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Optimization of Water Absorbing Exopolysaccharide Production on Local Cheap Substrates by *Bacillus* Strain CMG1403 Using One Variable at a Time Approach

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Optimum culture conditions, and carbon and nitrogen sources for production of water absorbing exopolysaccharide by *Bacillus* strain CMG1403 on local cheap substrates were determined using one variable at a time approach. Carbon source was found to be sole substrate for EPS biosynthesis in the presence of yeast extract that supported the growth only and hence, indirectly enhanced the EPS yield. Whereas, urea only coupled with carbon source could enhance the EPS production but no effect on growth. The maximum yield of EPS was obtained when *Bacillus* strain CMG1403 was grown statically in neutral minimal medium with 25% volumetric aeration at 30°C for 10 days. Under these optimum conditions, a maximum yield of 2.71±0.024, 3.82±0.005, 4.33±0.021, 4.73±0.021, 4.85±0.024, and 5.52±0.016 g/L culture medium was obtained with 20 g (sugar) of sweet whey, glucose, fructose, sucrose, cane molasses and sugar beet the most efficient one respectively as carbon sources. Thus, the present study showed that under optimum culture conditions, the local cheap substrates could be superior and efficient alternatives to synthetic carbon sources providing way for an economical production of water absorbing EPS by indigenous soil bacterium *Bacillus* strain CMG1403.

***Keywords*:** optimization, exopolysaccharide, OVAT, local cheap substrates

Introduction

The interest in bacterial exopolysaccharides (EPS) has increased considerably in recent years, as they are unique, biocompatible and environmental-friendly candidates for many commercial applications in different industrial sectors like food, petroleum, and pharmaceuticals. Biosynthesis of value-added biopolymers from bacteria serves as a promising alternative to harsh chemical processes that employ expensive, hazardous, and non-renewable raw materials. According to recent market reports, growing environmental concerns and increasing demand from end-use sectors are expected to increase the global market for microbial products to about 250 billion US dollars by 2016 (McWilliams, 2011). Though functional characteristics of EPS establish its market potential but because of their costly production processes, industrial bacterial EPS constitute only a minor fraction of the current polymer market. A useful biopolymer cannot find its proper place in the polymer market unless it can be produced economically. In order to reach high production titers at reasonable costs, fermentation medium and conditions should carefully be designed to make the end product compatible with its synthetic petrochemical counterpart. Therefore, since last two decades much effort has been devoted to the development of cost-effective and environmental friendly production processes by switching to optimum fermentation conditions and cheaper substrates. Fermentation is a very versatile process technology for producing value-added bacterial biopolymers and since fermentation parameters have a high impact upon the viability and economics of the bioprocess, their optimization holds great importance for process development. Bacterial EPS production is greatly influenced by fermentation conditions such as pH, temperature, oxygen concentration and agitation as well as by the composition of the culture medium (Sutherland, 2007; Kazak *et al.*, 2010; Nicolaus *et al.*, 2010). Moreover, fermentation feedstock has been the most expensive constituent in bacterial EPS production. Till the1990s, studies were generally focused on using defined culture conditions in order to recover ultra pure biopolymers with minimum batch-to-batch variation and free of impurities that would interfere with their chemical and biological characterization. However, to maximize the cost effectiveness of the process, a number of workers have used multi-component feedstock systems and the synthetic media were replaced by cheaper alternatives such as olive mill wastewater, syrups, and molasses (Salehizadeh and Loosdrecht, 2004; Sutherland, 2007; Nicolaus *et al.*, 2010). However, there are few reports on optimization of culture conditions and use of renewable raw materials as cheaper substrate for high production of value-added polysaccharides from *Bacillus* species at reasonable costs. Ebube *et al.* (1992) have reported the neutral pH, incubation for 80 h, sucrose as substrate and temperature of 30°C optimal for growth and polysaccharide biosynthesis in *B. licheniformis* NCIB 11634. While, Borgio *et al.* (2009) have examined the *B. subtilis* NCIM2063 for EPS producing ability at the laboratory level using milk medium and sewage water as nutrient source, and after 7 days of incubation at 37°C, 26 and 18 g/ml EPS was recoded respectively. Juan *et al.* (2011) optimized the medium and

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culture conditions for levan production by *B. licheniformis* 8-37-0-1 by single factor and orthogonal array experiments. After culture at 30°C for 24 h in a minimal medium prepared with tap water consisting of sucrose 100 g/L, a maximum levan production of 41.7 g/L was achieved. In another similar study, among *B. subtilis* (natto) strains, Takahashi strain was found to produce 40 to 50 mg of levan/ml after cultivation in minimal medium containing 20% (w/w) sucrose at 25 to 40°C, pH 6.0, with shaking at 150 to 200 rpm for 21 h. The levan yield by the Takahashi strain was comparable to that by *B. polymyxa* the known high levan-producer; however, this is the highest yield of levan first ever obtained in the least time (21 h) under the common cultivation condition (Shih *et al*., 2005).

