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Quantification of Exopolysaccharide Produced by *Bacillus subtilis* and the Effect of Different Factors on its Production

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ABSTRACT

The present study included the extraction and characterization of EPS production by a local soil isolate *Bacillus subtilis* and examine the effect of different factors pH, inoculums size, incubation periods, and carbon, nitrogen and phosphate sources on EPS production. The exopolysaccharide was recovered from the culture supernatant by using a cold ethanol precipitation. The total carbohydrate content was determined by phenol sulfuric acid method at 488 nm, and major structural groups were detected by Fourier transform infrared (FTIR) spectroscopy at frequency range of 400 to 4000 cm^{-1}. Basal salt solution (BSS) showed higher efficacy in supporting the bacteria to produce EPS (0.986 mg/ml) as dry weight. The study showed that glucose and fructose were gave maximum EPS production as a carbon source along with ammonium chloride as a nitrogen source. The optimal medium conditions were pH 7.0, inoculum size 5-6%, after four days of incubation to promote the maximum EPS production for the bacteria under study.

Keywords: *Bacillus subtilis*, Exopolysaccharide, FTIR spectroscopy.

التقدير الكمي لمتعدد السكريات الخارجي المنتج بواسطة *Bacillus subtilis* و تأثير عوامل مختلفة في إنتاجه

الملخص

تضمنت الدراسة استخلاص وتوصيف إنتاج متعدد السكريات الخارجي لبكتريا *Bacillus subtilis* المعزولة من التربة ودراسة تأثير عوامل مختلفة الاس الهيدروجيني وحجم اللقاح وفترة التحضين ومصادر مختلفة من الكربون والنتروجين والفسفور في إنتاجه. تم ترسيب متعدد السكريات الخارجي باستخدام الايثانول المبرد وقدر محتوى الكاربوهيدرات الكلي بطريقة الفينول-حامض الكبريتيك عند الطول الموجي488 نانوميتر، وتم تحديد المجاميع التركيبية باستخدام طيف الأشعة تحت الحمراء عند التردد 400-4000 سم$^{-1}$. اظهر وسط المحلول الملحي(BSS) كفاءة عالية لدعم البكتريا لإنتاج 0.986 ملغم/مل وزناً جافاً من متعدد السكريات الخارجي. لوحظ من الدراسة ان الكلوكوز والفركتوز اظهرا أعلى إنتاجية بوصفهما مصدراً للكربون مع كلوريد الامونيوم بوصفه مصدراً للنتروجين، وكانت ظروف الإنتاج المثلى عند الاس الهيدروجيني 7.0، حجم اللقاح 5- 6 % وبعد فترة تحضين أربعة أيام لدعم إنتاجية أعلى لمتعدد السكريات الخارجي للبكتريا قيد الدراسة.

INTRODUCTION

Microbial cells can persist in a free- living life style, while the majority of them establish a very complex and highly organized communities. For example, *Bacillus subtilis* forms high structured colonies on semi-solid surfaces and in non- agitated liquid culture usually forms a floating biofilm called a pellicle at the air-liquid interface as a consequence of extracellular matrix production. The main component of this matrix 90% or more is an exopolysaccharide (EPS) which aid binding the cells together in the biofilm (Nadell *et al.*, 2009; Lemon *et al.*, 2008; Türetgen *et al.*,

2012). EPS is a high molecular weight polymer consisting of monosaccharides and some non carbohydrate subunits such as protein; it may exists as tight capsules or loosely slimes or secreted to the surrounding environment (Rubinstein *et al*., 2012; Bragadeeswaran *et al*., 2011). The chemical composition of the EPS depends on the genetics of microbial cells and the physiochemical environment in which the biofilm matrix develops (Marvasi *et al*., 2010).

Substances within the EPS have multiple functions; some are energy and nutrient reservoir, play an important role in surface adhesion and interaction between bacteria and their environments, and may have many potential applications in a broad range of fields, for instance textiles, pharmaceuticals, oil recovery and metal removal in mining and industrial waste treatment (Marvasi *et al*., 2010; Orsod *et al*., 2012; Bragadeeswaran *et al*., 2011). They also have a vital role in biofilm formation, maintaining primary cellular functions and protection of bacterial cell from desiccation, predators and antibacterial elements (Penge *et al*., 2008).

