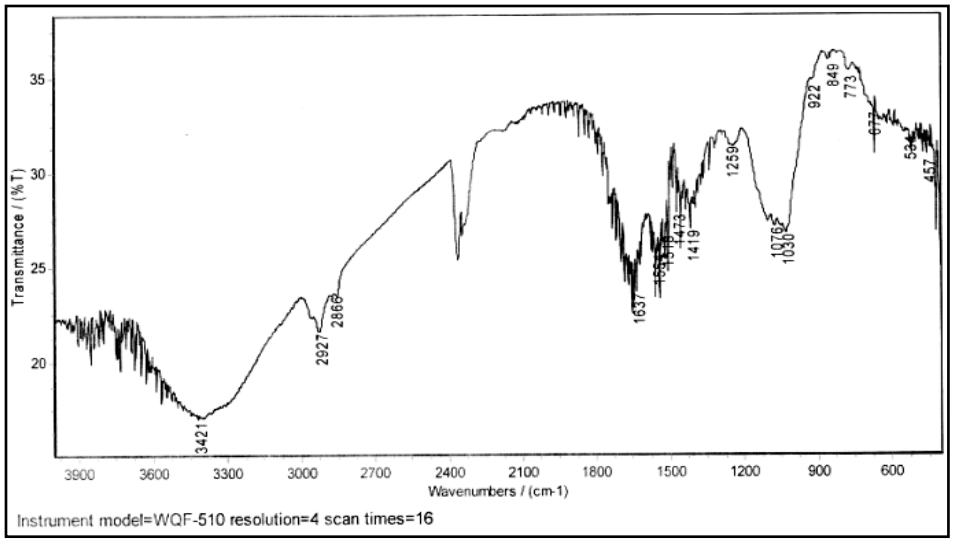

Fig. 2: FTIR spectra of the free EPS from NFM

The bands of EPS extracted from BSS did not show the characteristic peaks of O-H group, and the intense bands at 3462 cm^{-1} did not interrupted for alcoholic O-H group, it may appeared as a result of the presence of even low level of moisture (Fig. 3).

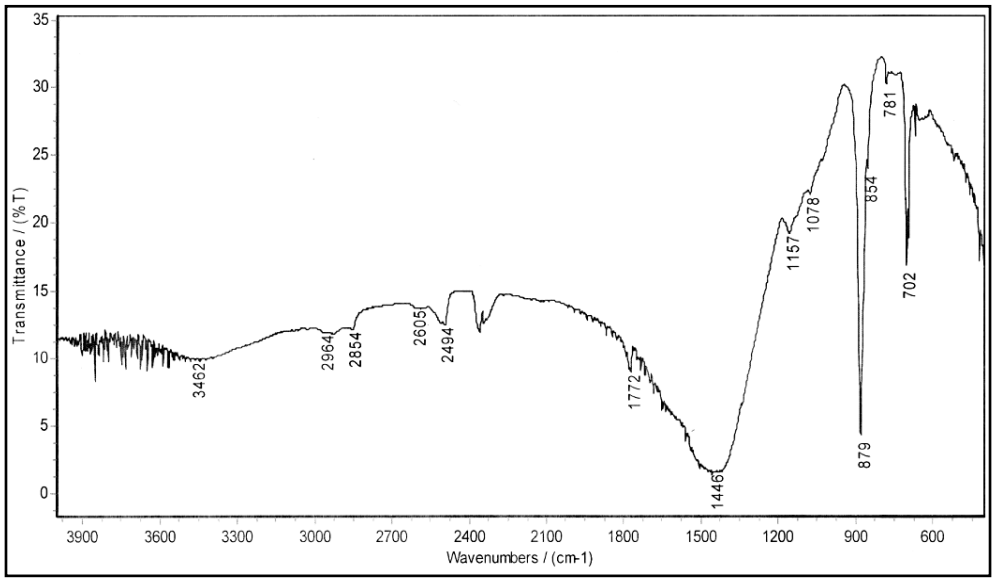

Fig. 3: FTIR spectra of the free EPS from BSS medium

Some researchers carried out their investigations on the cell-bounded EPS that belong to the environmental isolates of *Bacillus subtilis* and *Bacillus cereus* (Chai *et al*., 2012; Orsod *et al*., 2012) and from *Proteus vulgaris* (Myszka and Czaczyk, 2011). So, the current study also try to extract the cell-conjugated EPS depending on the previously used method according which, 30% NaOH solution was added to the bacterial cells then precipitating EPS with alcohol. According to the current results, there is no characteristic band of alcoholic O-H group referring to the presence of polysaccharide in the substances extracted from the bacterial cells grown in CDM nor NFM (fig.