In our previous study, EPS produced by *Bacillus* strain CMG1403 was characterized as novel heteropolysaccharide with nontoxic, biodegradable and environmental friendly water absorbing properties (Muhammadi and Ahmed, 2008). Now this requires cheap substrates and standardized culture conditions for the successful implementation of commercial production systems. Since, as agriculture country, Pakistan is well-sufficient in renewable natural resources such as sugar cane, sugar beet and milk byproducts. Therefore, to maximize the cost effectiveness of EPS production, recent work was shifted to optimize culture conditions and use local cheaper alternatives as substrates for production of water absorbing EPS from *Bacillus* strain CMG1403.

Materials and Methods

Bacterial strain and medium

Bacillus strain CMG1403 a facultative, aerobic and motile bacterium used in this study has previously been characterized as EPS producer (Muhammadi and Ahmed, 2008). The KN (Kurane and Nohata) medium (2% sucrose, 0.68% KH_2PO_4, 0.88% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.05% Yeast extract, 0.05% Urea) used for bacterial growth and production of EPS was prepared as described previously (Muhammadi and Ahmed, 2006).

Determination of optimal growth

Prior to experiments on optimization of EPS production, the optimum growth parameters such as pH, temperature, volumetric aeration, osmotic pressure and culture system were determined in 100 ml KN medium. A total of 0.5% (v/v) of standard inoculum was inoculated in medium of each experiment and performed in triplicate. Cell dry mass (CDM) (g/100 ml) was used as an indicator for growth after 120 h of incubation. The parameter that promoted the highest CDM was used for the subsequent steps of the investigation. KN medium without inoculation was used as a control.

pH: The influence of initial medium pH on bacterial growth was investigated at pH 4, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8, 8.5, and 9.

Temperature: The effect of temperature on bacterial growth was studied under incubation at 15, 20, 25, 30, 35, 40, and 45°C.

Volumetric aeration: To determine the effect of volumetric aeration, *Bacillus* strain CMG1403 was also grown in 100 ml blue cap Schott Duran (GL 45) bottles containing 100, 90, 75, 65, 50, and 25 ml medium representing 0, 10, 25, 35, 50, and 75% volumetric air concentration respectively (Kawai *et al*., 1992).

Culture system: In order to determine the effect of culture system (dissolved O_2), *Bacillus* strain CMG1403 was grown in an orbital shaking incubator at 0, 50, 100, 150, 180, and 200 rpm.

Osmotic pressure: The effect of osmotic pressure was determined by growing the *Bacillus* strain CMG1403 in KN medium supplemented with 0.01, 0.05, 0.1, 0.5, 0.89, 1.0, 1.5, and 2% NaCl.

Role of carbon and nitrogen sources in growth and EPS production

The role of carbon (2% each fructose or glucose or sucrose) and nitrogen (0.05% Urea and 0.5% Yeast extract) sources in EPS production was investigated with supplementation and absence criteria by growing the *Bacillus* strain CMG1403 under optimum pH and incubation temperature.

Cell dry mass determination

Each culture under different variables was separately centrifuged at 12,000 rpm. Hard compact pellet of bacterial cells was resuspended in saline, suspension was recentrifuged and supernatant was decanted, this process of washing was repeated two times. The resultant pellet was dried in a Wheaton dry-seal vacuum desiccator over $CaCl_2$. For complete desiccation, cell mass was subjected to evaporation at 100°C in an electric oven (OSK) until a constant weight was reached (Dlamini and Peiris, 1997a).

Preparation and analysis of local cheap substrates

Local sugar beet (*Beta vulgaris*) (containing 20% sucrose), sugar cane molasses and sweet whey were used as local cheap carbon sources. Sugar beet and sugar cane bagasse were crushed, stored at 4°C and required amount of each was autoclaved in 10 ml distilled water at 110°C for 15 min. The cane molasses (containing 50% sucrose) obtained from Habib Sugar Mills (Nawabshah, Pakistan) was clarified according to method of Panda *et al*. (1984) before use. Clarified molasses were filtered and autoclaved at 110°C for 15 min. Sample of sweet whey (containing 5% lactose) obtained from local milk market was prepared according to method described by Dlamini and Peiris (1997a, 1997b) and autoclaved at 110°C for 15 min.

Prior to utilization, sugar, nonsugar organic constituents and inorganic ions in prepared samples of local cheap substrates were determined by high performance liquid chromatography. Sugar components were determined according to method as described below for determination of monosaccharide composition of EPS samples. While the separation of nonsugar organic constituents and inorganic ions was made on Primesep B4 (150×4.6 mm) at 50°C with 60% 40 mM MeCN (pH 3) as the mobile phase, the elution rate was 1 ml/min and the injection volume of the sample was 20 μl. Quantification of components of the mixture was performed using an ELSD detector signal.

Optimization of culture conditions

Culture conditions (medium pH, incubation period, temperature, and volumetric aeration), and carbon and nitrogen sources were optimized using a one-variable-at-a-time (OVAT) approach (Van den Berg *et al*., 1995). Fructose, glucose and sucrose were used at concentration of 10, 20, 30, 40, and 50 g/L medium. While local cheap substrates (separately sterilized at 110°C for 10 min) sugar beet, cane molasses and sweet whey were used at certain concentrations that could provide one of carbon sources estimated as 10, 20, 30, 40, and 50 g/L medium. Based on this estimation 50, 100, 150, 200, and 250 g of sugar beet was added to 1 L KN medium. While cane molasses (20, 40, 60, 80, and 100 ml/L) and sweet whey (200, 400, 600, 800, 1000 ml/L) were enriched to 1 L by adding the KN medium of corresponding concentrations (% w/v). One millilitre of 24 h old seed culture was inoculated into 1 L of above mentioned media adjusted at different pH (4, 5, 6, 7, 8, and 9). *Bacillus* strain CMG1403 was grown at 10, 15, 20, 25, 30, 35, and 40°C for 3, 5, 8, 10, 12, 15, 20, and 25 days in an orbital shaking incubator at 0, 50, 100, 150, and 200 rpm.

