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PRODUCTION OF POLY-γ-GLUTAMATE (PGA) BIOPOLYMER BY BATCH AND SEMICONTINUOUS CULTURES OF IMMOBILIZED *BACILLUS LICHENIFORMIS* STRAIN-R

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ABSTRACT

Production of Polyglutamate (PGA) biopolymer by immobilized *Bacillus licheniformis* strain-R was intensively investigated. Preliminary experiments were carried out to address the most suitable immobilization methodology. Entrapment of *Bacillus* cells in alginate–agar led optimal PGA production (36.75 g/l), with 1.32- and 2.18-fold increase in comparison with alginate- or K-carrageenan-immobilized cells, respectively. During semicontinuous cultivation of agar-alginate gel-cell mixture, production of PGA by 10 ml mixture was increased from 2nd to 3rd run whereas, increased till the 4th run using 15ml mixture. Adsorption was the most suitable immobilization technique for production of PGA and the sponge cubes was the preferred matrix recording 43.2 g/l of PGA with the highest cell adsorption. Furthermore, no PGA was detected when *B. licheniformis* cells were adsorbed on wood and pumice. Although luffa pulp-adsorbed cells recorded the highest PGA production (50.4 g/l), cell adsorption was the lowest. Semicontinuous cultivation of *B. licheniformis* cells adsorbed on sponge led to increase of PGA production till the 3rd run and reached 55.5 g/l then slightly decreased in the 4th run. The successful use of fixed-bed bioreactor for semicontinuous cultivation of *B. licheniformis* cells held on sponge cubes (3 runs, 96 hours/run) provides insight for the potential biotechnological production of PGA by immobilized cells.

Key words: Poly-γ-glutamate, *Bacillus licheniformis*, immobilization, bioreactor, semicontinuous production

INTRODUCTION

Poly glutamic acid (PGA) is one of the few naturally occurring polyamides which are not synthesized by ribosomal proteins (29). It is produced by several *Bacillus* species as an extracellular polymer (6, 8, 14, 19, 20, 24, 26, 31, 40, 45). It serves as structural component in some bacteria such as *Bacillus anthracis* and *Bacillus megaterium* (17, 36, 41). In another bacterial species, e.g. *B. subtilis, B. licheniformis* and *Natrialba aegyptiaca*, PGA excreted into the medium to increase the survival of producing strains when exposed to environmental stresses (16, 18). Interestingly, it is completely biodegradable and nontoxic to human (45). Therefore, applications of γ-PGA and its derivatives have been of

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interest in a broad range of industrial fields such as food, cosmetics, medicine, plastics, oil recovery and water treatment (7, 11, 25, 30, 39).

For a broad application, the cost of bio-products is one of the main factors determining the economics of a process. Reducing costs of biopolymer production by the use of immobilized cells is one of the basic researches for industrial application. Although immobilized cells have received a lot of attention in production of various bio-poroducts, it is not possible to make a general statement about the behavior of microorganisms in gel matrix or adsorbed on support material. The use of immobilized *bacillus* cells for production of a broad range of bioproducts e.g. amylase, CGTase, prednisolone and applying fluidized and fixed-bed bioreactors, has been reported (10, 13, 22, 32, 38). Interestingly, other researchers have shown that polysaccharide biopolymer can be produced by immobilized bacteria, however production of PGA biopolmer by bacteria was rarely investigated. Anselmo *et al.*, studied the production of xanthan gum in immobilized cultures of *Xanthomonas campestris*, the immobilization was held using adsorption on three different vegetables (3). West and Strohfus studied the production of pullulan by sponge-immobilized *Aureobasidium pullulans* ATCC 42023 cells that was used 3 times for pullulan production (44). *Aureobasidium pullulans* cells were immobilized by adsorption on various solid supports and by entrapement in polyurethane foam for repeated reuse in pullulan biosynthesis (28).