4 and 5). The resulted bands from the substance extracted from cells grown in BSS may refer to the N-H that interfere with alcoholic O-H group (amide) at 3244cm^{-1}, the aliphatic C-H at 2927 cm^{-1}, and the C=O group at 1662 cm^{-1} (Fig. 6).

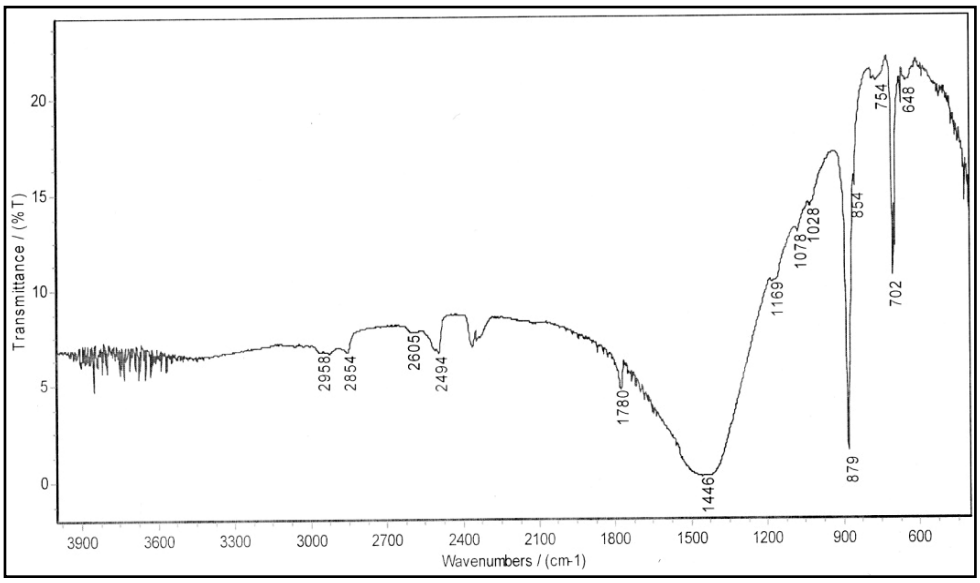

Fig. 4: FTIR spectra of the conjugated EPS from CDM

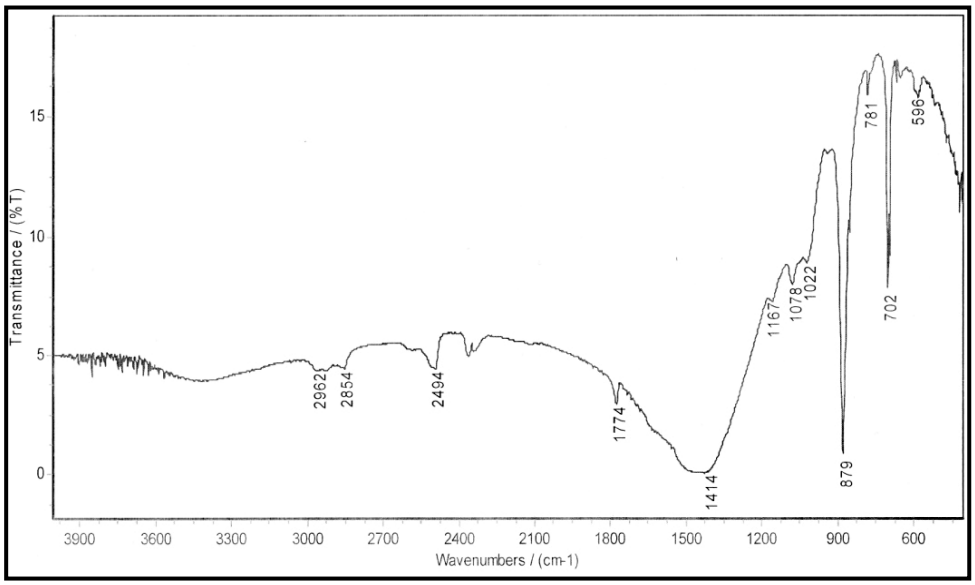

Fig. 5: FTIR spectra of the conjugated EPS from NFM

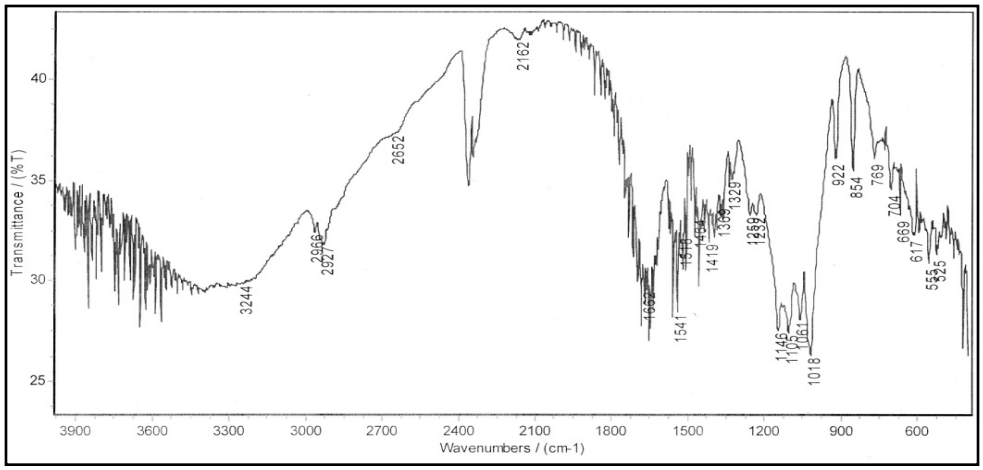

Fig. 6: FTIR spectra of the conjugated EPS from BSS medium

Recalling to the comparison between the primary media for production in correspondence to the amount of yield EPS, the bacterial isolate was able to produce valuable amount of free EPS in both CDM and NFM, although CDM showed higher efficacy to gain the larger amount of EPS than the free EPS of NFM and the conjugated EPS of BSS (Fig. 7), for this reason the next experiments of the current study will depend on free EPS produced in the CDM for further estimations and analysis. Fig.8 illustrates the evaluations of the total and crude EPS produced in CDM.