To determine the effect of volumetric aeration, *Bacillus* strain CMG1403 was also grown in 1,000 ml blue cap Schott Duran (GL 45) bottles containing 1000, 900, 750, 650, 500, and 250 ml KN medium representing 0, 10, 25, 50, and 75% volumetric air concentration respectively (Kawai *et al*., 1992).

Isolation and quantification of exopolysaccharide

After each stipulated incubation period the growth of culture was terminated, then EPS was isolated and quantified according to the method as described previously (Muhammadi and Ahmed, 2006, 2008). EPS yield obtained under each variable was expressed as g/L of culture.

HPLC analysis of EPS samples

The purified EPS samples obtained with different local cheap carbon substrates were hydrolyzed (Muhammadi and Ahmed, 2008), dissolved in methanol and then analyzed by HPLC (Shimadzu LC-20AT). The refractive index detector (RID-10A) system was used. The components were separated on Shodex ks–801 at 80°C with distilled water as the mobile phase, the elution rate was 1 ml/min and the injection volume of the sample was 20 µl. The model mixtures were prepared by pure sugars based on the HPLC guideline. The column was calibrated with different molecular mass standard and a standard curve was then established (Muhammadi and Ahmed, 2008).

Statistical analysis

The results obtained were analyzed statistically using the Statistix 8.1 software. The means of four repeated experiments were compared using one way ANOVA to indicate any significant difference among parameters and the variables. The result was considered significant followed by Tukey HSD test at $P<0.05$ significance level.

Results and Discussion

In order to improve the EPS production in *Bacillus* strain CMG1403, at first step of this study, optimum growth parameters, and the role of carbon and nitrogen sources in EPS production were determined under optimum temperature and medium pH for growth. Then in next step, growth conditions (medium pH, temperature and incubation period) for EPS production were optimized using local cheap carbon sources such as sugar beet, cane molasses and sweet whey.

Optimum growth parameters

In most cases, optimum values of growth parameters for biomass formation and EPS production differ considerably so that typical fermentations start with the growth phase followed by the production phase (Cheng *et al*., 2011).

The growth parameters including initial medium pH, incubation temperature, culture system and aeration, play important roles in enhancing biomass production (Fig. 1). Therefore,

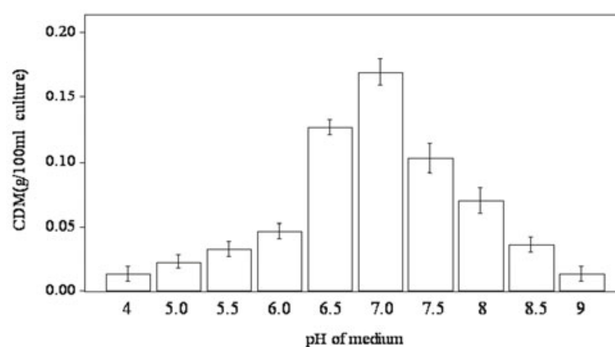

Fig. 1A. Effect of medium pH on growth of *Bacillus* strain CMG1421. *Bacillus* strain CMG1403 produced varying CDM when grown at different incubation temperatures in KN medium adjusted at pH 7 which showed significant difference at P value <0.01. These results showed that incubation temperature had a significant effect on growth. However, the maximum CDM (0.16 g/100 ml) was obtained from culture incubated at 30°C (Fig. 1B). This fell within the optimal incubation temperature range of 28–50°C specified for the *Bacillus* species for growth and metabolism (Ebube *et al*., 1992).

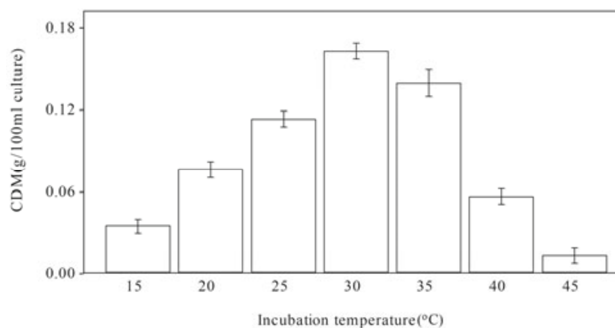

Fig. 1B. Effect of incubation temperature on growth of *Bacillus* strain CMG1421. The results in Fig. 1C showed that *Bacillus* strain CMG1403 was able to grow in volumetric aeration ranging from 0 to 75% with significant difference value at $P<0.01$. However, the maximum CDM (0.18 g/100 ml) was achieved at 25% volumetric aeration. Production of biomass in KN medium decreased at below and above 25% of volumetric aeration (Fig. 1C).

