

Production Conditions of Exopolysaccharide from *Bacillus megaterium* Identified by 16S rRNA Gene Sequencing

H. M. Abu Shady, M. M. S. Asker^{*1}, M. A. Gadalla^{*} and S. M. Abd Elnasser^{*}

Microbiology Department Faculty of Science, Ain Shams University and ^{*}Microbial Biotechnology Department, National Research Center, Cairo, Egypt.

THE *BACILLUS megaterium* exopolysaccharide (EPS) producer was isolated from the Egyptian soil and identified by molecular biology technique (16S rRNA). It was found that the exopolysaccharide showed a high yield after 72 hr, with an initial pH of 5.0, in 250 ml Erlenmeyer flasks containing 50 ml of culture, medium having 2% sucrose as a sole carbon source. The yeast extract (0.1%) and ammonium chloride (0.1%) were the best organic and inorganic nitrogen source, respectively, as they gave the maximum EPS production. Optimum incubation temperature was found to be 35° C. Pretreated molasses from sugarcane and sugarbeet were used as carbon source and they gave a considerable yield (~ tow fold) in relation to sucrose. The pretreatment of molasses by sulfuric acid showed high yield of polysaccharide and maximum yield coefficient.

Keywords : *Bacillus megaterium*, Exopolysaccharide, 16S rRNA, Molasses.

Microbial exopolysaccharides (EPS) are biosynthetic polymer mainly consisting of carbohydrates secreted by bacteria (Freitas *et al.*, 2009) and cyanobacteria (Chi *et al.*, 2007 and Parikh & Madamwar, 2006). However, it has also been reported to be produced by Cryptophyta (Bermudez *et al.*, 2004), mushroom (Zou *et al.*, 2006) yeast (Duan *et al.*, 2008) and basidiomycetes (Chi & Zhao, 2003 and Manzoni & Rollini, 2001). EPS constitute different classes of organic macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids and other polymeric compounds, thereby carrying different organic functional groups such as acetyl, succinyl or pyruvyl and some inorganic constituent like sulfate (Nielsen & Jahn, 1999). Depending on their location, EPS occur in two forms, either in capsular or slimy polysaccharides (Costerton, 1999). In recent years, interest in the exploitation of valuable EPS has been considerably increased towards polysaccharide producing bacteria and cyanobacteria, for various industrial applications (Mishra & Jha, 2009). Fermentation medium can represent almost 50% of the cost for a microbial fermentation (Van Hoek *et al.*, 2003). Employing complex media for growth is not economically attractive, because of the high amount of expensive nutrients such as yeast extract, peptone

¹Corresponding author, M.M.S. Asker
E-mail: mohsenmsa@yahoo.com

and salts. Hence, to achieve high production yields as well as to compete with synthetic petrochemical products in performance and cost, it is a prerequisite to design an optimal cost-effective production medium. Much effort in fermentation process optimization has been made to produce the biopolymers economically from several inexpensive waste substrates, thereby decreasing their production costs (Nicolaus *et al.*, 2010).

The genus *Bacillus* consists of more than 222 recognized species (<http://www.bacterio.cict.fr>) distributed widely across many terrestrial and aquatic habitats (Ivanova *et al.*, 1999 and Siefert *et al.*, 2000), including marine sediments (Miranda *et al.*, 2008). Conventionally, bacilli have been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (Bobbie & White, 1980 and Vaerewijck *et al.*, 2001). These are technically complex and labor intensive procedures, however, and the scarcity of reproducible and distinguishable phenotypic characteristics for several bacterial species often makes precise identifications difficult (Khamis *et al.*, 2003). To date, the development of gene amplification and sequencing, especially that of the 16S rRNA gene sequences, has simplified the identification and the detection of specific bacteria (Wang *et al.*, 2003 and Wu *et al.*, 2006), particularly those lacking distinguishable phenotypic characteristics. The gene commonly amplified in such cases codes for 16S rRNA, which is a highly conserved region both within a species and among species of the same genus, and is essential for the survival of living organisms (Hirano & Upper, 1983). The 16S rRNA gene is now used as a framework for the modern classification of bacteria, including those in the genus *Bacillus*.