In soil, the produced biofilm by *B. subtilis* on the plant root protects the plants from variety of pathogens (Kolodkin-Gal *et al*., 2012); members of the genus Bacillus are among the first successful biocontrol agents used against the insects and other pathogens as it can produce a variety of lipoproteins and other extracellular polymers which are potent biosurfuctant (Bais *et al*., 2004). They also play a role in degradation of organic polymers as they can form flocculation as a result of EPS synthesis which can be also a quick method for waste water treatment as their intermediates are harmless and biodegradable with no toxicity or secondary pollution (Bais *et al*., 2004; Wang *et al*., 2011). The current study focuses on EPS produced by *B. subtilis* because it is ubiquitous, present in almost all ecosystems and the EPS produced by it has significant ecological relevance (Earl *et al*., 2008). This study searches the production and extraction of the exopolysaccharide of the local soil isolate *B. subtilis* and determined the optimal culture condition that enhance maximum EPS production.

MATERIALS AND METHODS

Bacterial isolate and culture medium

The local soil isolate of *B. subtilis* was diagnosed in Bacterial Strains Bank Unit / Biology dept. / college of Science in Mosul University. Bacto® Nutrient broth was used for subculturing the bacterium at 37ºC.

Medium and conditions for primary EPS production

The basal salt solution (BSS) supplemented with 3% glucose was used as a basal medium for production with the initial pH value at 7.1±0.1. The medium was inoculated with 2ml of 18h culture of *B. subtilis* at 37ºC for 3 days as a primary condition for EPS production (Bragadeeswaran *et al*., 2011). Other basal medium was tested for the efficacy of EPS production, nitrogen- free medium (NFM) and chemically defined medium (CDM), (Borgio *et al*., 2009).

Isolation and extraction of EPS

Bacterial cells grown in the basal medium were precipitated by centrifugation (5000 rpm for 10min.); cooled ethanol alcohol was gently added to the supernatant in 1:2 (v/v) and incubated at 4ºC for 24 hr to precipitate the EPS from the supernatant. The sediment EPS was collected by centrifuge at 6000 rpm at room temperature for 20 min; then dried completely at 70ºC for 24- 48 hr and the powdered EPS was collected in an eppendroff tube and weighed (Ohno *et al.*, 2000).

Quantitative analysis of EPS

The quantificational estimates were done to assess the obtained EPS involved the weight of precipitated EPS in term of wet weight, and the weight of dried EPS as dry weight. The concentration of the carbohydrates by phenol-sulfuric acid method (Dubois *et al*.,1956) and proteins by Lowry's method (Lowry *et al.*,1951) was appointed by reading the optical density spectrophotometrically.

Qualitative analysis of EPS

The crude EPS was analyzed by the Fourier Transform Infrared (FTIR)-600 spectroscope (Biotech engineering management CO.LTD. (U.K.)) to screen for the presence of C-H, C=O and O-H groups in the sample by mixing one part of the crude EPS with nine parts of dried potassium bromide (KBr) and then compress them to prepare a thin salt disc. The discs were subjected to FTIR- spectra measurement in the frequency range of 400 to 4000 cm^{-1} (Vidhyalakshmi and Nachiyar, 2011).

Assessment of the effect of culture medium- associated factors on the yield EPS

Several selected factors were tested to study their effect on the amount of yield EPS using basal salt solution BSS as a basal medium. To find out the optimal carbon and nitrogen source, BSS medium supplemented with 3% glucose, 0.1%, 0.05% inorganic and organic nitrogen source respectively, was provided separately with different selected sources instead of the source employed in basal medium, as listed in Table (1). To find out the suitable concentration of Phosphate salts for EPS yield, different concentrations were separately provided instead of 0.07% K_2HPO_4 and 0.03% KH_2PO_4 employed in BSS medium. The amount of EPS by each factor was measured as carbohydrate concentration in µg/ml.

