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RESEARCH ARTICLE

Effective of Different Carbon Concentration on Yield of Extracellular *Polymeric* Substances (EPS) Produced by *Pseudomonas Putida* ATCC 11172

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ABSTRACT

The extracellular polymeric substances (EPS) extracted from *Pseudomonas putida* ATCC 11172 at different carbon concentration, where Luria broth used as medium growth with and without addition of 0.5% w/v (LBG 0.5%) and 1.0% w/v (LBG 1%) glucose. The Bound EPS were extracted by ethylene diamine tetra acetic acid (EDTA) methods and precipitated by cold ethanol 3:1; while free EPS extracted only by precipitate by cold ethanol 3:1. The study shows the clear effect of carbon concentration on the production of EPS. The amount of free EPS found 120.39±2.20, 111.16±1.75 and 100.38±2.0 mg g^{-1} dry cell for LB, LBG 0.5 and LBG 1 respectively. Where the yield of bound EPS shows fewer amounts from free EPS, the amount of bound EPS was 29.22±1.40, 21.18±0.90 and 17.10±1.10 mg g^{-1} dry cell for LB, LBG 0.5 and LBG 1 respectively.

KEYWORDS

Extracellular Polymeric Substances (EPS), *Pseudomonas putida* ATCC 11172

INTRODUCTION

Bacteria secrete extracellular polymeric substances (EPS) to facilitate attachment with each other and to solid surfaces[1,2,3]. EPS is categorized as either free or bound, where the free is released into surrounded media and the bound is attached to cell walls of the bacteria. Cell surfaces interact initially by bound and free EPS at the initial stages of biofilm formation. Extracellular polymeric substances include polysaccharides and proteins as major compounds where the minority is lipids, nucleic acids and other polymers, as will flagella, debris from lysed cells, outer membrane, pili, etc.[4,5,6]. The importance of EPS in the environment is well known. There is great interest in a wide range of applications.

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e.g. protection of drinking water supply from bacterial contamination[7]; bioremediation of oil-contaminated environments[8]; riverbank filtration[9]; in situ bioremediation of contaminated soil[10]; in wastewater treatment[11]; biotechnology used in the food and bioleaching fields due to their wide structural variety[12,13] and biopharmaceutical industries[14].

There is no universal method that can be applied to extract extracellular polymeric substances (EPS) without causing cell lysis[15]. A number of methods have been tested for extracted EPS. Extraction protocols in literature can be divided into three groups: i- physical which consist of the following : centrifuge[16,17,18], heating[19], sonication[20], cationic dowex resin[21,22], crown ether[23]; ii- chemical methods: EDTA[19], NaCl[11], NaOH[19], formaldehyde[11], Sulphuric acid[21,24], glutaraldehyde[25]; and iii- a combination of physical and chemical methods of extraction:

EDTA and centrifuge[21], centrifuge with formaldehyde[26], formaldehyde/ ultra-sonification[27], sonication with cation exchange resin[28], alternating current with EDTA[29]. Gehr and Henry[30] reported that there are five main steps of the EPS extraction:

1. Concentration of biomass and removal of extraneous matter

2. Stripping of the capsule from the cell (chemical stripping, physical stripping)

3. Precipitation., 4. Collection., 5. Purification. The chemical or physical stripping by blending followed by centrifugation was more successful at extracting EPS than chemical stripping or speed centrifugation alone.

The quantity and composition of EPS is strongly dependent upon the extraction method and analytical tools [31]. The procedure of extraction and extraction strategies both have a clear effect on EPS content and compositions. To find an efficient method a number of studies have compared different extraction methods[18,21,25,32,33]. The table 1 shows clear variation in EPS structure depends on used extraction method.

MATERIAL AND METHODS

All chemicals were purchased from Sigma-Aldrich (UK). UHQ water was used throughout this study. All experiments were conducted at least in triplicate, and the average of the results reported. Variation in the experimental results is presented as average ± standard deviation of the mean values. The genus *Pseudomonas* comprises a relatively large and important group of Gram-negative bacteria. Members of this genus are found in abundance as free-living organisms in soils, fresh water, marine environments, and in many other natural habitats. They may also be found in associations with plants and animals as normal flora or as agents of disease[34,35].