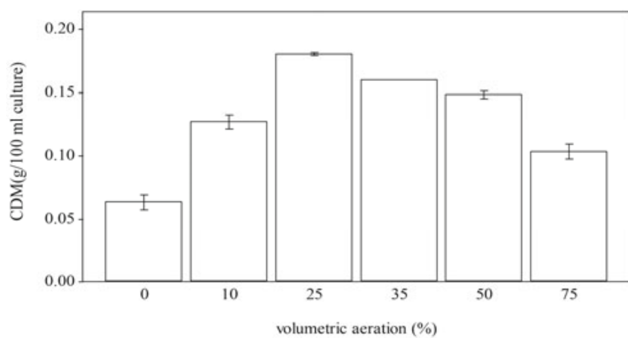

Fig. 1C. Effect of volumetric aeration on growth of *Bacillus* strain CMG 1421. In case of agitation, it was observed that with increasing speed of agitation, there was a decrease in CDM. Although the biomass produced at 0, 50, and 100 rpm had no statistically significant difference with *P* value at 0.54 but the maximum CDM was achieved from static (0rmp) culture (Fig. 1D). However, agitation did not favor the biomass production and as speed of agitation increased, did not CDM. These results suggested that for *Bacillus* strain CMG1403, incubation under static condition and with 25% volumetric aeration were optimum for maximum biomass production. On the other hands, a decreasing CDM was obtained as rate of volumetric aeration increased (>25%) and strain was grown under agitation as well. Since, *Bacillus* strain CMG1403 was a facultative, aerobe and motile bacterium therefore, 25% volumetric aeration might be enough and did not require more agitation for O_2 transfer. Also agitation might create shear which could damage bacterial cells and resulting a decreased biomass production.

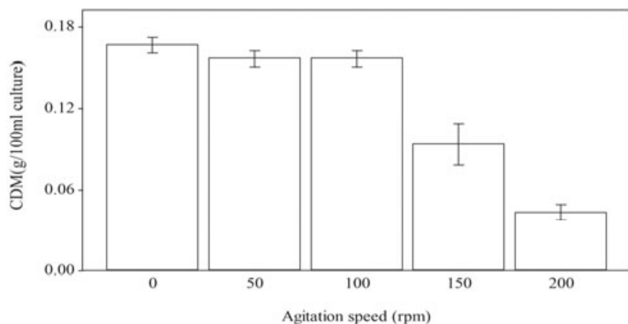

Fig. 1D. Effect of agitation speed on growth of *Bacillus* strain CMG1421. The means of CDM achieved under osmotic pressure developed at all NaCl concentrations were significantly different at *P* values <0.05 to 0.01 except that produced at 0.89 to 1.5% were found to be nonsignificant at *P* values >0.068. However, the maximum CDM (0.17 g/100 ml) was obtained from medium containing 0.05% NaCl (Fig. 1E).

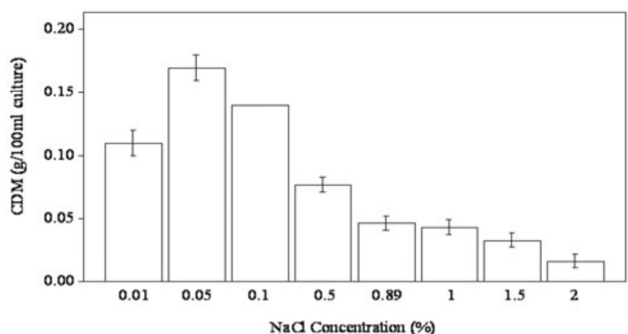

Fig. 1E. Effect of osmotic pressure on growth of *Bacillus* strain CMG1421. These optimum parameters for growth of *Bacillus* strain CMG1403 were used for optimization of EPS production in downstream steps of present study.

they need to be optimized. The optimization of initial medium pH for growth showed that *Bacillus* strain CMG1403 was able to grow at a wide range of pH (4 to 9). The means of CDM achieved at all pH were significantly differed at *P* vales <0.01 except that at 4 and 5, 4 and 9, 5 and 5.5, 5 and 8.5, 5 and 9, 5.5 and 8.5 were nonsignificant with *P* values at 0.49. The maximum CDM (0.17 g/100 ml) was obtained from neutral medium while at above and below the pH 7, there was a decreasing pattern of CDM (Fig. 1A). This result was in correlation with findings of other workers which have suggested the neutral pH optimum for various *Bacillus* strains to achieve maximum biomass (Combet-Blanc *et al*., 1995; Abdel-Mehawgoud *et al*., 2008; Younis *et al*., 2010).

Role of carbon and nitrogen sources in biomass and EPS production

In order to determine the role of carbon and nitrogen sources of the medium in the production of EPS, *Bacillus* strain CMG1403 was grown under different combinations of carbon source (CS: fructose, glucose and sucrose) and nitrogen (U: urea, YE: yeast extract). Figure 2 showed that CS solely could neither support the biomass nor EPS but with urea (CS/U) a trace CDM (0.01 g/100 ml) only, and in combination with yeast extract (CS/YE) both CDM (0.17 g/100 ml) and EPS (0.39 g/100 ml). Upon supplementation of YE solely, only CDM (0.02 g/100 ml) was produced and even along with U no EPS was obtained (Fig. 2). From these results it was found that CS acted as sole substrate for the biosynthesis of EPS and in coupled with nitrogen sources supported the growth as well (Dlamini and Peiris, 1997a; Dlamini *et al*., 2007). Further, YE was found to enhance the biomass only which indirectly participated in EPS synthesis (Muhammadi and Ahmed, 2006).