Table 1: Some Selected Culture- Associated Factors for their Effect on EPS Amount

Factors	Variables	Concentration (g) / 100ml
Carbon source	Glucose, xylose, fructose, sucrose, lactose	3
Inorganic nitrogen source	NH_4Cl, NH_4SO_4	0.1
Organic nitrogen source	Peptone, Beef extract, Yeast extract	0.05
Phosphate salts concentration	K_2HPO_4	0.07, 0.14, 0.28, 0.56, 1.12
	KH_2PO_4	0.03, 0.06, 0.12, 0.24, 0.48
pH value	5,6,7,8,9	

Assessment of the effect of bacteria- associated factors on the amount of yield EPS

1. **The inoculums size:** The basal medium was inoculated with graduated bacterial inocula (1 to 6% v/v) and incubated under standard conditions to estimate the concentration of EPS produced by each inoculums volume (Wang *et al.*, 2011).
2. **The bacterial growth phase:** The yield EPS in basal medium with maximal culture conditions was estimated over consequent incubation periods starting after 24 - 144h. (Czaczyk and Myszka, 2007).

RESULTS AND DISCUSSION

The amount of the yield EPS in basal medium

Based on the data listed in Table (2), the current study demonstrated that BSS was the most suitable medium that gave the larger amounts of EPS produced by the local isolate of *B. subtilis*. The weights of EPS, protein and carbohydrate concentrations obtained using BSS medium were greater than that of NFM and CDM. BSS was the excellent for production although the two other media were fitting to meet the high yield of EPS. BSS contains a nitrogen sources greater than CDM while NFM does not; also contains a greater concentration of carbon sources (3%) than the two other media. The percentages of carbon and nitrogen sources play the most important role in cell growth and production of exopolymer. Also, BSS contains 3.5% NaCl which enhanced larger amount of EPS up to 0.7% as it increases the osmotic pressure leading to detrimental effects on the cell (Abdul Razack *et al.*, 2013).

Table 2: The evaluation of EPS produced in basal medium

Basal medium	Estimations of EPS			
	Wet weight (mg/ml)	Dry weight (mg/ml)	Carbohydrate (µg/ml)	Protein (µg/ml)
Basal salt solution (BSS)	15.0	0.986	390	79
Nitrogen free medium (NFM)	12.8	0.958	320	84
Chemically defined medium (CDM)	13.3	0.630	320	87

Other comparison between the production media showed that *B. subtilis* MTCC121 isolate produced higher amount of EPS in basal medium than the other tested media (Vijayabaskar *et al.,* 2011), while the isolate *B. subtilis* NCIM 2063 of (Borgio *et al*., 2009) produce higher concentration of EPS in NFM rather than BSS, Minimum salts medium, Milk medium, CDM, sewage water sample. FTIR spectrophotometer of the EPS showed the presence of carbohydrates bands, sugar derivatives, amino groups in the structure of EPS. For the EPS extracted from BSS, the sharp peak of alcoholic O-H was at 3400 cm^{-1}, N-H or alcoholic O-H interfere with N-H (amines or amides) at 3327 cm^{-1}, the presence of aliphatic C-H was detected at the stretching bands 2935- 2960 cm^{-1}, of C=O at the peak 1655 cm^{-1} (Fig.1). In the EPS extracted from NFM, the carboxylic O-H peak was at 3408 cm^{-1}, N-H or alcoholic O-H interfere with N-H (amines or amides) at 3325 cm^{-1}, aliphatic C-H at 2927 cm^{-1}, the peak of 1653 cm^{-1} refers to C=O band and at 1739 may indicate the presence of ester bond (Fig. 2). To the EPS extracted from CDM, the peak at 3483 cm^{-1} indicates the carboxylic O-H and at 3290 cm^{-1}referrs to the N-H or (amines or amides), the presence of aliphatic C-H was detected at the stretching bands 2927-2980 cm^{-1}, the C=O band was at the peak 1647 cm^{-1}. The other peaks refer to the presence of sugar derivatives, alcohols, ethers, esters, and phenols groups. (Fig.3)

In general, bacterial EPS polymers are mainly composed of carbohydrates with glucose, galactose and mannose monomers. Neutral sugars, some uronic acids and aminosugars are also frequently present. They may contain several organic esters- linked substitutes and pyruvate ketals. The presence of some acyl groups confers the EPS an anionic character, increases its lipophilicity and affects its capacity to interact with other polysaccharides (Frietas *et al*., 2011).

