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 environmentally 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 the 1990s, 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 (Salehzadeh 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 recorded 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 (Muhammad 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 (Muhammad and Ahmed, 2008). The KN (Kurane and Nohata) medium (2% sucrose, 0.68% KH$_2$PO$_4$, 0.88% K$_2$HPO$_4$, 0.02% MgSO$_4$·7H$_2$O, 0.01% NaCl, 0.05% Yeast extract, 0.05% Urea) used for bacterial growth and production of EPS was prepared as described previously (Muhammad 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.