Preparation of Bacterial Inoculum

An inoculum of *P. putida* ATCC11172 was prepared from overnight culture cultivated in LB medium containing different concentrations of 0.5% w/v and 1.0% w/v glucose. Cells were washed three times with 0.9% NaCl, by centrifugation at 4000 g (Hermle Z 300 K centrifuge, HERMLE Labortechnik, Germany) for 10 minutes and re-suspended using 50 mL plastic tubes (Sarstedt, Germany).

Table 1: Composition of EPS of *R. Acidophila* by four extraction methods (reproduced from)[19]

Component Mg g^{-1} dry cells	EDTA	NaOH	H_2SO_4	Heating	Control (centrifuge)
Carbohydrate	6.5	7.7	10.6	10.3	4.1
Protein	58.4	126.6	6.2	37.7	6.2
Nucleic acid	5.4	24.9	4.6	23.6	2.6
Total EPS	70.3	159.2	21.4	71.6	12.9
Carbohydrate/protein	0.11	0.06	1.71	0.27	0.66

The inoculum was adjusted to OD 600nm 0.5 and 1 mL of inoculum was applied per 100 mL LB medium in a 500 mL conical flask. In addition, a working stock of *P. putida ATCC* 11172 was maintained in nutrient agar plates at 4ºC. However, long-term maintenance of bacteria was performed by cultivating *P. putida ATCC* 11172 overnight on LB medium containing different concentrations of glucose at 30ºC. 1 mL aliquots were distributed into 1.5 mL microtubes (Sarstedt AG & Co, Nümbrecht, Germany) in sterile conditions and stored at -20ºC.

Glucose Uptake Assay

The amount of glucose present in cell free cultures was analysed using a glucose assay kit (Kit GAGO-20 Sigma, UK). During the growth phase, the cell free cultures were obtained by centrifugation of the cells at 15,000 rpm at 4°C for 15 minutes. The supernatant was filtered through a 0.22 µm cellulose membrane filter. The samples were stored at -20°C. The amount of glucose present in the supernatant was analysed as described by the manufacturer's protocol.

Extracellular Polymeric Substances (EPS) Extraction

Free and bound EPS was extracted using the ethylene diamine tetra acetic acid (EDTA) method[19]. This method showed better results than other extraction methods including heating, alkaline, sulphuric acid and high speed centrifugation, because of high extraction efficiency and low amount of cell lysis[36]. Centrifugation 12,000 rpm at 4^0C for 10 minutes was used to separate cells from their solution. The cell pellets were transferred to 10 ml double-distilled water in 25ml tubes, followed by the addition of EDTA at 4°C, the extraction time was 3 hours (2% EDTA produced approximately 3.2 g g^{-1} dry cells). The supernatant was then recovered after filtration through a 0.22µm cellulose membrane filter. To remove residual cells the supernatant was centrifuged at 12000g for 30 minutes at 4°C. The "free EPS" (from supernatant) and "bound EPS" (from washed cells) were precipitated by adding three volumes of cold reagent-grade ethanol, and stored at -20°C for 18 hours. Crude EPS was collected by centrifugation at 12000 g for 30 minutes at 4°C. To remove ethanol and entrained media residue, the pellet was dissolved in ultra-pure water and dialysed against the ultra-pure water using regenerated cellulose membranes (3500 MWCO)[37] Dialysis was carried out for 72 hours with two changes of ultra-pure water per day then the dialysed EPS was freeze-dried for storage.

Yield of Extracellular Polymeric Substances (EPS)

The yield of EPS was calculated against the amount of one gram of dry cells. The dry cells' weight was calculated from taking the certain weight of wet cells and freeze-dried cells after being washed three times with 0.9% NaCl.