The objective of this study was to investigate the possible production of PGA biopolymer by immobilized *Bacillus licheniformis* strain-R. Preliminary experiments have been conducted to address the most suitable immobilization methodology that can be used for PGA production. The choice of the most suitable supporting matrix used for immobilization was also investigated. Special emphasis was given to the semicontinuous production of PGA in fixed-bed bioreactor using *Bacillus licheniformis* cells adsorbed on sponge. To our knowledge, this is the first report provides insight for possible production of PGA biopolymer by application of different immobilization techniques.

MATERIALS AND METHODS

Microorganism

The *Bacillus* strain used throughout this study was isolated from an agriculture farm in ABIS rectorate in Egypt, characterized by previous history of fertilizer's use, as previously described (6). Further morphologically and physiologically characterization by the help of Fermentation Biotechnology and Applied Microbiology (FERM-BAM) Center, Azhar University revealed that the bacterium was closely related to *Bacillus licheniformis*.

Fermentation and PGA production

The bacterium was allowed to grow in 50ml aliquot of nutrient broth medium composed of (g/l): Peptone, 5 and beef extract, 3 and NaCl, 3, dispensed in 250ml Erlenmeyer flask and incubated at 37°C for 12h at 120 rpm. 1% inoculum of the overnight culture was used to inoculate the basal salt production medium of the following composition (g/l): K_2HPO_4; 14, KH_2PO_4; 6, $MgSO_4$; 0.2, ammonium sulfate; 4, glucose; 20 and 2 ml of trace element solution ($FeSO_4.4H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnCl_2$ 1 mM each) at 37°C. For semicontinuous production of PGA in flasks or trickle flow bioreactor, the fermentation medium was decanted at the end of four days incubation period, and fresh medium was added under aseptic conditions.

Growth and PGA determination

During fermentation process growth was estimated as OD at 600_{nm} and PGA was determined in culture supernatants after clarifying cultures by centrifugation. For recovery, PGA biopolymer was precipitated by the addition of 2 volumes of ice-cold ethanol 95% and separated by centrifugation at 5,000 rpm for 20 min in a cooling centrifuge then dried at 60^{o}C to constant weight in a weighed vials (4, 5, 40).

Measurement of relative viscosity

Polyglutamic acid (PGA) is a water soluble highly hygroscopic white powder, leading to highly viscous solutions even at low concentrations (33). In this study, the relative viscosity of the culture filtrate, after cell removal, was measured using a conventional Ostwald viscometer at 30°C (37, 42). The apparent relative viscosity δ (app) was determined as follows:

$$\delta \text{ (app)} = t_s/t_o$$

Where t_s is the falling time of the sample at 30°C and t_o is that of water under the same conditions.

Immobilization of *B. licheniformis* cells by entrapment

Entrapment in Ca-alginate and agar-alginate

Cells were entrapped in 4% calcium alginate gel beads as described by Eikmeier *et al.* (12), four percent gel solution (w/v) was prepared by dissolving 4 g Na-alginate in 90 ml distilled water (for preparation of alginate beads) or 2g agar and 2g alginate (for agar-alginate beads) in 90 ml distilled water, then autoclaved at 108°C for 10 min. Ten ml cell suspension was added to the sterile gel solution. Ten ml of the gel-bacterial cell mixture were drawn with the aid of a sterile syringe, and allowed to drop through a hypodermic needle into a cross-linking solution (100 ml of 2% $CaCl_2$ solution in 250 ml Erlenmeyer flask) to obtain spherical beads (about 3mm in diameter). The beads were left in calcium chloride solution for one hour and then washed several times with sterile distilled water. Beads resulted from 10ml alginate were added to 50 ml sterile medium in a 250 ml Erlenmeyer flask. The flasks were incubated on a rotary shaker (120 rpm) at 37°C.