The main EPS component of *B. subtilis* isolated from Asian Sea Bass by Orosd and other (2012) was 50% carbohydrate, 26% protein and 24% fatty acid; the functional groups presented were N-H, alkenes, ketons, in addition to peak bands of alcohols, ethers, esters carboxylic acids, phenols groups. The presence of peak bands of these functional groups revealed the presence of many protein- related amine and amide groups indicating the possible presence of bacterial toxin, which deduce that EPS may contribute in pathogenesis; also, the presence of some phenolic and carboxylic groups may account for antibacterial activity. The chemical EPS composition of the fouling marine *B. cereus* GU812900 strain was 540.124 µg/ml sugar and 18.521µg/ml protein (Bragadeeswaran *et al*., 2011).

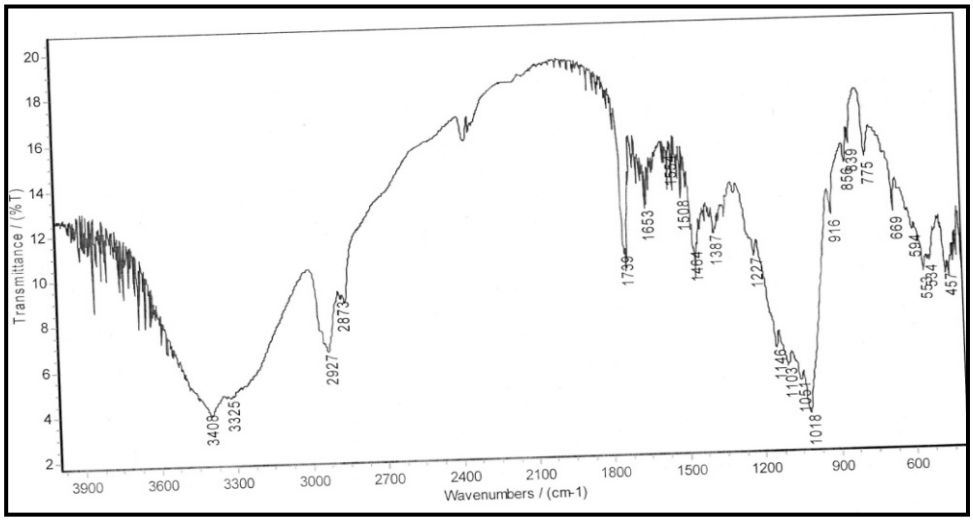

Fig. 1: FTIR spectra of the EPS by using BSS medium

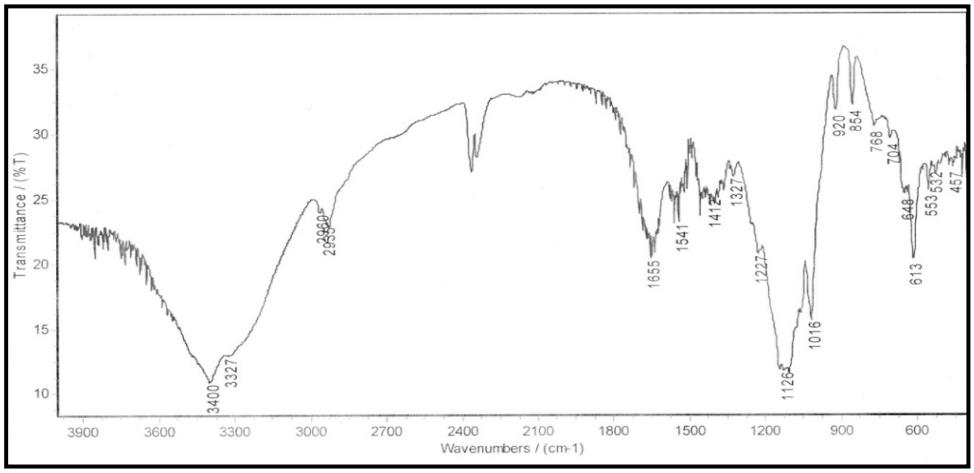

Fig. 2: FTIR spectra of the EPS by using NFM medium

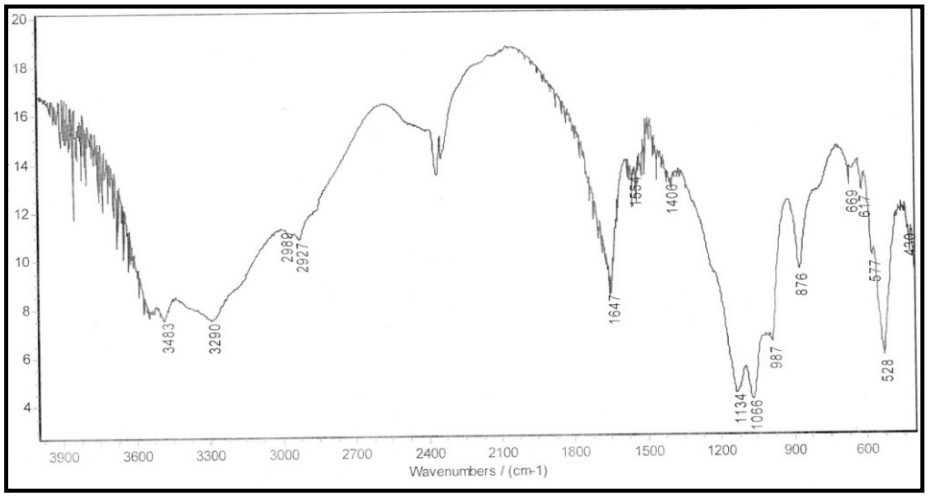

Fig. 3: FTIR spectra of the EPS by using CDM medium

The effect of culture medium- associated factors on the EPS yields

The extracellular biopolymer 's synthesis by microbial cells depended on the carbon and nitrogen availability in the culture medium, most exopolymer producing microorganisms utilize carbohydrates as their carbon and energy source, sugars are the most commonly used carbon source for the production of bacterial EPS, (Czaczyk and Myszka, 2007; Frietas *et al*., 2011). So, effects of various nutrient sources like carbon and nitrogen and phosphate were determined in this study to find out the optimal source for maximum EPS production by *B. subtilis*, the concentrations used as their concentrations in basal medium BSS.