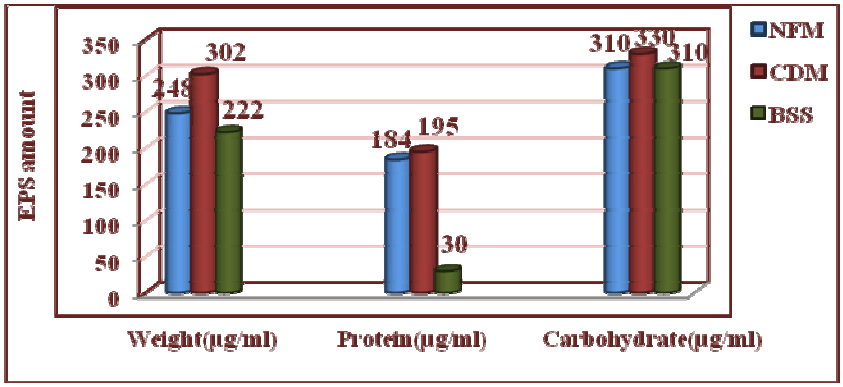

Fig. 7: Comparison between the amounts of EPS produced in CDM, NFM, and BSS

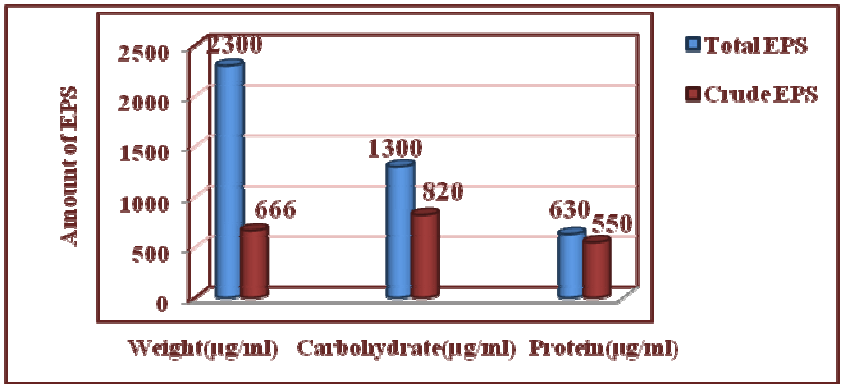

Fig. 8: Evaluation the amounts of EPS produced in CDM

EPSs are very hydrated polymers with more than 90% of their wet weight comprised of water. Most bacterial EPSs are synthesized intracellularly and exported to the extracellular surrounding as macromolecules, so the diffused EPS in the culture medium was obtained in the culture supernatant and collected by absolute alcohol which precipitates polysaccharide but not monosaccharide. Then it was dehydrated under 75ºC. This procedure is proficient for the extraction and isolation of bacterial EPS as the obtained EPS of high purity gave an orange- yellow color in the phenol sulfuric acid assay; it is the commonly used in the studies on EPS from bacterial isolates from clinical cases or in various environmental applications (Qiang *et al*., 2013; Jiang *et al*., 2011) and in the investigations on EPS in microbial industrial field (Abednego *et al*., 2007; Savadogo *et al*., 2004).

We choose the nutrient minimal media to enhance *Klebsiella pneumoniae* to make up the EPS as the production occur in response to poor nutrients under some selected different medium conditions such as carbon source, pH value, and some physiological bacterial properties such as inoculum size and growth phase. The yield and amount of microbial EPSs are greatly affected by the nutritional and environmental conditions and it is possible to increase the EPS production by manipulating the culture conditions. CDM was suitable medium for choice to build up EPS because it contains simple concentrations of salts, minerals, and nitrogen and carbon source. The suggested environmental factors that affect the synthesis of EPS include: increased oxygen, limitation of nitrogen and cations, desiccation, growth on minimal media, and growth phase. For instance, in such nutrients limitations when essential cations are required, anionic EPS would act as the driving force that concentrates the helpful metal ions to the microenvironment around the cell (Quin *et al*., 2007; Qiang *et al*., 2013).

Polymerization of EPS also increases the gradient across the cell membrane offering excess reducing power to drive high energy uptake system (Quin *et al*., 2007). CDM contains K_2HPO_4 and $MgSO_4$ salts higher than NFM and BSS; also contains the inorganic nitrogen source, NH_4SO_4 while NFM contains any nitrogen sources; these components seem to be of enhancing role for the production. Similar to the current outcome that was deduced for the activated sludge isolate *Klebsiella* sp. H-207 where these mineral salts were proved to improve EPS production with 5 g/ L of K_2HPO_4 and 0.2- 0.4 g/L of $MgSO_4$, but lower and higher concentration inhibited the production; it was decided that the deficiency of phosphate may inhibits the glycometabolism, while the scarcity of magnesium can influence the oxidation of the carbon source. For the nitrogen sources, it was seen that the higher yield was achieved by the addition of either organic and inorganic nitrogen source that promote the growth rate and EPS production (Qiang *et al*., 2013; Poli *et al*., 2010). Other study on *Lactobacillus delbrueckii* and *Streptococcus thermophilus* found that the low concentration of nitrogen was favorable in the buildup of EPS (Zehra and Belma, 2008). BSS medium contains higher percentages of chloride salts particularly NaCl (3.5 %) and KCl and NFM contains divalent salts which may affect the amount of the yield EPS by the current isolate as it was deduced by Abednego *et al*., (2007) for the polysaccharide produced by *K. oxytoca* that was also affected by such salts which may cause contraction of the polysaccharide chain as a result of electrolyte- induced charge shielding. On the other hand, they were favored by other species of the environmental isolates of Bacillus as claimed by Borgio *et al*., (2009) and Bragadeeswaran *et al*., (2011).

