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Production and Optimization of Extracellular Polysaccharides from *Paenibacillus* sp.

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Abstract

Extracellular polymeric substances (EPSs) are produced by a wide array of bacteria for protection against aggregation, expression of virulence and adhesion. Growth associated EPS production was determined by *Paenibacillus* sp. in submerged fermentation. To improve the productivity and efficiency of EPS from *Paenibacillus* sp. the physical factors and nutrient components were optimized. The bacterial growth (2.093 OD at 600 nm) was maximum after 36 h of incubation and EPS production (1.9 mg/ml) was high after 3 days of incubation at 37 °C. EPS production was found to be maximum at pH 7.0 (2.31 mg/ml), and at 40 °C (2.49 mg/ml). Supplementation of 1% sucrose (4.30 mg/ml) supported more EPS production. Among the nitrogen sources, addition of yeast extract (0.1%) supported maximum EPS production (5.36 mg/ml). Among inorganic sources, NH_4Cl significantly influenced on EPS production.

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Paenibacillus sp.
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Emulsifying agents

Introduction

In recent years there has been a continuous search for novel polysaccharides from microorganisms. These biopolymers have been attracting much interest due to their potent application in cosmetic, food, pharmaceutical and oil industries, where they are used as stabilizing, thickening and emulsifying agents (Hayashi and Hayashi, 1996). Extracellular polysaccharides (EPSs) are extracellular biopolymers that are produced during the metabolic process of microorganisms such as fungi, bacteria and blue-green algae (Amjres *et al.*, 2014). The EPSs could be either covalently associated with the cell surface forming a capsule, or be loosely attached, or totally secreted into the surrounding environment during the microbial cell growth (Yang *et al.*, 2010). EPSs are either heteropolysaccharide or homopolysaccharide or biologically synthesized by varieties of bacteria. EPSs show many biological functions such as immuno-stimulating activity, antitumor activity (Vijayabaskar *et al.*, 2011).

Production of EPS by various microbial groups including isolates belong to genus *Bacillus* have been widely investigated (Kim *et al.*, 2002). Microorganisms are highly suited for EPS production than plants or algae, exhibiting good growth rates and being more amenable to manipulate, optimize the process conditions for better growth and EPS production (Moreno *et al.*, 1998). Moreover, the high cost of the carbon sources such as sucrose, glucose and fructose and the cost of nitrogen sources directly influence on EPS production costs, which directly limit the potential of EPS market (Kumar *et al.*, 2007). To obtain high EPS production, it is significant to optimize bioprocess conditions, which require an understanding of the various process parameters involved (Velasco *et al.*, 2006). The productivity of EPSs has been found to vary with

medium composition, environmental conditions including nitrogen source, carbon source and pH of the culture medium (Papagiannai, 2004).

Biopolymers have many applications in the pharmaceutical, food and other industries due to their physical properties and unique structure. The EPS produced by bacteria have been explored for various biotechnological applications such as anticoagulants, antitumor agents and wound healing properties (Sutherland, 1985). Modern pharmacological studies revealed that microbial polysaccharide possesses anti-radiation, anti-oxidation, hyperglycemic, hypolipidemic activities, anti-tumour, anti-fatigue and other biological activities, which are hotspots of research on functional factors of health food and drugs (Tseng *et al.*, 2008). The genus *Paenibacillus* consists of more than 89 species of neutrophilic, facultative anaerobes, periflagellated heterotrophic, endospore forming, and low G + C content, Gram +ve bacilli, which were previously included within the genus *Bacillus* and further reclassified as a genus *Paenibacillus* (Ash *et al.*, 1993). Bacteria belonging to the genus *Paenibacillus* have been isolated from variety of environments such as soil, water, insect larvae, food, vegetable matter, rhizospheres as well as clinical samples (Raza *et al.*, 2011; Vijayaraghavan *et al.*, 2016). *Paenibacillus* species produced a range of exopolysaccharides with diverse physiological and biotechnological functions. *Paenibacillus* sp. EPSs has attracted great interest because of their biotechnological potential in the treatment of wastewater and various industrial processes. Microorganisms are good and cheaper sources for EPS production compared with algae or plants because of their ability to grow in cheaper nutrient media within a few days, high growth rate, ease of manipulation and lower space requirement (Raza *et al.*, 2011). Hence, there has been an increasing interest in producing EPSs from any new microbial isolates. Therefore, the aim of the present study was to isolate and screen EPS producing bacteria from the marine environment and to optimize the process parameters to enhance the EPS yield.