Though exopolysaccharides are produced by large number of microorganisms, reports from *Bacillus megaterium* are few (Yuan *et al.*, 2011 and Chowdhury *et al.*, 2011). Therefore in the current investigation the bacterial strain was first identified at the species level by sequencing the 16S rRNA gene. Optimum culture conditions for exopolysaccharide production by *Bacillus megaterium*, as well as some of its chemical properties and antioxidant activity *in vitro* including scavenging activity on hydroxyl radical are discussed.

Materials and Methods

Isolation of bacteria

Soil samples taken from Al-Fayoum Governorate in Egypt were serially diluted by repeated serial dilution technique (Nicolaus *et al.*, 2000) and plated on agar medium containing (g/L), NH₄SO₄,2; sucrose,10; KH₂PO₄, 2.5; Na₂HPO₄, 0.9; MgSO₄.7H₂O, 0.05; yeast extract, 1.0; and agar 20.0, pH of the medium was adjusted to 7.0 with 1N NaOH. The medium was sterilized by autoclaving for 20min at 121°C. After 24 hr of incubation at 37°C, mucoied bacterial colonies were selected, purified and stored on agar medium.

Assessment of EPS production

Bacterial cultures were grown in the same medium wherein sucrose was replaced by trisodium citrate (10.0 g/L) for 72 hr. Cells were removed by *Egypt. J. Microbiol.* **46** (2011)

centrifugation at 5,000 rpm at 4°C for 15 min and discarded. One milliliter of the supernatant in replicate was used to assess the production of EPS. The EPS was estimated by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Of the ten isolates examined, isolate SS-1 produced the highest amount of EPS. This isolate was used for further work as described below.

Identification

The isolate SS-1 had been identified as *Bacillus megaterium* on the basis of its morphological characteristics (Bergey *et al.*, 1994) and partial 16S rRNA gene sequencing. The genomic DNA of the pure *Bacillus* strain was extracted from the bacterial cells using the GeneJet™ Genomic DNA purification Kit (Fermentas), according to the manufacturer's instructions. Amplification of 16S rRNA gene was performed with a thermocycler using 35 cycles of 94°C for 1min, 48°C for 1min and 72°C for 2 min. The PCR reaction (50 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 150 µM each of dATP, dCTP, dGTP and dTTP, 25 pmol each of universal bacterial primers DG74 and RW01 corresponding to positions 1522-1540 and 1-15170-1189 of the *Escherichia coli* small subunit rRNA sequence (Greisen *et al.*, 1994), 2.5 units of Taq DNA polymerase (Fermentas) and 15 µl template DNA of the colony SS-1. Sequencing was performed in Macrogen Service Center (Korea).

Culture condition for EPS production

Flask cultures were performed in 250 mL flasks containing 50 mL of the seed culture (PNR) (g/L): sucrose, 20; K₂HPO₄, 7.5; MgSO₄, 2.0; NH₄NO₃, 1.5; CaCl₂, 0.33; peptone, 0.5-2.0. After inoculating with 6% (v/v) (OD₆₀₀=1.58, 24hr old) of the seed culture, and cultivated at 150 rpm for 72 hr. Factors affecting cell growth and EPS production were investigated using one factor at a time method. Different carbon and nitrogen (organic and inorganic) sources were chosen to substitute the corresponding components in the PNR medium also sucrose, yeast extract and ammonium chloride (NH₄Cl) at different concentrations were studied. The effect of pH and temperature were studied from (4, 5, 6 and 7) and (30, 35 and 40°C), respectively on the production of EPS were studied.

Determination of cell growth and EPS production

Samples collected at various intervals from shake flasks were centrifuged at 5,000 rpm for 15 min. The biomass was obtained by washing, the resulting precipitated cells with distilled water repeatedly and drying at 80°C to a constant weight. Deproteinization takes place with 5% (w/v) trichloroacetic acid for overnight at 4°C. After centrifugation at 5,000 rpm for 15 min the clear solution was adjusted to pH 7.0 with NaOH solution, then was dialyzed against distilled water for 48 hr, the supernatant was then mixed with four volumes of absolute ethanol, stirred vigorously and kept overnight at 4°C. The precipitate was collected by centrifugation at 5,000 rpm for 15 min, resuspended in distilled water and lyophilized to afford the crude EPS.