2. The factors that affect the amount of EPS:

(1) The effect of carbon source type and its different concentrations: In comparing the best carbon source that enhance the EPS production, we found that all of the tested carbon sources stimulated the production with the max yield with fructose and xylose, 2510 and 1990 µg / ml respectively, while lactose led to the lowest yield (Fig. 9). When we estimated the amount of EPS produced under different fructose concentrations, it was obvious that the production was raised along with the increased fructose concentrations up to 6% (Fig.10). Bacterial EPS consists mainly of a polysaccharide, so the increased carbohydrate precursor lead to increased EPS yield; also, the increasing sugar concentration per volume of the medium means the increasing of unfavorable

osmolality which can regulate gene expression up to build a protective barrier of extracellular matrix. Based on these issues, fructose with the best concentration was used in CDM instead of glucose in performing the following next experiments.

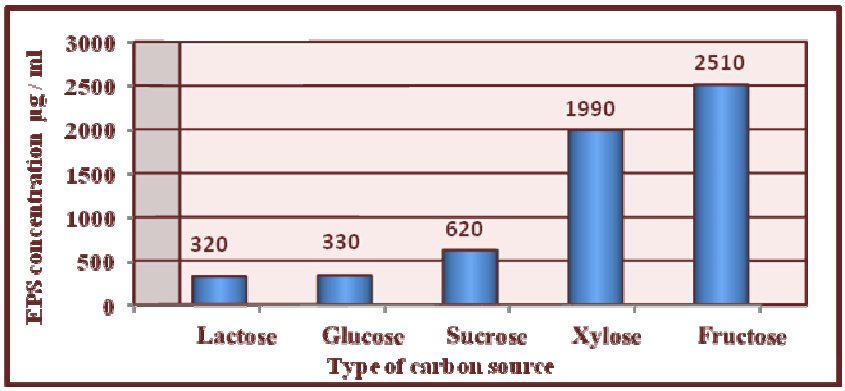

Fig.9: Effect of different carbon sources on the amount of EPS

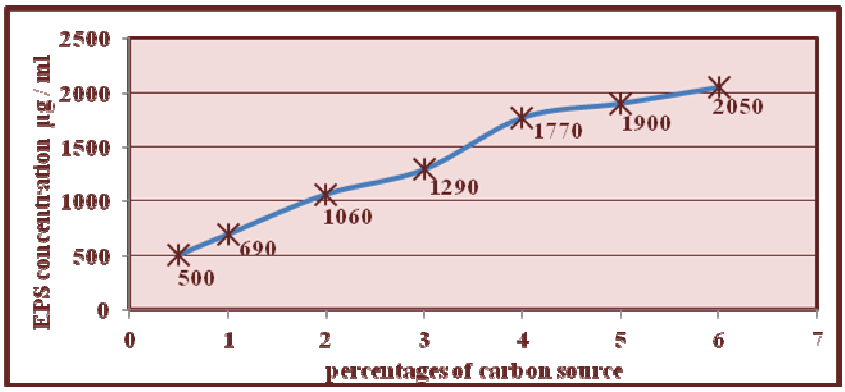

Fig.10: Effect of different fructose concentrations on the amount of EPS

The current finding that the amount of EPS production by *K. pneumoniae* is apparently affected by the type and concentration of the carbon source is in the same purport of that reported by Qiang and his colleagues (2013) which also emphasized the dependence of the EPS production by the activated sludge isolate *Klebsiella* sp. H207 on the carbon sources and their concentrations. According to that study, glucose, soluble starch, maltose, and lactose were also favorable carbon sources for the production and the highest yield was obtained when sucrose was used as the sole carbon source with the optimal concentration of 3%; whereas the lowest yield occurred when dextrin was used as the sole carbon source. Other findings that the biosynthesis of extracellular compounds in *Acetobacter xylinum* cells was determined by the availability of fructose, sucrose, and starch at level between 2.5 – 10%, while lactose and xylose induce the lowest production (Czaczyk and Myszka, 2007). Others reported that the synthesis of extracellular polymer by *Citrobacter* sp. was not dependant on limitation of nitrogen or phosphate sources, but with the type and concentration of carbon source, as the highest exopolymer level was obtained with lactose (contrary to the current results) while with glucose the amount was decreased (Allan *et al*., 2002).