Materials and Methods

Bacterial strain and cultural conditions

Paenibacillus sp. IND8 was isolated previously from food and was used throughout the present study (Accession no: KF250416). This organism was grown on nutrient agar slants at 37 °C for 24 h and sub cultured regularly at 15-day interval.

Inoculum preparation

A loopful culture of *Paenibacillus* sp. IND8 was inoculated into the nutrient broth medium ((g/l); beef extract 1.5; peptone digest of animal tissue 5.0, sodium chloride 5.0, and yeast extract 1.5. The culture medium pH was adjusted to 7.0 using 1N HCl/NaOH. Further, the Erlenmeyer flask was incubated for 18 h at 37 °C in an orbital shaker (150 rpm). The culture was then stored at 2 – 8 °C and used as the inoculums for further studies.

Effect of fermentation period on the growth and EPS production

About 0.2 ml inoculum was introduced into a 100-ml Erlenmeyer flask containing basal medium ((g/l); yeast extract-0.1 g; glucose-0.5 g; $MgSO_4$-0.01 g; peptone-0.25 g; KH_2PO_4-0.05 g; and NaCl-1.0 g). The culture was incubated at 37 °C in an orbital shaker (150 rpm) for 4 days. At every 24 h, 5 ml culture was withdrawn and centrifuged at 10,000 rpm for 15 min at 4 °C. The EPS content of culture supernatant was assayed.

Estimation of EPS

The fermented medium was centrifuged (10000 rpm, 10 min, 4°C) to separate the cell pellet and supernatant. The EPS in the cell free supernatant was precipitated with double volumes of acetone, kept overnight at 4°C and then separated by centrifugation (10000 rpm, 4°C, 20 min). Further, the precipitated EPS was completely dissolved in double distilled water and quantified. To 0.1 mL of EPS sample, 1 ml of 5% (w/v) phenol solution was added and mixed completely. Then, 5 mL H_2SO_4 (concentrated) was added in the vial and the optical density was measured at 490 nm using a UV-vis spectrophotometer. The amount of EPS was then determined from the calibration curve using glucose as the standard.

Effect of pH on EPS production

Effect of pH on EPS production by *Paenibacillus* sp. IND8 was evaluated by adjusting the culture medium pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) using 1N HCl/NaOH. After sterilization, 500 µl of inoculum was introduced into an Erlenmeyer flask. Then these flasks were incubated for 3 days at 37 °C. After 3 days of incubation, the cells were centrifuged and harvested. The

culture supernatant was precipitated with acetone and EPS content was assayed.

Effect of different temperature

Effect of temperature on EPS production was carried out by inoculating 500 µl of inoculum into the nutrient broth medium at various temperatures for 3 days at 37 °C. The temperatures maintained were 25 to 50 °C. After 3 days of incubation, the cell free extract was precipitated with acetone and EPS content was assayed.

Effects of different carbon sources

The effect of carbon sources (1%) on the production of EPS was carried out by supplementing carbon sources such as dextrose, sucrose, maltose, lactose and glucose. After 3 days of incubation, the cell free extract was precipitated with acetone and EPS content was assayed.

Effect of nitrogen sources on EPS production

The effect of nitrogen sources (0.1%) on EPS production was evaluated by supplementing nitrogen sources such as yeast extract, gelatine, oat meal, skimmed milk, casein and ammonium chloride in the nutrient broth medium. The medium was inoculated with 500 µl inoculum. After 72 h, the culture was centrifuged (10,000 rpm, 10 min). The culture supernatant was precipitated with acetone and EPS was assayed.

Effects of amino acids on EPS production

The effect of amino acids at 0.1% (w/v) concentration (Glutamine, Glycine, Methionine Cysteine and Alanine) was supplemented into the production medium individually to determine the effect of amino acids on EPS production. After 72 h, the culture was centrifuged (10,000 rpm, 10 min). The culture supernatant was precipitated with acetone and EPS was assayed.