Molasses

The sugar beet molasses (BM) and sugar cane molasses (CM) was supplied from Delta Company for Sugar and Refinery (Kafrelshiekh) and Sugar and Integrated Industries Company, El-Hawamdia, respectively. Sugar cane and sugar beet molasses were subjected to different pretreatment methods including, removal of suspended matter by centrifuging, Potassium ferrocyanide 0.07% (Hamissa, 1970), sulfuric acid 1N (Forage & Righelato, 1978) and Calcium phosphate 1% (Reda, 1990). The total sugars concentration following every pretreatment step was determined according to Dubois *et al.* (1956) and then diluted with distilled water to an appropriate final carbohydrate concentration.

Results and Discussion*Identification of the EPS highly-producing strain*

The identification of strain SS-1 was based on the morphological and physiological characteristics and the 16S rDNA sequence. The colonial appearance of strain SS-1 on the agar plate was a circular, smooth, mucoied, yellow-white. Gram stains showed a gram-positive rod and spore forming. This strain was non-motile, aerobic, and catalase negative (Table 1). On the other hand, the strain SS-1 identified by amplification and sequencing of its 16SrRNA gene. The amplified DNA molecule was 350bp long. Phylogenetic analysis of the nearly complete sequence data was done by BLAST search. Alignment revealed that the bacterium has a similarity of 99% to *Bacillus megaterium* the sequence of the matching strain can be found in the Gen Bank database by its accession number HQ124332.1 (Fig. 1–4).

TABLE 1. Morphological, cultural and physiological characteristics of the bacterial isolate.

Characteristics	Bacteria colony
Morphology	Gram-positive, rods, spore forming
Motile	Non-motile
Cultural	Circular, smooth, mucoied, yellow-white
Physiological	Aerobic, catalase negative

Effect of carbon and nitrogen sources on cell growth and EPS production

In general, carbon source is the most critical nutrient and energy source for cell growth and different carbon sources may have different effects of catabolic repression on cellular secondary metabolism (Kim *et al.*, 2005). A substantial change in the polysaccharide production was observed with different carbon sources. To select the suitable carbon source for cell growth and EPS production, various carbon sources were separately added at 20 g/L instead of sucrose in the PNR medium. As shown in Table 2, sucrose supported maximum EPS production. This is consistent with the finding of



File: 1-primer_2.ab1 Run Ended: 2010/10/17 0:15:36 Signal C:2963 A:2365 C:3010 T:2064
 Sample: 1-primer_2 Lane: 66 Base spacing: -16,163063 769 bases in 9279 scans