(2) The effect of pH value of the medium: CDM with the 6% fructose was depended as a more favorable condition for production and tested with different pH values. This medium was suitable for inducing *K. pneumoniae* to produce the maximal yield with pH values set between 6– 8, above or less this range the production will decrease especially with the alkaline pH values (Fig.11)

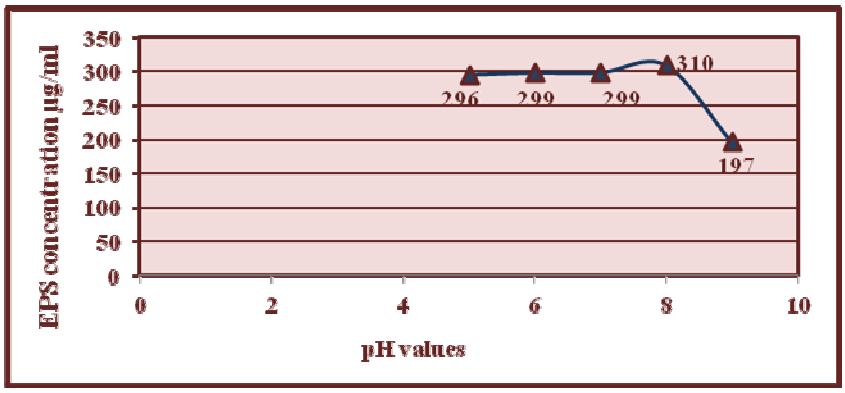

Fig. 11: Effect of different pH values on the amount of EPS

The extreme pH profile of the medium (2-3 or ≥ 10) was verified to inhibit microbial growth and extracellular polymer biosynthesis; this was true also for *Aureobasidium pulluans* where the polymer biosynthesis was decreased with the dropped pH value to 2; and for *Antrodia camphorate* which showed maximum production with pH value of 5, while higher and lower pH values significantly inhibited the biosynthesis (Czaczyk and Myszka, 2007). Similar to the currently pH range for *K. pneumoniae* that was deduced for *Klebsiella oxytoca* which persisted constructing the exopolymer under the pH range of 2-8, above that a drop in the production was observed (Abednego *et* al., 2007). Contrary to these settings that was published by Qiang and his colleagues (2013) who tested the effect of various initial pHs (3.0-12.0) and found that an initial alkaline pH range among 7.0- 12.0 was more favorable than acid pH values, and the maximum yield was obtained at the initial pH between 8-12.0.

(3) The effect of the bacterial inoculums size: the correlation between the resulted EPS with the bacterial cell concentration is planed in Fig.12. The higher yield was obtained with the increased bacterial inoculums which means the increased bacterial cell density that consume the available substances in the medium to make up the exopolymer, this was obvious with a sharp raise when the percentage of inoculum increased from 1 to 4 %; above that the raise was little and became nearly constant. The inoculated bacteria consume the medium and no further supplements are offered to the batch culture used in the recent experiment, so the increased bacterial cell will not produce additional amount in the same incubation period. The increased inoculum may be useful to minimize the period of incubation as a rapid production rate will be gained; it is also a benefit to gain a large amount of EPS when continuous cultures are used in which the product is drained out and a new medium is introduced continuously. Most other studies used an inoculum size of 2% (Bragadeeswaran *et al*., 2011) or 3% (Borgio *et al*., 2009), some preferred 8% bacterial inoculum (Wang *et al*., 2011), while few others preferred the use of 10-12% inoculum (Qiang *et al*., 2013).