Entrapment in K-Carrageenan

Cells were entrapped in K-carrageenan under sterile conditions as described by Wada *et al*. (43). About twenty ml cell suspension containing an equal cell inoculum as that used for the preparation of alginate beads were added to 80 ml K-carrageenan solution (3 g/80 ml of the desired concentration) previously autoclaved and kept in water bath at 40°C. The solution was well mixed and 10ml fractions were introduced dropwise with sterile syringe into 100 ml of well-stirred 2% KCl solution. The hardened gel particles (about 3mm in diameter) were isolated from the KCl solution and washed several times with a sterile physiologic solution (0.85% NaCl), then transferred aseptically to 50 ml of the cultivation medium.

Entrapment in agar

The gel was prepared by dissolving 2g agar in 80 ml water. After sterilization, about 20 ml cell suspension was added and mixed well (at about 45.50°C). 10 ml of this mixture were aseptically poured into a Petri-dish. After solidification, the gel was cut with a sterile cutter into small cubes of about 0.5 cm in length that was subsequently transferred to 50 ml of the cultivation medium.

Immobilization of *B. licheniformis* cells by adsorption

Two ml cell suspension was added to the Erlenmeyer flasks containing 50 ml sterilized culture medium and sponge cubes (about 20 cubes), luffa pulp, pumice and wood (about 20 particles). The flasks were incubated in a rotary shaker (120 rpm) at 37°C.

Trickle Flow Column bioreactor

The trickle flow column used in the present work consisted of a glass tubing (75 cm long and 3 cm inner diameter). The column was connected by a quick fit to a reservoir (250 ml conical flask). The column was filled with adsorbent granules for adsorption and 4% cell solution was used to inoculate the support. After adsorption of cells on the packed support-matrix approximately 200 ml of the mineral medium was recycled through the support by the aid of a peristaltic pump at a flow rate of about 120 ml/h or 240 ml/h. The column was also aerated with a sterile air pump from the upper part of the column at a flow rate of about 0.21l/min.

RESULTS

Production of PGA biopolymer by entrapped *B. licheniformis* strain-R cells

Different gel materials namely; Na-alginate, agar-alginate, k-carrageenan and agar were used for immobilization of *Bacillus licheniformis* cells by entrapmet. The gel-cell beads (or cubes) were used to inoculate 50 ml production medium. Results shown in Table 1 indicated that immobilization by entrapment using alginate-agar as the gel material show the highest polygutamate production (36.75 g/l), and relative viscosity (2.2) but lower than those of free cultures (45.8 g/l and 2.4, respectively). Furthermore, application of different levels of gel-cell mixture showed that the optimal PGA production (36.75 g/l) and relative viscosity (2.2) were obtained by the use of 10 ml gel-cell mixture, higher or lower number of beads decreased polyglutamate production and relative viscosity (Figure 1).

Table 1. Effect of different gel matrices on the production of PGA by entrapped *B. licheniformis* strain-R cells.

Gel Material	Optical density at 600 nm	Dry wt of PGA (g/l)	Relative viscosity
Alginate	1.0345	25.31	2
Alginate - Agar	0.991	36.75	2.2
Agar	1.004	27.75	1.8
K-carrageenan	1.310	16.85	1.4
Free cells	3.092	45.8	2.4