The means of CDM obtained with U/YE and YE were found to be statistically nonsignificant at *P*=0.1161. Similarly, the means of CDM obtained with CS/YE and CS/YE/U were found to be statistically nonsignificant at *P*=0.8745. These results suggested that urea solely and even with CS or/and YE did not support the biomass production. It was previously reported that certain essential amino acids can't be synthesized from inorganic nitrogen components (Chiu-Yeh *et al*., 2008), because of which bacterial cells might neither be

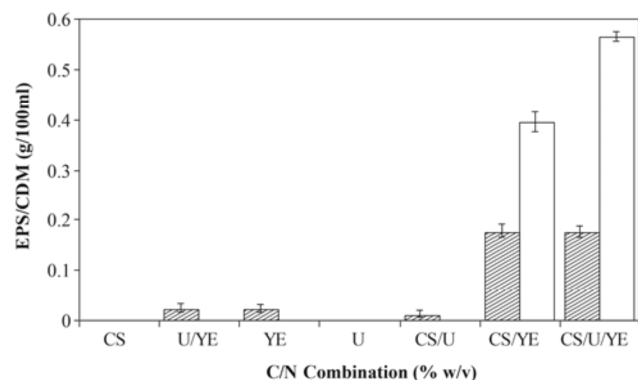

Fig. 2. Role of carbon (CS) and nitrogen sources (Yeast Extract and Urea) in biomass and EPS production.

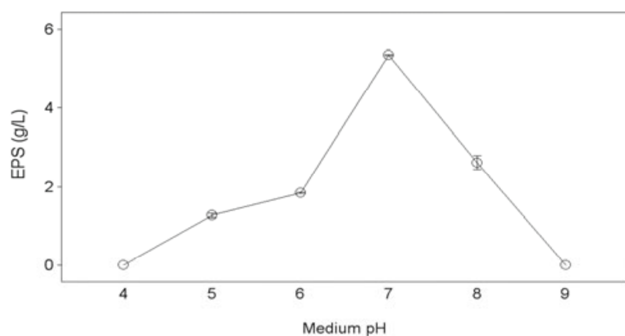

Fig. 3. **Effect of medium pH on EPS biosynthesis by *Bacillus* strain CMG 1421.**

fully grown nor undergone metabolism. However, in the absence of U, the yield of EPS was reduced and when added to medium, EPS was significantly increased (Fig. 2). Therefore, urea might enhance the EPS synthesis in the presence of carbon source as reported by Kurane and Nohata (1995). Upon supplementation of CS/U/YE combination, both the biomass and EPS were increased to maximum (Fig. 2). It was reported that ratios of carbon and nitrogen sources play the most important role in cellular growth and exobiopolymer biosynthesis by *Bacillus* species (Gandhi *et al*., 1997; Lee *et al*., 1997). Once the role of carbon and nitrogen sources was identified, local cheap carbon sources such as, sugar beet, cane molasses and sweet whey were also used as substrate for EPS production.

Effect of medium pH on EPS production

Result has shown that the medium pH significantly influenced EPS production at *P* values <0.01 (Fig. 3). The extreme pH profiles of the medium (pH<4.0 and pH>9) reduced not only the bacterial growth but also inhibited the biosynthesis of EPS. Both increasing and decreasing pH value of the culture medium significantly influenced EPS biosynthesis by *Bacillus* strain CMG1421. However, the optimal pH profile of the medium for the highest productivity (5.35 g/L) of EPS was found 7 (Fig. 3). These results were matched with those of several reports on optimum growth and maximum EPS production in *Bacillus* species obtained from media adjusted at neutral pH (Gandhi *et al*., 1997; Lee *et al*., 1997; Jie *et al*., 2008)

Effect of temperature on EPS production

The effect of cultivation temperature on EPS biosynthesis by *Bacillus* strain CMG1421 was found to be statistically significant at *P* values <0.05 (Fig. 4). It was found that as temperature increased to 30°C, the yield of EPS was proportionally increased but after 30°C (at 35 and 40°C) the EPS yield was decreased gradually. According to Sutherland (2001) reduction of the cultivation temperature by 10°C below optimal level inhibits the exopolysaccharide biosynthesis by microbial cells. The optimum temperature for maximum EPS (5.38 g/L) production was found to be 30°C which was in agreement with that for maximum growth of *Bacillu*s strain CMG1421 (Figs. 1B and 4). This optimum temperature (30°C) also fell within the optimal incubation temperature range of 28 to 50°C and 26 to 31°C specified for the *Bacillus* species for optimum growth and metabolism (Ton-That *et al*., 2004), and for the production of the most EPS (Ebube *et al*., 1992) respectively. Further, according to Gandhi *et al.* (1997) for EPS biosynthesis and growth, the temperature is often a critical factor in *Bacillus* species which ranges 25–35°C. This was true for optimization of cultivation temperature for EPS production by *Bacillus* strain CMG1421 in current study.