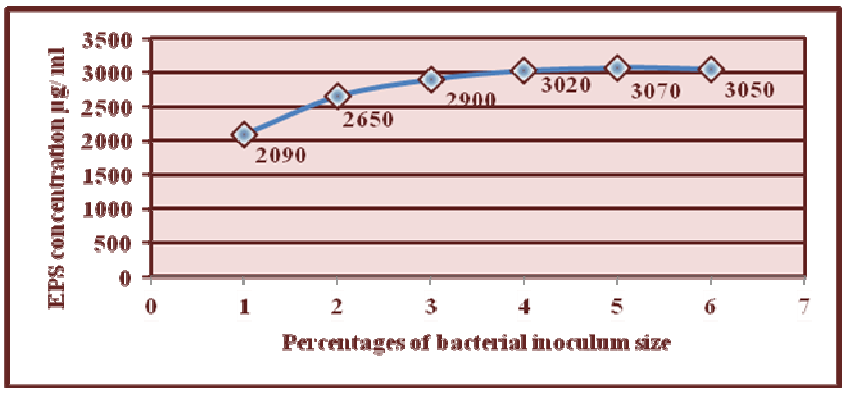

Fig. 12: the effect of bacterial inoculum size on the amount of EPS

(4) Evaluating the concentration of EPS in correlation with different incubation periods: the concentration of produced EPS over 7 days of incubation is pointed up in Fig.13.

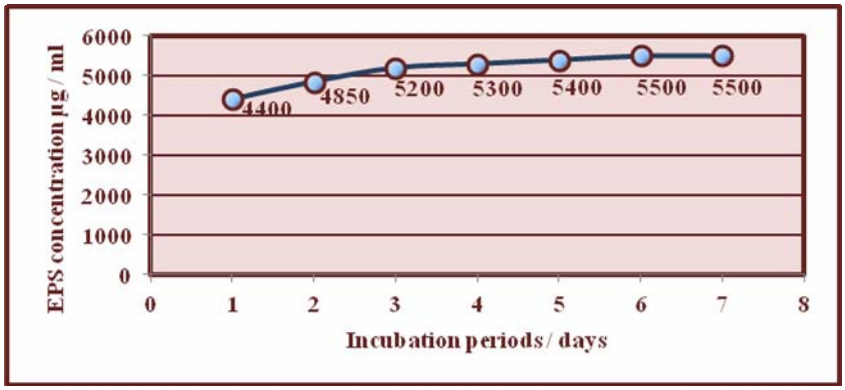

Fig. 13: the amount of EPS over different incubation periods

The constant medium component inoculated with constant bacterial inoculum was tested for the amount EPS concentration over different periods of incubation. It is possible to deduce that the EPS production was increased with the extent of incubation period, it increased sharply after 3 days of incubation as a response to the depletion of the available substances in the medium; beyond that the amount of EPS was increased in a constant rate after 4, 5, 6 days; finally reached a constant level that was not increased after 7 days; instead the produced EPS will be utilized in such starvation conditions where the available substances were depleted by the inoculated bacterial cells and no further EPS will produced as no additional supplements or bacterial cells were added to the incubation vessel. Previously, it was reported for several bacterial species that the exopolymer synthesis initiate during exponential growth phase (growth- associated synthesis), but for others the polymerization began only in the post- stationary growth phase (growth- dissociated synthesis) (Freitas *et al*., 2011). For *Klebsiella* sp. H207 production was gradually increased after 42 hr. and maintained at a constant level after 96 hr., which indicates that the synthesis of the EPS occurred only in the post-stationary growth phase (Qiang *et al*., 2013). For *Citrobacter* sp. the synthesis of EPS began after 96 h of incubation (Allan *et al*., 2002). In strains of *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*, the high production of EPS molecules was observed during late logarithmic and early stationary phase. It was also noted that the ceasing of exponential growth phase induce losing the integrity of the microbial cell surface which was caused by the reduction or even lack of extracellular molecules produced by *Pseudomonas* sp. and *Escherichia* sp. (Czaczyk and Myszka, 2007).

CONCLUSION

For the optimum isolation and investigation of an EPS produced by the pathogen *Klebsiella pneumonia* under study, CDM with 3-6% fructose or xylose as a sole carbon source and pH value set between 6-8 can be inoculated with 4-5% (v/v) of a freshly growing bacterial cells and incubated for 4-5 days, under these circumstances a valuable amount of EPS can be precipitated from the culture supernatant using absolute ethanol which can then be dehydrated as a crude EPS to perform number of promising investigations especially those with concerned screening for useful approaches to control the biofilm formation in human infections.

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