RESULTS AND DISCUSSION

Growth Phase and Effective of Different Concentration of Glucose

The growth phase occurred under batch conditions with two clearly different phases, i.e. exponential phase 3-9hours and the stationary phase after 9hours. Samples of bacteria were taken at different times, 6, 12 and 24 hours, to determine the pH and bacterial growth, which can be used to demonstrate the relative biofilm formation capacity. The final pH value decreased gradually when the glucose concentration increase (Fig. 1). The degradation of glucose may lead to the formation of some acidic compounds, which may decrease the pH of medium. The pH values were 5.9±0.27, 5.4±0.22 and 4.9±0.30 for LB, LBG 0.5% and LBG 1% respectively. Our results are consistent with the results obtained by Jaradat and Bhunia[38] who performed similar experiments on *Listeria monocytogenes* bacteria. The findings showed that the pH decreased with values of 6.59, 6.28, 6.1, 5.26, 4.99 and 4.3 for glucose concentration of 0.0, 0.2, 0.4, 0.8, 1.8 and 3.

The concentration of glucose used as a carbon source by *P. putida* ATCC 11172 under batch conditions at 0.5 and 1 % w/v glucose is shown in Figure 2. Over 95% of the glucose present in the LBG 0.5% media was taken up by the cells

within the first 5 hours of growth with no glucose remaining after 6hours. Vicente et al.[39] observed that *Pseudomonas putida* is capable to uptake of both glucose and gluconate by up to 92% under similar growth conditions. Bacterial biomass was measured as a function of its optical density (OD).

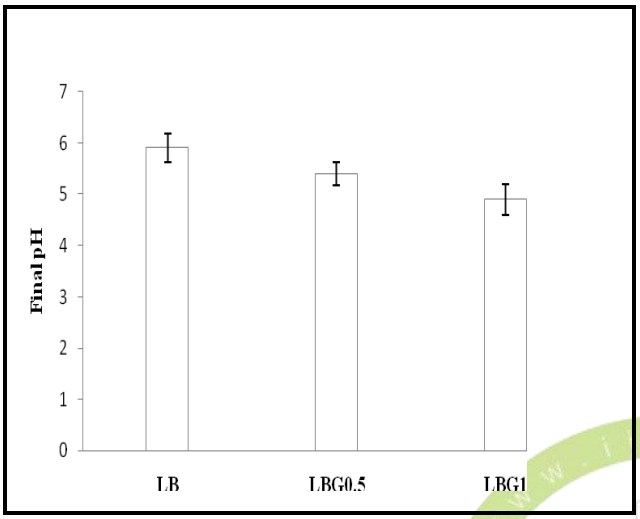

Figure 1: Final pH value of the *P. putida* ATCC 11172 cultivated in LB and LB supplemented with (0.5 and 1 w/v) % glucose

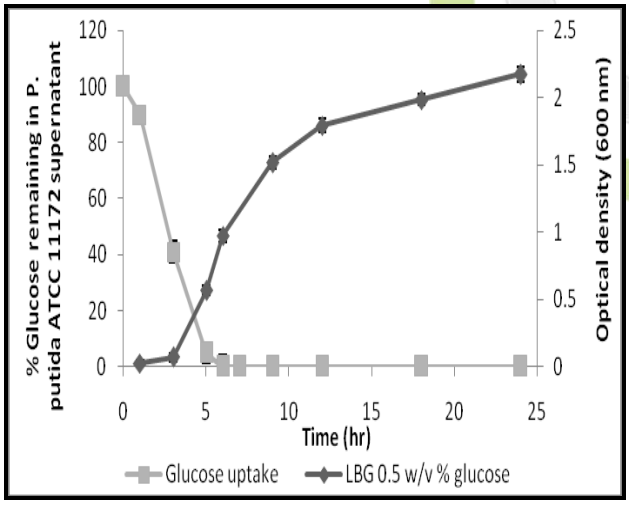

Figure 2: Percentage of glucose uptake during the growth of *P. putida* ATCC 11172 in LBG 0.5 % w/v glucose

The consumption of glucose during *P. putida* grown at 1.0 % w/v is shown in Figure 3. Within the first 5h, the bacteria have used approximately 80% of glucose; and 97% of the glucose present in the LBG 1% media; was taken up by the cells within 7 hours of growth with no glucose remaining after 9hours.