Effect of incubation period on EPS production

Means of EPS yields obtained after different lengths of incubation significantly differed from each other at *P* values <0.05 except between those obtained from 8th and 20th old culture were considered to be not quite statistically significant at P=0.0567 (Fig. 5). These results indicated that incubation periods had significant effect on biosynthesis of EPS by *Bacillus* strain CMG1421. However, the maximum yield of EPS (5.37 g/L) was obtained from 10 days old culture only (Fig. 5). Up to 10th the yield was found to be a positive function of incubation period but later on (>10 days) a reduction pattern was observed. This has suggested the availability of carbon source till 10th day and after that due to prolonged incubation starvation stimulated the EPS depolymerases to liberate consumable carbon source from some

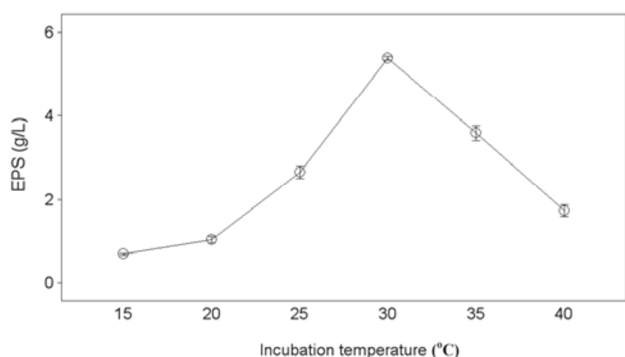

Fig. 4. **Effect of incubation temperature on EPS biosynthesis by *Bacillus* strain CMG1421.**

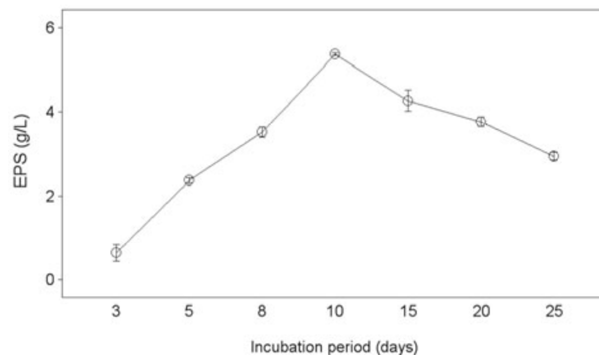

Fig. 5. **Effect of incubation period on EPS biosynthesis by *Bacillus* strain CMG1421.**

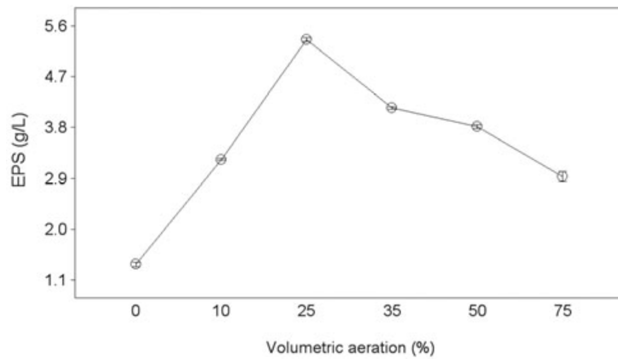

Fig. 6. Effect of volumetric aeration on EPS biosynthesis by *Bacillus* strain CMG1421.

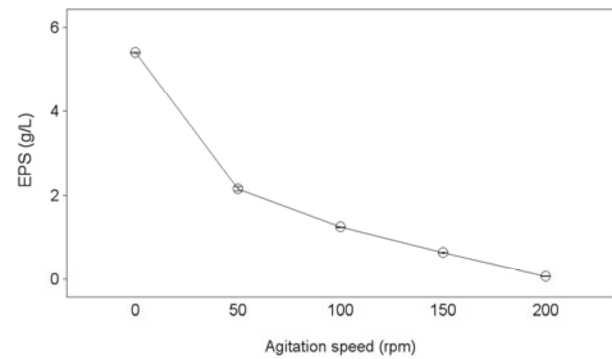

Fig. 7. Effect of agitation speed on EPS biosynthesis by *Bacillus* strain CMG1421.

of synthesized EPS for bacterial growth (Pham *et al.*, 2000). In another similar study, Szumigaj *et al.* (2008) have reported that low-nutrient environment might accelerate biodegradation of EPS from *Bacillus* species. Further, after 10th day of incubation, the accumulation of metabolic wastes might also alter the optimum pH 7 of culture medium for EPS biosynthesis towards acidic or basic pH (Pham *et al.*, 2000). Perhaps, this change in pH most probably coupled with scarcity of carbon source might bring about a reduction in yield of EPS. Therefore, it was suggested that the under studied EPS was biodegradable which substantiated the previous report on its biodegradability (Muhammadi and Ahmed, 2008).

Effect of rate aeration on EPS production

The results in Fig. 6 showed that yields of EPS obtained from cultures with different volumetric aerations were found to be statistically significant at *P* values <0.01. Growth with volumetric aeration profile <25% and >25% brought about a reduced EPS yield and maximum yield of EPS (5.36 g/L) was favored by growth with 25% volumetric aeration only (Fig. 6).