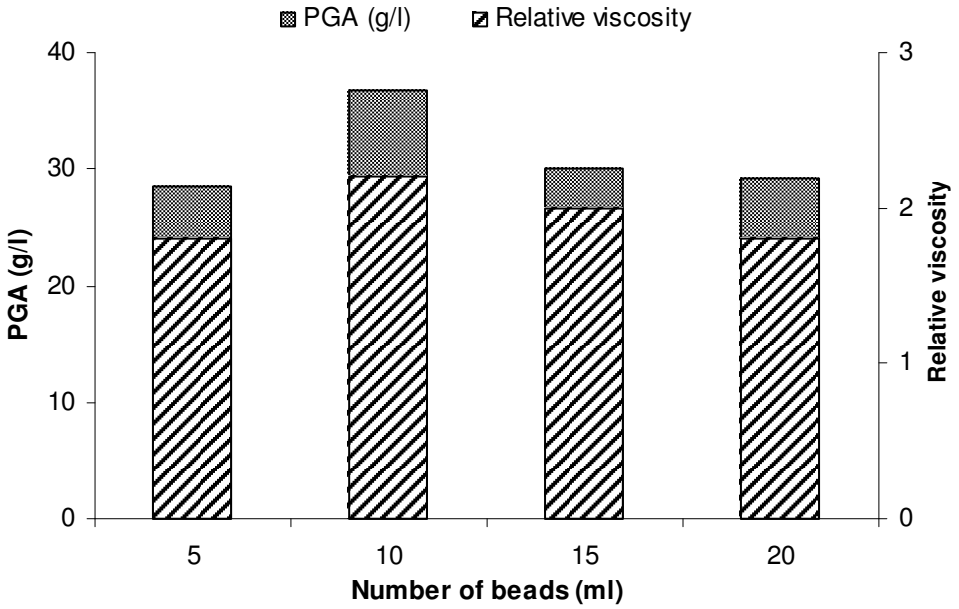

Figure 1. Effect of different alginate-agar beads concentration on production of PGA by entrapped *B. licheniformis* strain-R cells.

Semicontinuous production of PGA by entrapped *B. licheniformis* strain-R cells on alginate-agar beads

The production of PGA by the use of *B. licheniformis* cells entrapped in alginate-agar was investigated using different number of immobilized beads (10 and 15 ml gel-cell mixture). The gel-cell mixture was incubated for 4 days in the production medium. At the end of each reuse, medium was decanted and fresh medium was added. Results in Figure 2 indicated that the reuse of 10 ml gel-cell mixture led to increase in PGA production from the 2nd till the 3rd run and then decreased at the 4th run. On the other hand, PGA production continued to increase till the 4th run by the use of 15 ml gel-cell mixture, however it was still lower than the batch fermentation by free cells.

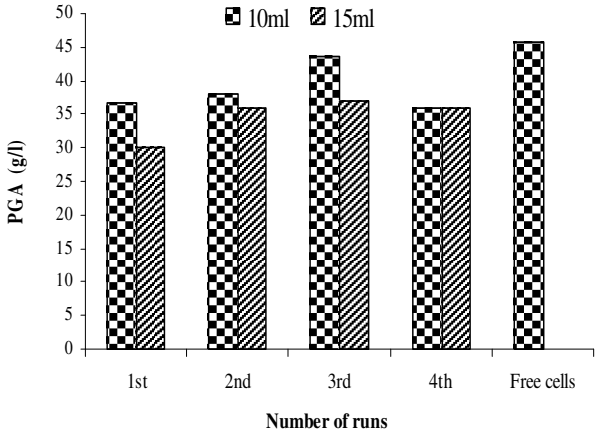

Figure 2. Semicontinuous production of PGA using diffferent concentrations of *B. licheniformis* strain-R cells entrapped on alginate-agar beads.

Production of PGA by *B. licheniformis* strain-R cells adsorbed on different solid supports

In a trial to investigate the possible production of PGA by adsorption technique *B. licheniformis* cells were adsorbed on different solid porous supports such as luffa pulp, sponge cubes, wood pieces and pumice particles. Production medium containing porous supports were inoculated with *B. licheniformis* strain-R cell suspension, at the end of incubation period PGA was determined. Results recorded in Table 2 indicated that cultures containing adsorbed cells on sponge cubes and luffa pulp showed an optimal cell adsorption as well as PGA production. However, PGA was not detected in cultures containing adsorbed cells on wood pieces and pumice particles. Although, cultures containing luffa pulp as solid support exhibited the highest PGA production (50.4 g/l), even higher than that of free cultures, however cultures containing sponge cubes as solid supports exhibited the highest cell adsorption.

Table 2. Effect of immobilization on different solid porous supports on the production of PGA by *B. licheniformis* strain-R.