According to the results in Fig. 4, all the tested carbon sources were suitable to produce the EPS by *B. subtilis* under study; the most encouraging one was glucose and fructose as they cause the higher concentration of the yield EPS (2950µg/ml) while lactose led to the lowest yield (2430µg/ml). The inorganic nitrogen form, NH_4Cl was the best in inducing the production (3750µg/ml) than NH_4SO_4. The organic form of nitrogen, peptone was the best (3690µg/ml) among the other tested forms, beef extract and yeast extract. The most favorable pH values of the medium that promote the greatest production were between 7.0- 8.0. (Fig. 4) also shows clearly that with the increasing phosphate salt concentrations the yield EPS will decrease obviously from 3690 to 1350µg/ml with the increasing K_2HPO_4 concentration and from 3690 to 1340 µg/ ml with increasing KH_2PO_4 concentrations. Medium and growth conditions are important factors for EPS production. The production and chemical characteristics of EPS are controlled by nutrient dynamics, microbial physiology, phytoplankton species, age of phytoplankton bloom, etc. It was cited that marine microbes grown in laboratory cultures will produce EPS when nutrients such as nitrogen, phosphorous, sulfur, and potassium are limited in the medium (Bragadeeswaran *et al*., 2011). Others cited that the presence of organic nitrogen is preferred if the bacterium is unable to build up particular essential amino acids and the organic nitrogen sources gave the higher amount of EPS than inorganic ones as the role of the heterotrophic *B. subtilis* in soil is decomposing and mineralizing the organic nitrogen present as a dissolved particle, this is one of the possible causes for EPS production by this bacterium; hence; when nitrogen source decreases, growth rate will ascend but EPS alleviation will occur (Abdul Razack *et al*., 2013).