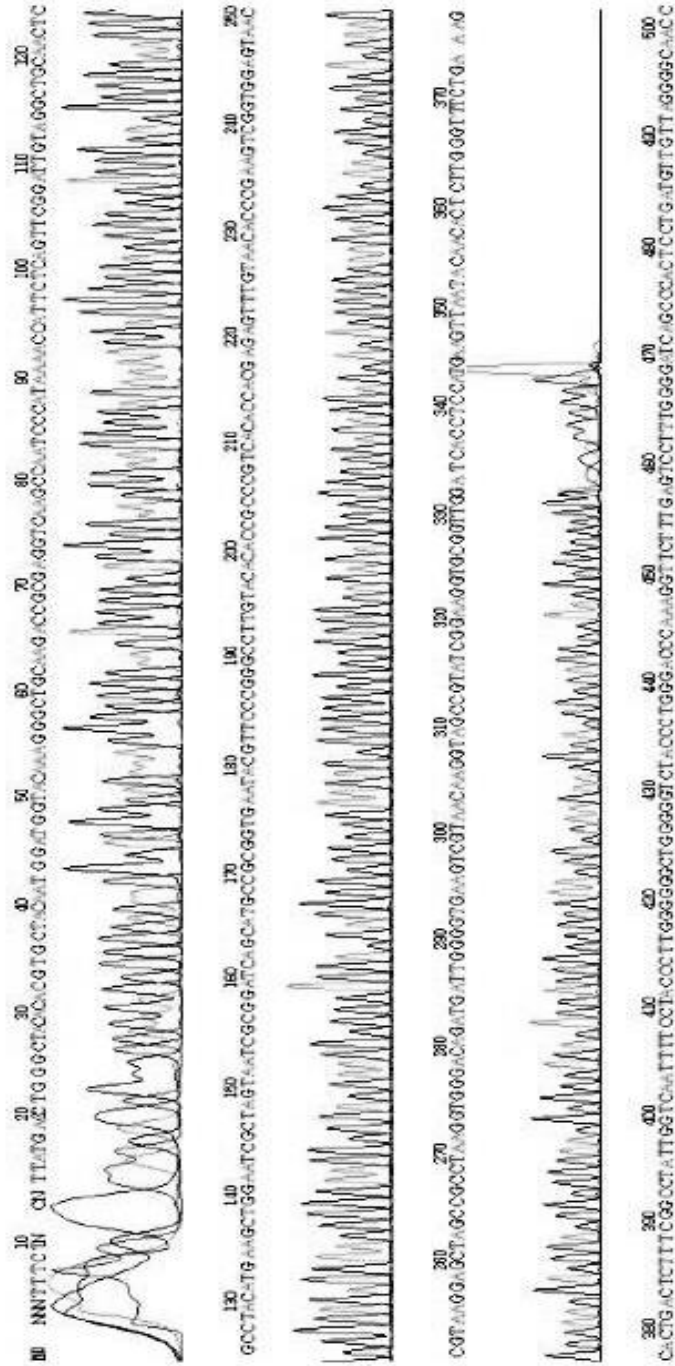


Fig.1. Sequencing chromatogram of forward sequence of 16s rRNA gene of *B. megaterium*.

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>|_qb|HQ124332.1| Bacillus megaterium strain SW1:2 16S ribosomal RNA gene, par
sequence
Length=684

Score = 603 bits (326), Expect = 7e-169
Identities = 328/329 (99%), Gaps = 0/329 (0%)
Strand=Plus/Plus

Query 9 TTATGACCTGGGCTACACACGTGCTACAAATGGATGGTACAAAGGGCTGCAAGACCGGAG 68
      |||
Sbjct 350 TTATGACCTGGGCTACACACGTGCTACAAATGGATGGTACAAAGGGCTGCAAGACCGGAG 409

Query 69 GTCAAGCCAATCCCATAAAACCAATTCAGTTCGGATTGTAGGCTGCAACTCGCCTACAT 128
      |||
Sbjct 410 GTCAAGCCAATCCCATAAAACCAATTCAGTTCGGATTGTAGGCTGCAACTCGCCTACAT 469

Query 129 GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGGTGAATACGTTCCCGGGCCT 188
      |||
Sbjct 470 GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGGTGAATACGTTCCCGGGCCT 529

Query 189 TGTACACACCGCCGTCACACCACGAGAGTTTGTAAACACCCGGAAGTCGGTGGAGTAACCG 248
      |||
Sbjct 530 TGTACACACCGCCGTCACACCACGAGAGTTTGTAAACACCCGGAAGTCGGTGGAGTAACCG 589

Query 249 TAAGGAGCTAGCCGCTAAGGTGGACAGATGATGGGGTGAAGTCGTAACAAGGTAGCC 308
      |||
Sbjct 590 TAAGGAGCTAGCCGCTAAGGTGGACAGATGATGGGGTGAAGTCGTAACAAGGTAGCC 649

Query 309 GATCGGAAGGTGGGTGGATCACCTCC 337
      |||
Sbjct 650 GATCGGAAGGTGGGTGGATCACCTCC 678

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Fig.2. Identities percentage of 99%.

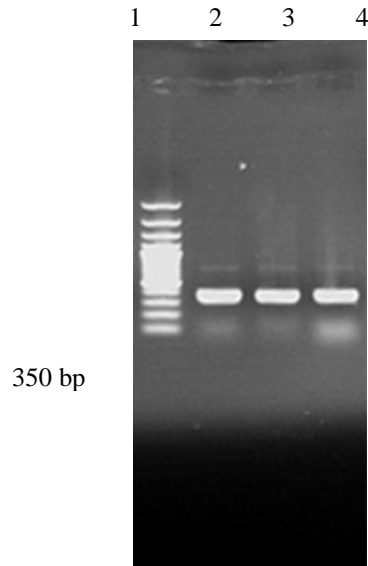


Fig. 3. 0.8% Agarose gel electrophoresis of Polymerase Chain Reaction of strain *Bacillus megaterium* genomic DNA using *Bacillus* specific primers of 16S rRNA gene. Lane 1: Molecular weight marker ladder (1kb); Lane 2 and 4: PCR amplified product of stander control (*Bacillus sp.*), 350 bp; Lane 3: PCR amplified product of 16S rRNA gene of strain *Bacillus megaterium* (Size of band approximately 350 bp) with DG74 and RW01 primers.