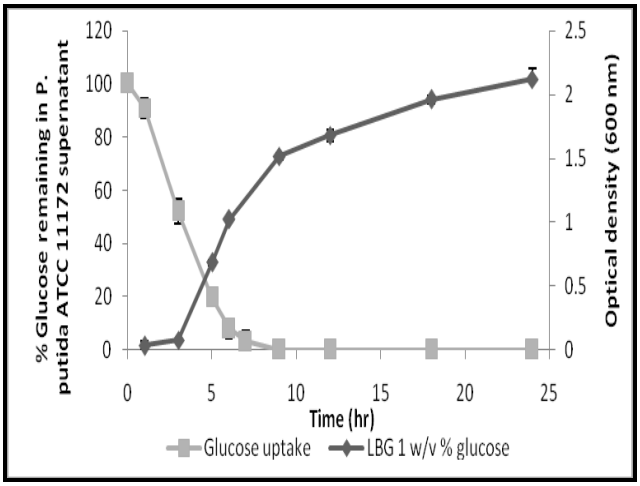

Figure 3: percentage of glucose uptake at the growth phase of *P. putida* ATCC 11172 in LBG1% w/v glucose

Yield of Extracellular Polymeric Substances (EPS)

The amount of EPS produced by *P. putida* ATCC 11172 depend on the amount of glucose added to the medium and due to the effect of the cells growth with an increase for glucose by decreasing the pH value, which in turn reduces the rate of yield of EPS. Figure 4 shows the yield of EPS depending on the carbon concentration added to LB media, where the amount of free EPS found was 120.39±2.20, 111.16±1.75 and 100.38±2.0 mg g^{-1} dry cell for LB, LBG 0.5 and LBG 1 respectively. The amount of bound EPS was 29.22±1.40, 21.18±0.90 and 17.10±1.10 mg g^{-1} dry cell for LB, LBG 0.5 and LBG 1 respectively. Similar results have been observed by Celik et al.[40] who grew two different strains of *P. putida* G12 and *P. aeruginosa* G1in different carbon sources and concentrations where both strains produce a different amount of EPS. Similar results were found by Geogheganet al.[41] who grew *E. coli* MG1655, and observed that the production of EPS decreased with an increase in the concentration of glucose, and attributed that to the glucose effect on the biological activities, governed at the genetic level influencing the cell surface. The results obtained by Cerninget al.[42] were inconsistent with our results; they studied *Lactobacillus sacei* CG11in basal minimum

medium with different sugars and different concentrations, they reported that the yield of the EPS produced by *Lactobacillus sacei* CG11 increased with an increase in the concentration of glucose in medium. They declared that it seems likely that glucose stimulates EPS production. The difference, that the small amount of added glucose in their experiments did not reach the limit, which begins with inhibiting the increased production of EPS.

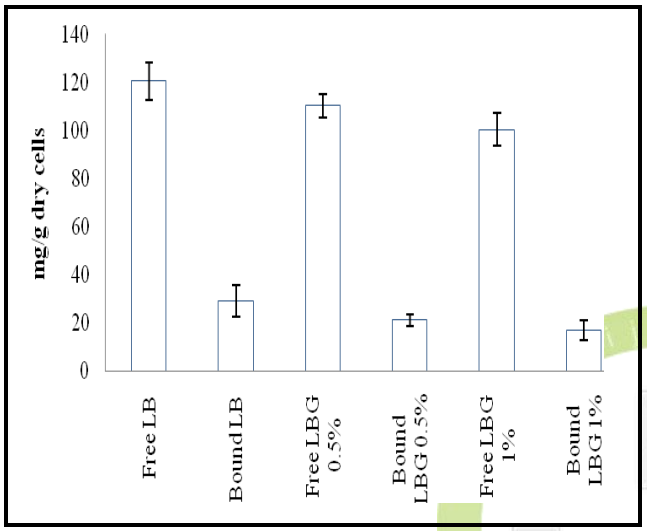

Figure 4: Yield of EPS (free and bound) at different media composition

CONCLUSION

The results showed that the yield of EPS of *P. putida* ATCC 11172 was affected by the concentration of carbon source. Additionally, the increase of glucose concentration resulted on a decrease on the pH value of the solution to a level that may have had an effect on the growth of bacteria. Bacteria grown using different carbon concentrations showed different yield of EPS. The findings provide the foundation for future studies on the effect of different concentrations of carbon source on the structure of EPS.

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