Wang and his colleagues (2011) analyzed culture conditions for EPS production from *B. thuringiensis* 27 isolated from sand biological soil crusts; a sizeable change in EPS production was observed with different carbon sources; a high level of EPS obtained with maltose and glycerol as the carbon source with the maximum yield with 3% maltose, the organic nitrogen was preferred than inorganic and peptone caused maximum production. Organic nitrogen sources are absorbed easier by the cell than the inorganic ones. The inorganic ions affect the EPS production via combining with enzymes. Also, NaCl was the most preferred among the tested mineral elements.

Other studies have shown that the EPS produced by GU812900 strain of the fouling marine *B. cereus* was affected by environmental conditions, especially availability of nitrogen which affected the carbohydrate and protein content of bacteria; in that nitrogen starvation may enhance carbohydrate production (Al-Nahas *et al*., 2011; Bragadeeswaran *et al*., 2011). Carbon sources are also of particular important, in that sucrose enhanced the elevated EPS concentration along with increasing sucrose elevation; among the tested nitrogen sources, ammonium sulfate produced the highest quantities of EPS; production was also continuously increased from 227.08 to 498.04 mg/ L with gradual elevated phosphate concentration from 0.25 to 2 µg/ml. (Bragadeeswaran *et al*., 2011).

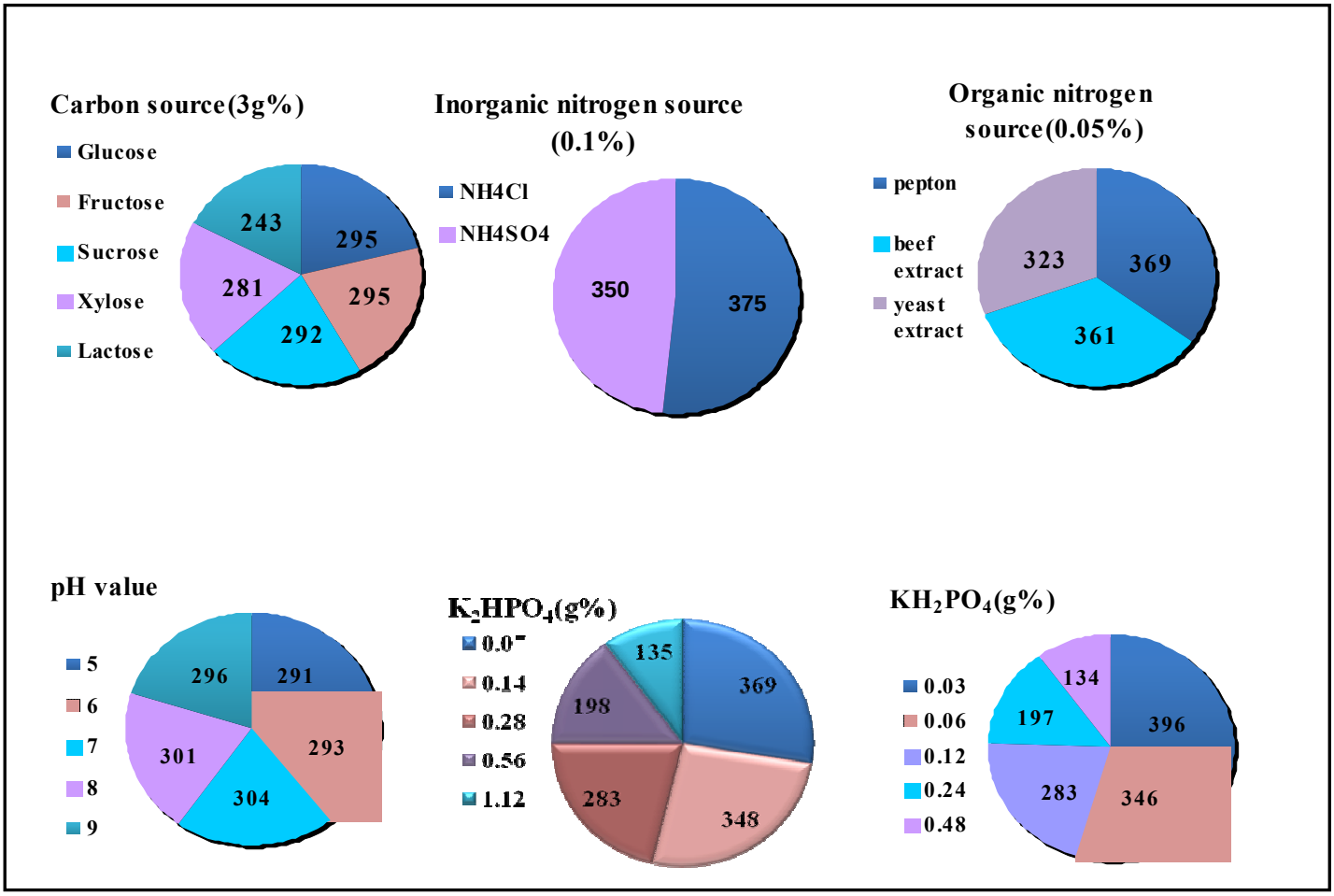

Fig. 4: Pie chart illustrates the effect of culture- associated factors that affect the amount of EPS pointed in concentration ×10 µg/ ml.