many other investigators (Han & Clarke, 1990 and Lee *et al.*, 1997). Sucrose supported maximum EPS production production of 7.5 g/L, cell dry weight of 1.75 g/L, whereas, the yield coefficient ($Y_{p/x}$) of 4.2, while galactose gave maximum yield coefficient ($Y_{p/x}$) of 6.8 and lower cell dry weight of (0.7 g/L). Mannose gave maximum cell growth of 1.85 g/L but EPS of 7.0 g/L. To select the optimal sucrose concentration for cell growth and EPS production, 15–30g/L sucrose was added into the PNR medium. As shown in Fig. 5, the increase of the initial sucrose concentration in the medium led to the increases of both cell growth and EPS production. However, only small amounts of sucrose residues (less than 10% of initial sucrose) could be detected in the final culture broth, indicating that most of the sucrose in the medium had been utilized for cell growth and EPS production. A similar conclusion has been drawn from culture studies of various *Paenibacillus polymyxa* strains (Han & Clarke, 1990 and Lee *et al.*, 1997). The highest EPS production (19.38 g/L) was obtained at the sucrose concentration of 160 g/L. and it should be noted that the cell growth increased continuously even when sucrose concentration reached 200 g/L (Han & Clarke, 1990 and Lee *et al.*, 1997). This was also probably due to the specific growth condition within the living tissues of plant that *Paenibacillus polymyxa* EJS-3 could survive in such high sucrose concentration as mentioned above. To investigate the suitable nitrogen source for cell growth and EPS production, various organic nitrogen sources replace peptone at 2.0 g/L in the PNR medium also different inorganic nitrogen sources were separately provided at

1.5 g/l in place of ammonium nitrate in the same medium. As shown in Table 3, yeast extract and ammonium chloride maximum yield coefficient ($Y_{p/x}$) were 3.9 and 1.3, respectively. The stimulatory effect of yeast extract may be due to its rich contents of protein, amino acid and vitamin (Pokhrel & Ohga, 2007). The EPS yield from *B. megaterium* decreased as yeast extract or ammonium chloride concentrations increased in the range of 1-2.5g/L. Cell dry weight, however, increased as the concentrations of yeast extract increased. The highest EPS yield (7.3 g/L) was achieved at 1.5 g/L yeast extract compared to 1g/L at 2.5 g/L yeast extract. On the other hand, the EPS yield (4.2g/L) was achieved at 1g/L ammonium chloride compared to 3g/L at 2.5g/L ammonium chloride and this is clear in Fig. 6 and 7, respectively.