On the other hands, statistical analysis showed that the agitation speed was also been proven to be a critical factor influencing the EPS biosynthesis significantly at *P* values <0.01. But like CDM, the maximum yield of EPS (5.4 g/L) was obtained only from static culture and EPS decreased with increasing agitation speed (Fig. 7). Therefore, it was suggested that the biosynthesis of EPS by *Bacillus* strain CMG1403 could not require any expensive shaking instrument or mechanical method, so from industrial point of view it was simple. Economically, static culture system on the other hands, is also more likely to succeed because the low shear forces in static culture promote higher productivity (Saxena *et al.*, 1990, 1991). Further, it was found that the pattern of effect of volumetric aeration profile and agitation speed on yield of EPS was in agreement with that on CDM. Therefore, it was inferred the growth-dependent EPS biosynthesis by *Bacillus* strain CMG1403 in the presence of 25% volumetric O_2 without agitation as substantiated above in case of CDM. Lee *et al.* (1997) have correlated the rate of aeration with cell growth and acidic EPS biosynthesis by *B. polymyxa*.

Effect of carbon substrate on yield of EPS

Statistical analysis showed that sole carbon sources, local cheap substrates and their different concentrations significantly influenced the EPS biosynthesis by *Bacillus* strain CMG1403 at *P* values <0.01 (Table 1). For each carbon source, at a concentration of 2% of sugar, EPS was at its maximum due to the facilitated carbon uptake but as the concentration of supplied sugars increased >2%, the cell growth and the yield were found to decline. This is mostly due to the elevation of osmotic pressure in the cellular system, thereby causing plasmolysis leading to cell death (Kuntiya *et al.*, 2010). In terms of weight (g/g), a maximum of 27.6, 24.25, 23.65, 21.65, 19.1, and 13.55% of supplied sugar from sugar beet, cane molasses, sucrose, fructose, glucose and sweet whey res-

Table 1. Yield of EPS obtained with different concentrations of carbon sources

Carbon source	EPS (mean±SD g/L) obtained with different concn of CS (sugar g/L)					*P* value
	10	20	30	40	50	
SCS						
Fructose	3.13±0.020	4.33±0.021	3.84±0.025	3.73±0.011	3.25±0.010	<0.01
Glucose	2.72±0.012	3.82±0.005	3.54±0.015	3.44±0.010	2.82±0.015	<0.01
Sucrose	3.62±0.020	4.73±0.021	4.54±0.025	4.12±0.017	3.84±0.015	<0.01
LCS						
Cane molasses	3.74±0.013	4.85±0.024	4.39±0.007	3.83±0.007	3.43±0.007	<0.01
Sugar beet	3.83±0.018	5.52±0.016	4.84±0.011	4.23±0.016	3.97±0.045	<0.01
Sweet whey	0.84±0.011	2.71±0.024	2.36±0.009	2.09±0.036	1.87±0.016	<0.01

SCS, sole carbon source; LCS, Local cheap substrate

Table 2. Chemical composition of prepared local cheap substrate determined by HPLC

Constituents	Concentration (%) of constituents contained by local cheap substrates		
	Sugar beet	Molasses	Sweet whey
Sucrose	20.02	50.02	-
Glucose	1.02	0.02	0.03
Galactose	-	-	0.04
Fructose	1.02	0.02	-
Raffinose	-	2.25	-
Lactose	-	-	5.03
Citric acid	0.095	-	-
Lactic acid	0.072	-	0.032
Fumaric acid	-	0.026	-
Succinic acid	0.068	0.037	-
Aconitic acid	0.68	1.372	-
Glycolic acid	0.042	0.076	-
Acetic acid	0.029	0.052	-
Oxalic acid	0.015	-	-
Formic acid	-	0.57	-
Protein	9.46	5.73	2.46
Amino acids	4.55	3.22	-
Polyphenols	0.08	0.1	-
Non-protein nitrogen	1.49	1.56	0.16
Betaine	-	5.23	-
Niacin	0.004	0.031	-
Sodium	0.05	1.43	0.04
Magnesium	0.03	0.002	0.006
Phosphorus	0.06	0.14	0.04
Chloride	0.04	1.33	0.11
Potassium	1.33	1.55	0.12
Calcium	0.08	0.56	0.043
Iron	0.08	0.003	0.002

–, Not detected.

pectively was converted into EPS. With sweet whey the yield of EPS was found to be minimum (Table 1) because the most of *Bacillus* species utilize the lactose slowly (Nakamura, 1987) therefore, lower growth and EPS biosynthesis (Cerning *et al*., 1994). Among all tested carbon sources, sugar beet and cane molasses were found to be superior and efficient local cheap substrates for EPS production by *Bacillus* strain CMG1403. Although, recently Razack *et al*. (2013) have obtained a maximum yield (4.86 g/L) of EPS from *B. subtilis* by one factor at a time method using 2% cane molasses. However, in present study the highest yield of EPS was obtained upon 100 g of sugar beet (20% sucrose/L) in the culture medium. There are few reports on utilization of sugar beet as substrate for EPS production by *Bacillus* species. However, in a similar study made by Han and Watson (1992) reported 38.0 g/L of levan from *Paenibacillus polymyxa* NRRL B-18475 using sugar beet as efficient cheap substrate. Mao *et al*. (2011) have reported 500 mg/L of EPS from *B. cereus* B-11 replacing glucose with sugar beet molasses wastewater. Taking into consideration that sugar beet is carbohydrate-rich with a high carbon-to-nitrogen ratio (C/N, 35/40), that sugar beet farming is a widespread and already mature industry, and that sugar beet is abundant and cheap, this has potential for use as a renewable feedstock for EPS production by *Bacillus* strain CMG1403.