Results and Discussion

EPSs are synthesized by the bacterial cells and excreted out to the outer environment. Little information is available on the biosynthesis of EPSs from microbes (Sutherland, 1996). In the present study the process parameters were optimized to enhance the production of EPSs. To improve the productivity and efficiency of EPS from *Paenibacillus* sp. the physical factors and nutrient components were optimized. Many researchers have evaluated the effects of process parameters on the maximal production of EPSs to optimize the process conditions, such as temperature, pH and medium composition (Rafigh *et al.,* 2014; Raza *et al.,* 2011). Studies of EPS have demonstrated that the composition of the culture medium plays an important role in EPS production (Liu *et al.,* 2009).

It was previously reported that the production of EPS was associated with cell growth (Raza *et al.,* 2011; Wang *et al.,* 2011; Liang *et al.,* 2014). In the present study the bacterial growth (2.093 OD at 600 nm) was maximum after 36 h of incubation and EPS production (1.9 mg/ml) was high after 72 h incubation at 37 °C. Rafigh *et al.,* (2014) reported that when the initial pH of the culture medium was increased from 5.5 to 7.0, there was a dramatic increase in curdlan gum and biomass production in *Paenibacillus* sp. approximately 39.0% and 5.0%, respectively. Moreover, higher pH values (>pH 8.5) caused a decrease in EPS production. In the present study, EPS production was found to be maximum at pH 7.0 (2.31 mg/ml). The EPSs from *Paenibacillus* sp. IND8 were in agreement with earlier study that for EPS production by *P. polymyxa* KCTC 8648P, the optimum pH value of 7.0 was previously reported (Lee *et al.,* 1997). In the present study EPS production was high when the organism was incubated at 40 °C (2.49 mg/ml). Fermentation temperature is one of the critical factors for the production of EPS (Liu *et al.,* 2009). Rafigh *et al.,* (2014) reported that EPS production increased rapidly when the incubation temperature varied from 30 to 40 °C and then slightly increased as the fermentation proceeded from 40 to 50 °C.

Many carbon sources namely, glucose, sucrose, maltose, lactose, and dextrose were used to determine the production of EPS. Supplementation of 1% sucrose (4.30 mg/ml) supported more EPS production. The other carbon sources such as maltose, glucose lactose, dextrose supported 2.4 mg/ml, 3.9 mg/ml, 3.2 mg/ml, and 2.9 mg/ml, respectively (Fig. 1). This result was in consistent with the findings of earlier findings (Lee *et al.,* 1997). Among the nitrogen sources, addition of yeast extract (0.1%) supported maximum EPS production (5.36 mg/ml). Addition of other nitrogen sources such as oat meal (1.2 mg/ml), skim milk (3.4 mg/ml), and casein (2.87 mg/ml) also supported EPS production (Fig. 2).

Various nitrogen sources were employed for their effects on EPS production from the isolate. It was previously reported that organic nitrogen sources increased high amount of EPS than inorganic nitrogen substrates.

Fig.1 Effect of fermentation period on EPS production by *Paenibacillus* sp.

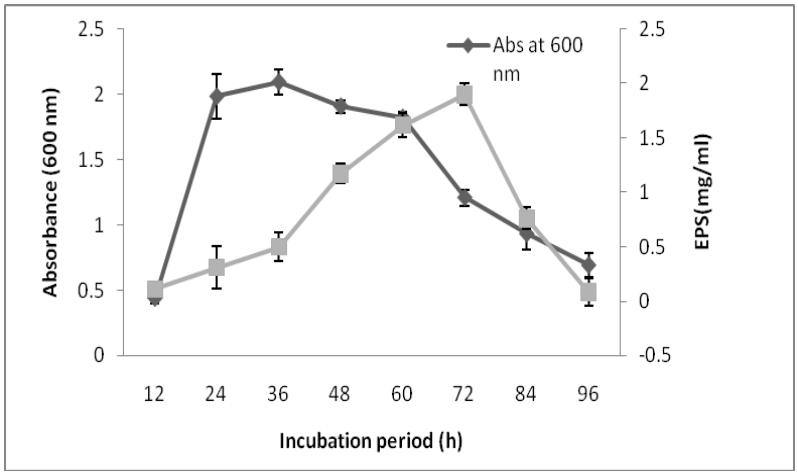


Fig.2 Effect of pH on EPS production by *Paenibacillus* sp.