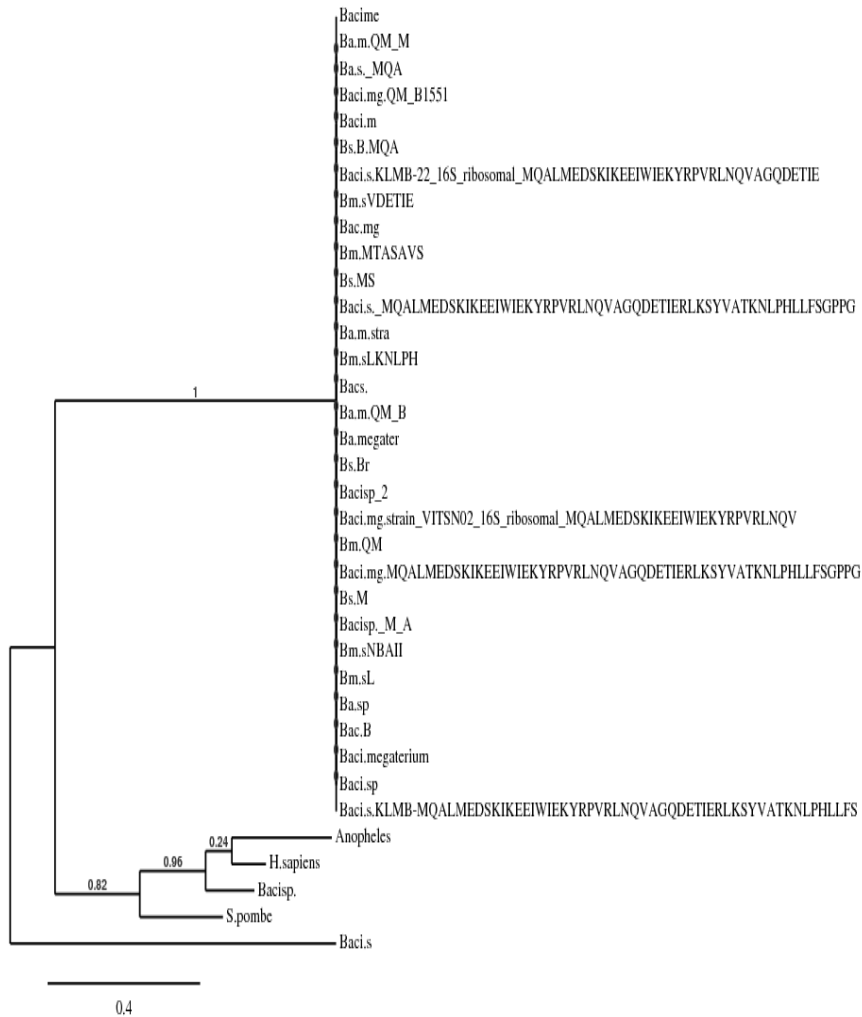


Fig.4. Phylogenetic tree, Genetic relationships in the 16S rRNA sequence.

TABLE 2. Effects of carbon sources on biomass and EPS production by *B. megaterium*.

Carbon sources	CDW(g/L)	EPS (g/L)	EPS/Biomass ($Y_{p/x}$)	Final pH
Sucrose	1.75	7.5	4.2	5.5
Xylose	2.60	7.1	2.7	5.5
Mannose	1.85	7.0	3.7	6.0
Galactose	0.70	4.8	6.8	5.5
Glucose	0.95	3.8	4.0	5.5
Lactose	1.84	5.9	3.2	5.5

• Initial pH: 7 • $Y_{p/x}$ = g EPS/g dry biomass
 CDW, Cell dry weight. • EPS, Exopolysaccharide

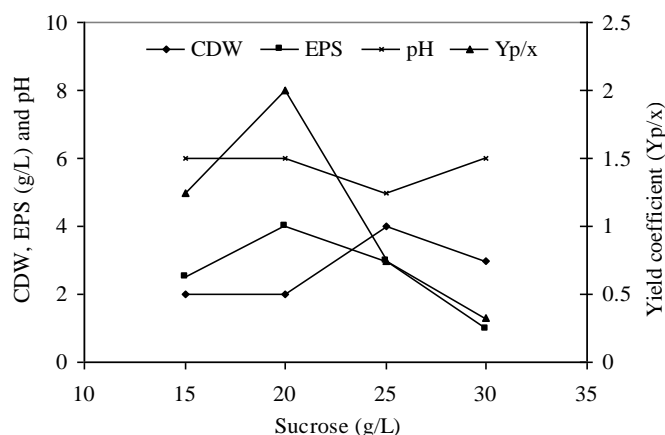


Fig.5. Effect of sucrose concentration on cell growth and EPS production from *B. megaterium* ($Y_{p/x}$ = g EPS/g dry biomass, CDW, Cell dry weight and EPS, Exopolysaccharide).

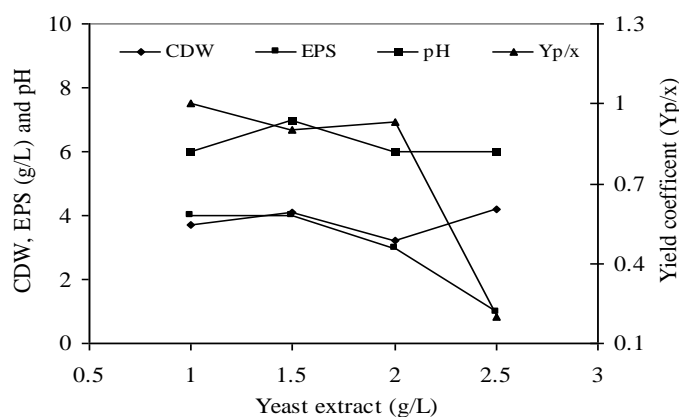


Fig. 6. Effect of yeast extract concentration on cell growth and EPS production from *B. megaterium* ($Y_{p/x}$ = g EPS/g dry biomass, CDW, Cell dry weight and EPS, Exopolysaccharide).