Other researchers showed that the EPS production by *Bacillus subtilis* was generally favored by high carbon and low nitrogen ratio in the medium (Borgio *et al*., 2009). The pH value of the medium is another critical factor that affects the production of EPS; the preferred pH values of the Bacillus isolate under study was between 7.0-8.0; dissident this range causes lowered amounts of produced EPS. This was fitted to pH values recorded to other Bacillus isolates as the production occurred at 5.0-9.0 pH range with the maximum at 7.0 (Wang *et al*., 2011).

Bacteria- associated factors that affect the amount of EPS

The bacterial inoculum volume:

The correlation between the resulted EPS with the bacterial cell concentration is plained in (Fig. 5). The results indicated that 5% and 6% inoculums size gave the maximum EPS yield 2950 µg/ml and 3010 µg/ml respectively. With the increased bacterial lawn, the amount of EPS will increase, that means the increase of bacterial cell that construct the EPS substance in a given period.

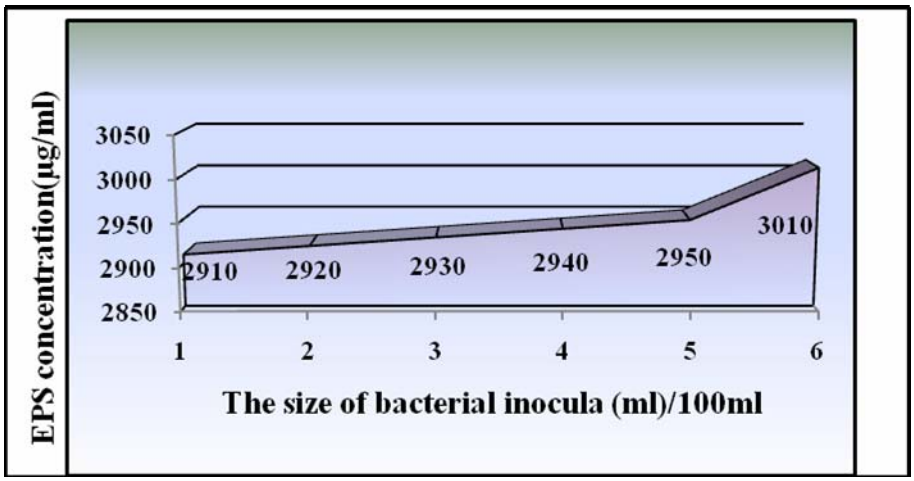

Fig. 5: The effect of different bacterial inoculums size on the amount of the yield EPS

Among several bacteria physiological properties, inoculum size may play an important role in cell reproduction and EPS production; it was recorded that 8% inoculum size in 25 ml of medium was best for EPS production (Chen *et al*., 2008). The same conclusion was observed with *B. thuringiensis* 27 where Wang and his colleagues (2011) recorded that inoculum size of 8% was fit for maximal EPS production.