HPLC analysis showed that local cheap substrates were found to contain a pool of nutrients, vitamin, minerals and growth factors in varying concentration (Table 2) that have the potential to stimulate the growth of *Bacillus* spp. and with certain concentration also EPS production (Razack *et al*., 2013) but in present study, the yields of EPS obtained with the molasses and sugar beet were comparable to those of media containing pure sucrose and fructose except with glucose and sweet whey. These results were substantiated by earlier reports that the suitability of substrates for EPS production highly depends on the ability of the microorganism to utilize carbon source especially the lactose (sweet whey) (Nakamura, 1987; Silva *et al*., 2009). The higher yield with sugar beet compared to cane molasses and other substrates might be due to higher concentration of protein and amino acids content in sugar beet (Table 2). It was reported that in the presence of certain essential amino acids and protein bacterial cells could undergo maximum growth and metabolism, hence the elevation of EPS yield (Chiu-Yeh *et al*., 2008; Razack *et al*., 2013).

Monosaccharide composition of EPS

HPLC was used for determining any possible variation in

Table 3. Monosaccharide composition of EPS determined by HPLC

Standard monosaccharides	Retention time (min)	Monosaccharide composition (%) of EPS from different substrates					
		EPS_{Suc}	EPS_{Fru}	EPS_{Glu}	EPS_{SB}	EPS_{CM}	EPS_{SW}
D-Glucose	13.22	-	-	-	-	-	-
D-Xylose	14.4	-	-	-	-	-	-
D-Galactose	15.05	58.02	57.97	58.01	58.02	57.98	58.01
D-Galacturonic acid	15.33	-	-	-	-	-	-
L-Rhamnose	15.71	-	-	-	-	-	-
D-Arabinose	16.01	-	-	-	-	-	-
D-Glucuronic.acid	17.03	4.05	4.12	4.11	4.13	4.17	4.03
D-Mannose	17.08	34.12	34.13	34.14	34.13	34.12	34.13
D-Glucuronolactone	20.03	3.81	3.73	3.75	3.73	3.68	3.83
Gal : Man : GluA		7.38:4.34:1	7.38:4.34:1	7.38:4.34:1	7.38:4.34:1	7.38:4.34:1	7.38:4.34:1

EPS_{Suc}, EPS_{Fru}, EPS_{Glu}, EPS_{CM}, EPS_{SW}, and EPS_{SB}: EPS obtained from sucrose, fructose, glucose, sugar beet, cane molasses and sweet whey as substrate respectively; – : Not detected.

previously reported (Muhammadi and Ahmed, 2008) composition and concentration of monomers in EPS obtained from different sole carbon and local cheap substrates. In the analysis of the hydrolysate of the EPS samples by HPLC four different independent peaks were identified and molecular mass was determined with retention time (Table 3). The observed peaks in the chromatogram were assigned to D-galactose, D-glucuronic.acid, D-mannose and D-glucuronolactone according to retention time of the monosaccharide standards (Table 3). Glucuronolactones were not structural monomeric units of any EPS sample but were formed from glucuronic acids released during the hydrolysis. Because during acid hydrolysis, the monomeric uronic acids are in equilibrium with their lactones. Therefore, in EPS from all substrates the ratio of D-galactose, D-mannose and D-glucuronic.acid was calculated to be 7.38:4.34:1 (Table 3). These results showed that EPSs produced by *Bacillus* strain CMG1403 from different sole carbon and local cheap substrates were found to contain same monosaccharide composition in similar ratio as reported previously (Muhammadi and Ahmed, 2008). Hence, it was suggested that the change of carbon substrates could not affect the composition and ratio of monosaccharides in EPS produced by *Bacillus* strain CMG1403 but did affect the EPS yield and bacterial growth only (Mao *et al*., 2011; Razack *et al*., 2013).

Conclusion

It is concluded that there is correlation of EPS biosynthesis by *Bacillus* strain CMG1403 with culture conditions and growth. The optimum culture conditions for maximum EPS productivity are static cultivation at medium pH 7.0, temperature 30°C with 25% aeration rate for 10 days. Growth-associated EPS synthesis suggests that a sufficient supply of nutrients is required for a high production of the EPS. Further, for EPS biosynthesis by *Bacillus* strain CMG1403, a carbon source as substrate, organic nitrogen source for growth and an inorganic source as EPS synthesis enhancer are essential. Under optimum culture conditions, a significant yield of EPS (5.52 and 4.85 g/L) was obtained with sugar beet and cane molasses respectively which are superior and cheap alternative substrates than other tested synthetic carbon sources. Interestingly, with the change of carbon substrate, could affect the yield of EPS but the composition and ratio of monosaccharides in EPS remain unchanged. These lab-scale results provide essential information needed for scaling up biosynthesis of EPS by *Bacillus* strain CMG1403 for environmental friendly water absorbing and sanitary purposes.

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