Immobilization material	Optical density at 600 nm	Dry wt of PGA (g/l)	Relative viscosity
Sponge	1.014	43.2	2.4
Luffa	1.320	50.4	2.6
Wood	0.306	ND	ND
Pumice	2.018	ND	ND
Free cells	3.092	45.8	2.4

Semicontinuous production of PGA by adsorbed *B. licheniformis* strain-R cells

The effect of reusing adsorbed *B. licheniformis* cells on the production of PGA using different solid supports as sponge cubes and luffa pulp was investigated. For this purpose, cultures containing different solid supports were used and after time interval of 4 days for repeated reuse, PGA was determined. Results in Figure 3 show that by reusing cells adsorbed on sponge cubes, a slow cell leakage was observed up to the 4th run (data not shown), while PGA production increased till the 3rd run and reached 55.5 g/l then slightly decreased in the 4th run, however it was still higher than that of batch fermentation cultures by free cells. On the other hand, by reusing cells adsorbed on luffa pulp PGA production decreased in the cultures of the 2nd run and continued to decrease up to the 4th run till it was nearly the same as that of batch fermentation cultures via free cells. The

results collectively indicated the possible semicontinuous production of PGA by *B. licheniformis* cells adsorbe on sponge.

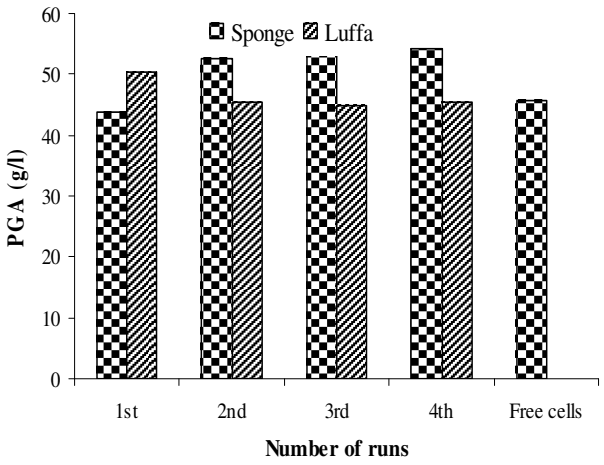

Figure 3. Semicontinuous production of PGA by *B. licheniformis* strain-R cells adsorbed on luffa pulp and sponge.

Cultivation of adsorbed *B. licheniformis* cells on sponge in a trickle flow bioreactor

In a trial to test the possible production of PGA in bioreactor, a trickle-flow column was used in cultivation of *B. licheniformis* cells adsorbed on sponge. About 200ml of the production medium was recycled through the support at a flow rate of about 120ml/h or 240ml/h and the column was incubated at 37^{o}C for 5 days. Results in Figure 4 showed that the optical density decreased gradually during incubation indicating a good adsorption of the cells to the sponge. Also, results showed an increase in PGA production from 24 to 48 h incubation, then slowly increased till 72h followed by subsequent increase with maximum PGA production (31.25 g/l) after 96h of incubation. Subsequent decrease in PGA and relative viscosity was finally recorded. Interestingly, similar growth and PGA production were recorded by increase of the medium flow rate during feeding process to 240 ml/h. Polyglutamate production reached 45 g/l after 96h incubation i.e.1.44-fold as compared with PGA produced applying flow rate of 120ml/h at the same incubation period.