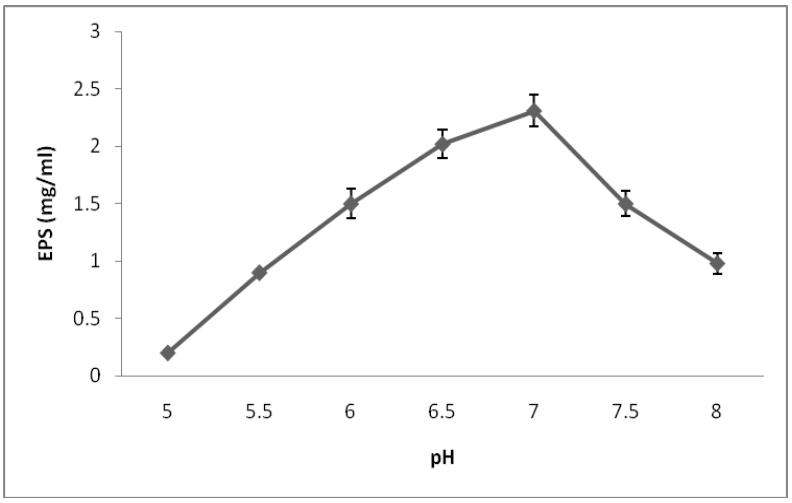


Fig.3 Effect of temperature on EPS production by *Paenibacillus* sp.

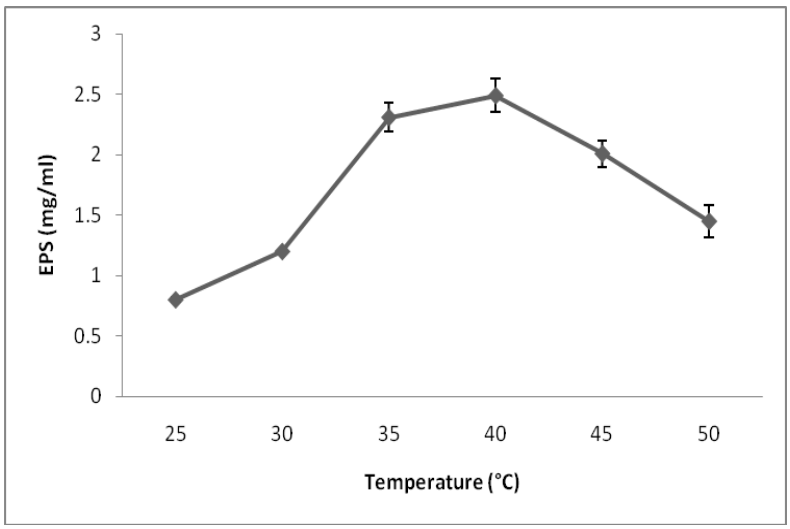


Fig.4 Effect of carbon sources on EPS production by *Paenibacillus* sp.

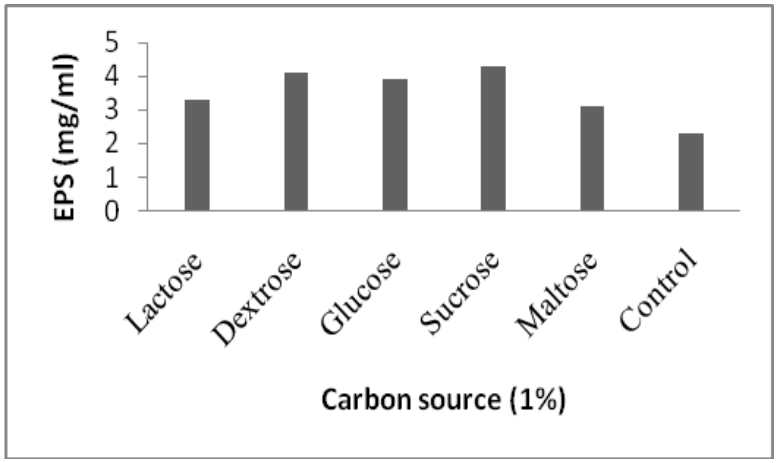


Fig.5 Effect of nitrogen sources on EPS production by *Paenibacillus* sp.