Relationship of the amount of produced EPS with the incubation periods:

The concentration of produced EPS over 7 days of incubation is pointed up in (Fig. 6). The concentration of EPS increased along with extension of incubation period to reach its max level after 4 days of incubation 3230 µg/ml, beyond that the yield gradually decreased to reached 2190 µg/ml after 7 days. Along with incubation period, the bacteria will utilize carbon and nitrogen sources leading to increasing cell number; after deletion the existing sources, it will be subjected to scarcity of nutrients which stimulate the production of EPS as a response to starvation conditions; this was noted after 4 days of incubation. After that the amount of EPS was observed to lower as the bacteria began to consume the EPS component during starvation conditions.

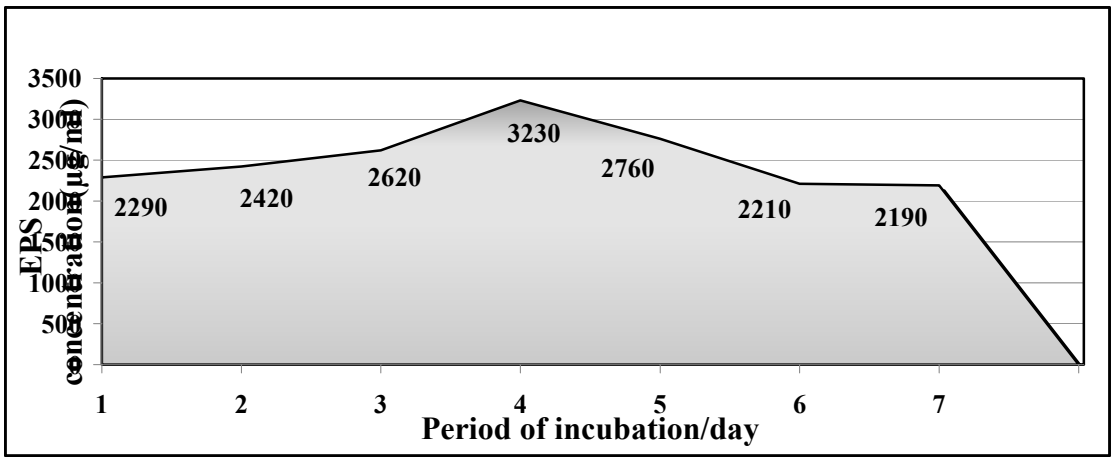

Fig. 6: The amount of EPS produced at different incubation periods

Maturation of the pellicle formed by *B. subtilis* occur after three days of incubation, after that disassembling and releasing individual planktonic cells will occur after 8 days of incubation (Kolodkin-Gal *et al*., 2012). It was reported that after few days the onset of EPS production from bacteria, its level in the culture medium declined which might be due to the secretion of β1, 3-glucanases (Vijayabaskar *et al*., 2011). Other observation on EPS in aqueous phase from *B. subtilis* showed that the composition of the functional groups of the matrix depends on the cell growth phase (e.g. exponential vs. stationary) (Omoike and Chorover, 2004). The EPS produced by GU812900 strain of the fouling marine *B. cereus* was observed at all stages of culture growth and was higher during the stationary phase. The release of EPS by bacteria is generally low during exponential growth and it accumulate during the stationary phase (Bragadeeswaran *et al*., 2011).

CONCLUSION

Medium conditions are important factors for EPS production and the maximum yield of EPS produced is known to be influenced by the composition of the nutrients in the media in which the bacterium is grown. When compared the three culture media used in the study, basal salt solution (BSS), nitrogen- free medium (NFM) and chemically defined medium (CDM) for their ability to induce the primary EPS production, it was concluded that EPS extract was higher in BSS basal medium than the other in which the dry weight was 0.986 mg/ml. From a FTIR analysis to EPS produced display presence of different functional groups such OH, C=O, COOH. Various factors influencing EPS production such as pH, inoculums size, incubation periods, carbon, nitrogen and phosphate source.

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