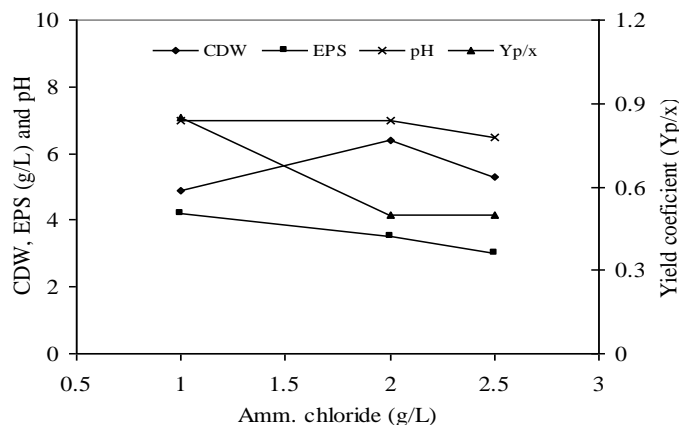


Fig.7. Effect of ammonium chloride concentration on cell growth and EPS production from *B. megaterium* ($Y_{p/x}$ = g EPS/g dry biomass, CDW, Cell dry weight and EPS, Exopolysaccharide).

TABLE 3. Effects of nitrogen sources on biomass and EPS production by *B. megaterium*.

Nitrogen sources	CDW (g/L)	EPS (g/L)	EPS/Biomass ($Y_{p/x}$)	Final pH
(NH ₄) ₂ SO ₄	3.6	2.7	0.7	5
NH ₄ Cl	2.7	3.5	1.3	7
NH ₄ NO ₃	3.9	3.5	0.9	7
Peptone	3.0	2.5	0.8	5
Yeast extract	2.9	3.5	1.2	6
Meat extract	4.1	2.0	0.4	6

• Initial pH: 7 • $Y_{p/x}$ = g EPS/g dry biomass.
CDW, Cell dry weight. • EPS, Exopolysaccharide.

Effect of pH and temperature on cell growth and EPS production

The initial culture pH is an important factor that may affect cell membrane, cell morphology and structure, the uptake of various nutrients and EPS biosynthesis (Kim *et al.*, 2005). To find out the optimal pH for *B. megaterium*, various pH values ranging from 5 - 9 were applied. As shown in Fig. 8, the optimal pH value for cell growth and EPS production was 5, with the corresponding cell biomass and EPS production of 3.2g/L and 7.00 g/ L, respectively. Unlike *Panibacillus polymyxa* EJS-3 strains from soil (Han & Clarke, 1990 and Lee *et al.*, 1997), the optimal pH for EPS production by *P. polymyxa* EJS-3 was slightly alkaline. This might be due to the specific growth condition of *P. polymyxa* EJS-3 within the living tissues of plant. Incubation temperature is also a critical factor in EPS biosynthesis. As shown in Fig. 9, the optimal temperature for cell growth and EPS production were 40°C and 35°C with the corresponding cell biomass and EPS of 2.8 g/L and 7.7g/L, respectively. This is consistent with the report that bacteria favor lower temperature for EPS production comparing with that for cell growth (Fett, 1993).

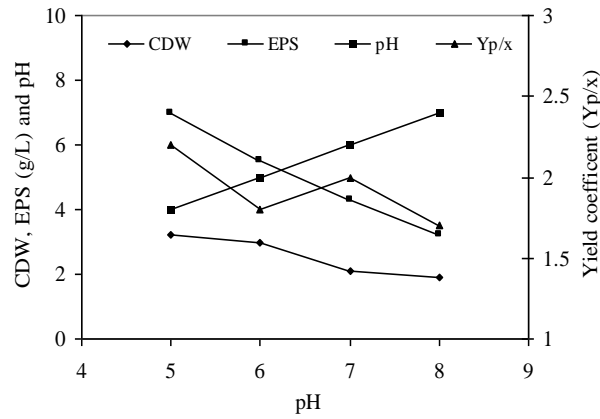


Fig. 8. Effect of pH on cell growth and EPS production from *B. megaterium* ($Y_{p/x}$ = g EPS/g dry biomass, CDW, Cell dry weight and EPS, Exopolysaccharide).