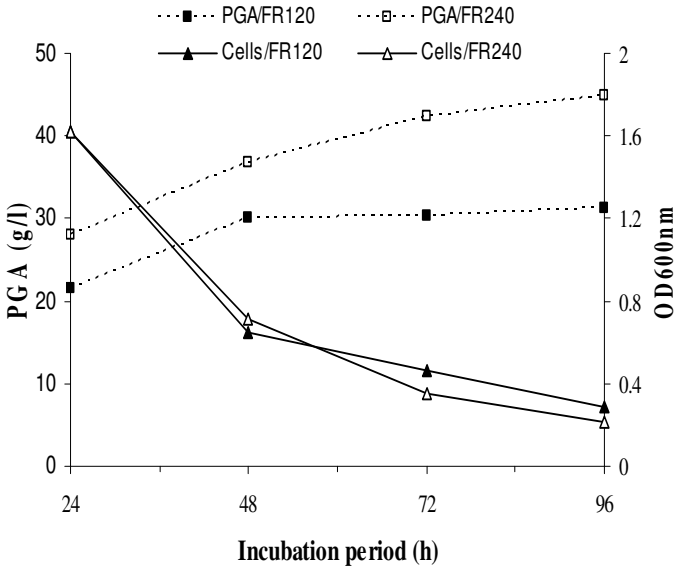

Figure 4. Production of PGA by *B. licheniformis* cells adsorbed on sponge and cultivated in a trickle flow bioreactor applying two different flow rates.

Semicontinuous production of PGA in the trickle flow bioreactor

In order to investigate the possible reduction in costs of PGA production, *B. licheniformis* cells adsorbed on sponge cubes were cultivated in a trickle flow column in semicontinuous manner. For this purpose, cells were cultivated in the production medium and reused after a time interval of 5 days. Samples were taken every 24h to trace the amount of PGA produced. Results shown in Figure 5 indicate that the optical density showed a gradual decrease during the incubation period of each run reflecting adsorption of *B. licheniformis* cells on the support. On the other hand, PGA production showed a gradual increase for each run till 96h of incubation, and decreased after wards (data not shown). Also, the results clarified that there was an increase in polyglutamate production and relative viscosity (data not

shown) till the 3rd run, in which the polyglutamate production and relative viscosity reached 37.5 g/l and 2.4, respectively.

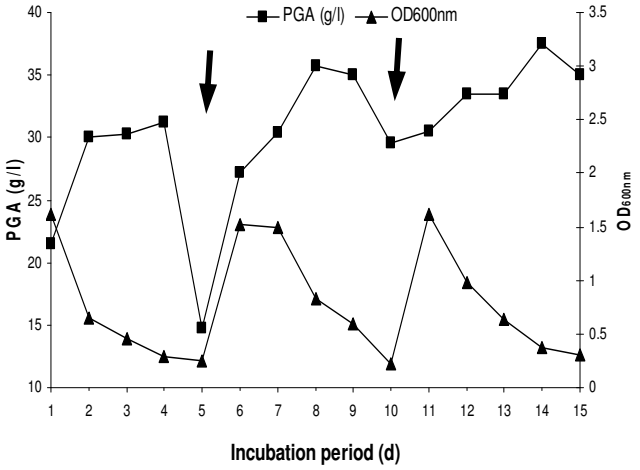

Figure 5. Semicontinuous production of PGA by *B. licheniformis* cells adsorbed on sponge and cultivated in the trickle flow bioreactor. Arrows representing the decantation of used medium and feeding with fresh medium.