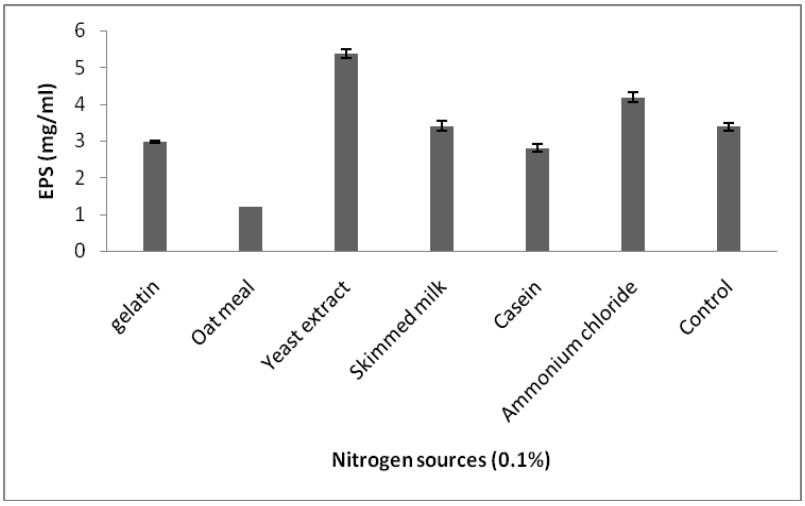
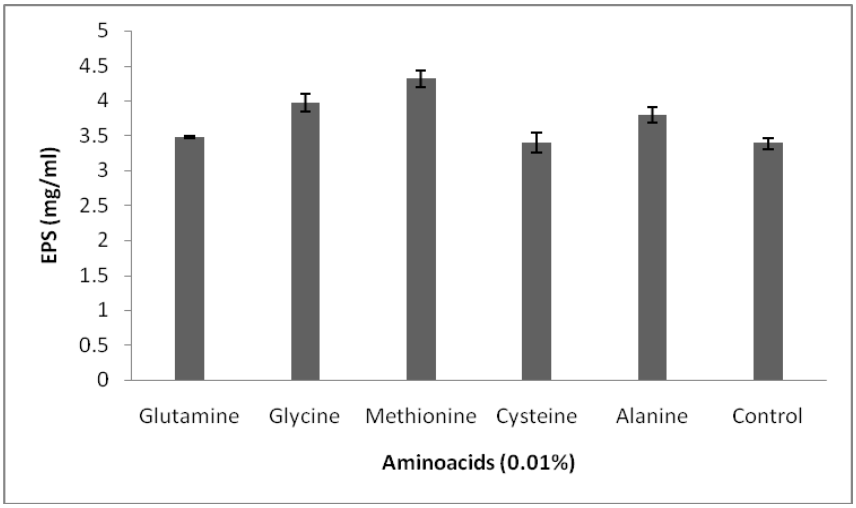


Fig.6 Effect of aminoacids on EPS production from *Paenibacillus* sp.



Yeast extract was found to produce the maximum amount of EPS than inorganic nitrogen sources. Among inorganic sources, NH_4Cl significantly influenced on EPS production. Reports suggest that nitrogen limitation and maximum amounts of carbon in the medium could yield a maximum amount of EPS in the culture medium (Degeest and de Vuyst, 1999). The carbon sources such as, glucose, sucrose were mainly used for the production of EPS (Liu *et al.,* 2009). Many authors have reported that the production of EPS is the culture medium containing carbon and nitrogen sources. In addition, the initial pH of the culture medium and incubation temperature are both important and may significantly affect the uptake of different nutrients, the cell growth, an EPS production (Rafigh *et al.,* 2014).

In the present study, EPS production was found to be maximum in the culture medium containing methionine, followed by glycine. Vitamins showed a significant effect on EPS production. Aminoacids and vitamins are required for growth of bacteria and EPSs formation, and might have a significant role in the cellular metabolism, especially as precursors for EPS synthesis (Grobben *et al.,* 1998). In *B. subtilis* strain 51, the growth and EPS production have been significantly influenced by methionine, isoleucine, leucine, glycine, tryptophan, cystine and alanine (Osadchaya *et al.,* 1997).

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