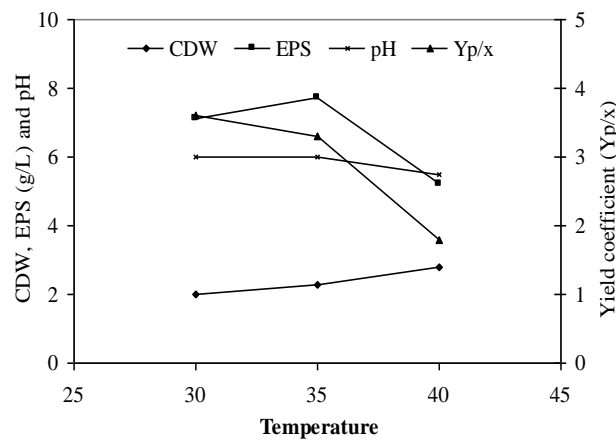


Fig. 9. Effect of temperature on cell growth and EPS production from *B. megaterium* ($Y_{p/x}$ =g EPS/g dry biomass, CDW, Cell dry weight and EPS, Exopolysaccharide).

Molasses and treated molasses

Treated molasses (four treatments) from sugarcane and sugarbeet were used as carbon sources for EPS instead of sucrose (Table 4). Molasses from sugarcane and sugarbeet treated by sulfuric and calcium phosphate had maximum production of EPS 13.4g/L and 13.7g/L, respectively from sugarcane and 10.8g/L and 14.6g/L, respectively from sugarbeet. The treatment sugarcane and sugarbeet used as carbon source for dextransucrase *Leuconostoc mesenteroides* NRRL B-512F (Abdel-Aziz & Asker, 2008).

TABLE 4. Effect of pretreated molasses from sugarcane and sugarbeet on (EPS) production.

Molasses treatment	CDW (g/L)	EPS (g/L)	EPS/Biomass ($Y_{p/x}$)	Final pH
Control	4.5	8.0	1.8	5
Sugarcane				
A	3.1	0.8	0.3	7
B	3.1	0.8	0.3	5
C	3.4	13.4	3.9	5
D	5.4	13.7	2.5	5
Sugarbeet				
A	3.7	8.3	2.2	7
B	5.1	8.3	1.6	5
C	0.8	10.8	12.8	5
D	6.8	14.6	2.1	5

• Volume each treatment containing equivalent 2 % sucrose

A:removal of suspended matter; B:potassium ferrocyanide treatment; C: sulfuric acid treatment; D: calcium phosphate treatment.

• $Y_{p/x}$ = g EPS/g dry biomass

• CDW, Cell dry weight. • EPS, Exopolysaccharide.

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الظروف الانتاجية للسكر العديد المنتج من *Bacillus megaterium* المعرفة بالتسلسل الجيني 16S rRNA

هالة محمد أبو شادي ، محسن محمد سليم عسكر*، مجدى على جاد الله*
وسلمى مجدى عبد الناصر*

قسم الميكروبيولوجى- كلية العلوم - جامعة عين شمس و *قسم التكنولوجيا
الحيوية الميكروبية - المركز القومى للبحوث- القاهرة - مصر.

السلالة البكتيرية *Bacillus megaterium* المنتجة للسكر العديد تم عزلها من التربة المصرية و تعريفها بالتسلسل الجيني 16S rRNA وقد وجد أن الظروف البيئية المثلى لهذه السلالة لتعطي أعلى إنتاجية من السكر العديد هي تحضين لمدة ٧٢ ساعة على درجة حرارة ٣٥ °م في البيئة الانتاجية التي تحتوى على ٢٪ من سكر السكروز كعنصر وحيد للكربون ، ٠,١٪ من مستخلص الخميرة و ٠,١٪ من كلوريد الامونيوم كمصدر للنيتروجين العضوى و الغير عضوى على التوالي. ودراسة تأثير المولاس من (قصب السكر- بنجر السكر) المعرض لمعاملات تنقية عديدة كعنصر للكربون مقارنة بسكر السكروز على الأنتاجية وجد أن معاملة التنقية باستخدام حمض الكبريتيك كانت ذات اعلى انتاجية .