DISCUSSION

In this study, the production of a useful biodegradable polyamide biopolymer, polyglutamate (PGA), by immobilized *Bacillus licheniformis* strain–R was closely investigated. In practical utilization of bacterial cells entrapped in gel matrix, diffusion of essential nutrients, oxygen transfer, physical and chemical properties of the gel and immobilization procedure are the important factors affecting microbial metabolism and effeciency of the system. It was found that PGA production reached its maximun yield (36.75 g/l) after 96 h when using 10 ml agar-alginat gel beads mixture. This result is in concordance with the finding of Shih *et al*. (39) and Berekaa *et al*. (6), where the maximum PGA production during batch fermentation using free cells was obtained after 96h. The results also revealed the possible production PGA biopolymer by entrapped cells. However, the fluctuation in the amount of PGA produced by *Bacillus* cells entrapped in various gel matrices as well as during the use of different gel beads concentrations attributed to variation in aeration and diffusion of nutrient in the immobilized cell system (9, 27, 34, 35). Interestingly, polysacchrides biopolymers were successfully produced by entrapped microbial cells. Indeed, immobilization of *A. pullulans* by entrapement in polyurethane foam was investigated and immobilized cells could be repeatedly used for pullulan biosynthesis (28). Measurement of relative viscosity during biopolymer production can be an important factor of a successful bioprocess. Furthermore, the change in medium viscosity during fermentation by *B. licheniformis* cells is attributed to the production of PGA of different molecular weights. Berekaa *et al*. (6) reported that the produced PGA polymer by *B. licheniformis* strain-R during batch fermentation by free cells is in the range of 80 to 180 Kda. The result is supported by the finding that the PGA from bacilli are usually produced as mixture with molecular weights ranging from 10 to 1000 Kda, due to PGA depolymerases activity (2). Generally, the final molecular weight of PGA is dependent on many factors, and can decrease as fermentation time increases, owing to enzyme that catalizes the hydrolytic breakdown of PGA (15, 23).

Given the industrial importance of Bacilli, we examined semicontinuous production of PGA by adsorbed *B. licheniformis* strain-R cells in more details. Adsorption was the most suitable immobilization technique for production of PGA biopolymer and the sponge cubes was the preferred matrix recording 43.2 g/l of PGA with the highest cell adsorption. Moreover, no PGA was detected when *B. licheniformis* cells were adsorbed on wood and pumice. Although luffa pulp-adsorbed cells recorded the highest PGA production (50.4 g/l), cell adsorption was the lowest. The use of adsorbed cells in production of biopolymer was investigated by several scientists. *Aureobasidium pullulans* was immobilized on various solid supports in a liquid medium as diatomaceous earth, fiber glass mat, cellulose fiber mat, ceramic, polyurethane foam for polysaccharide production. The immobilized cells could be repeatedly used

for pullulan biosynthesis (28). Furthermore, application of fed-batch cultivation of *Bacillus* cells adsorbed on sponge led to increase of production till the 3rd run and reached 55.5 g/l then slightly decreased in the 4th run. In concordance with these results, *B. licheniformis* ATCC 9945a produced 35g/l PGA during fed-batch cultivation of free cells in 2.5L bioreactor with pulsed feeding of citric acid and glutamic acid (45). Similarly, remarkable increase in PGA production was recorded by Abdel-Fattah and his co-workers (1). They managed to optimize PGA production by free cells of *B. licheniformis* SAB-26 strain from 33.5 g/l to 89 g/l by fed-batch and pulsed feeding experiments. Interestingly, West and Strohfus (44) studied the production of pullulan by sponge-immobilized *Aureobasidium pullulans* ATCC 42023 cells, the immobilization was held on sponge cubes in media containing either corn syrup, sucrose or glucose as a carbon source, the cells could be used 3 times for pullulan production. Immobilized cells revealed the highest polysaccaride levels in the culture medium after 5-7 days of growth at 30°C. Indeed, Anselmo *et al*. (3) studied the production of xanthan gum in immobilized cultures of *Xanthomonas campestris*, the immobilization was held using adsorption on three different vegetables.

The practical use of trickle flow bioreactor for semicontinuous cultivation of *Bacillus* cells held on sponge cubes (3 runs for 96 hours/run) for PGA poduction was successfully established in this work. There was an increase in polyglutamate production and relative viscosity till the 3rd run, in which the polyglutamate production and relative viscosity reached 37.5 g/l and 2.4, respectively. The successful use of immobilized *B. cereus* cells employing packed- or fixed bed bioreactor to continuously synthesize several bioproducts of industrial interest was reported by many working groups (22, 32, 38). Finally, results obtained in this study provide insight for the successful cultivation of *B. licheniformis* cells in trickle flow bioreactor and reflects the potential biotechnological production of PGA by immobilized cells.

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