

NOVEL POLYMER-BASED Ca-ALGINATE MICROBIAL ENCAPSULATION WITH CHITOSAN COATING FOR DEGRADATION OF HIGH CONCENTRATION OF BISPHENOL A

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Abstract

*This paper represents our finding on new formulation of Ca-alginate beads preparation to support cell survival in the BPA biodegradation by extracellular laccase from *Pseudomonas aeruginosa* NR.22. The microencapsulation of the cells in Ca-alginate coated with 0.40% (w/v) chitosan has proved to enhance better survival in high BPA concentration (1200 ppm) with high activity and stable laccase production with BPA degradation over 80%. The coating has sized up the beads diameter to $116 \pm 0.01 \mu\text{m}$ from $98 \mu\text{m}$ (without the chitosan coating). The specific growth (μ) of the *Pseudomonas aeruginosa* NR.22 in the Ca-alginate was 0.19 hour⁻¹ suggesting that wellbeing of cells was excellent. The microcapsule yield (EY) was recorded as $94 \pm 0.01\%$ proved that the bead's mechanical strength has been enhanced. Scanning Electron Microscope (SEM) resulted in very smooth non-pitting surface of Ca-alginate beads with chitosan coating. The coating process has increased the mechanical strength by 40% compares to non-coated beads which have ruptured slowly after 120 min. We are looking forward to research the mechanical properties of Ca-alginate over the residual stress and deformation of beads structure as an expansion of our academic report.*

Keywords: Microbial coating; Biomaterial surfaces; *Pseudomonas aeruginosa* NR.22; Microencapsulation; chitosan Coating

Introduction

Alginate, a linear heteropolysaccharide composed of β -D-mannuronic acid and α -L-guluronic acid [1] is a naturally occurring polyanionic linear biopolymer that has been widely used in various applications such as biomedical, pharmaceutical, textiles and extensively used in wound dressings because of its low toxicity, biocompatibility and relatively low cost. Alginate beads, as described by Rathore *et al.* [2] are used for protection against harsh surrounding features and to retain viability of encapsulated microbial cells against adverse environmental conditions such as changes in temperature, pH and pressure, damaging metabolic products and osmotic stress. Alginate encapsulation of *Pseudomonas* species with chitosan by using calcium chloride has been enhancing the ability of the microbial cell to degrade phenol. Based on findings reported by Thanh Le *et al.* [3], alginate beads have been used to treat a highly toxic- polluted wastewater where laccase is immobilized in core-shell beads containing alginates and iron oxides. Chitosan, a derivative of chitin which composed of β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine has a vast range of application and uses in many areas such as pharmaceutical, cosmetic, medical, food, textile, agricultural applications and

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medical. Chitosan coats improve stability of alginate microspheres and subsequent improvement in the viability of the encapsulated cells. It contains probiotics to reduce porosity of alginate beads that will eventually decrease cell leakage from the microspheres [2]. Encapsulation of bacteria in calcium alginate beads is a must study system for immobilization and protection of cells [4]. This paper focuses on encapsulation of *Pseudomonas aeruginosa* NR.22 with chitosan coating to degrade bisphenol A (BPA), which is widely used as monomers in polymer industry such as production of epoxy resins, polycarbonates, phenol resins, polyacrylates and polyesters [5]. After the encapsulation of the strain in the alginate beads, various changes to the strain characteristics were analyzed and recorded.

Material and Methods

Reagent, chemicals and pH. All material and chemicals were collected from various sources and quality as listed. Bisphenol A (BPA, R&M Chemicals, 98%), bacteriological peptone (ultrapure, protein = $N \times 6.38 \geq 76.5\%$), ammonium phosphate monobasic (79546, $\geq 98\%$, Sigma-Aldrich), glycerol (Merck), *Pseudomonas* agar (Microbiology), nutrient agar (Merck), nutrient broth (Merck), potassium dihydrogen phosphate (KH_2PO_4 , V90004, Vetec™ reagent grade, 99%), sodium hydroxide (NaOH, Fluka), yeast extract (Merck Milipore, granulated, for microbiology), 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich), chitosan (low molecular weight, Sigma-Aldrich 448869), sodium chloride (NaCl, Sigma), sodium alginate (Sigma-Aldrich, W201502), calcium chloride (granular, ≤ 7.0 mm, $\geq 93.0\%$, Sigma-Aldrich), glucose (Sigma-Aldrich, CAS Number: 50-99-7), peptone solution (Sigma-Aldrich, 42587), glacial acetic acid (Sigma-Aldrich, $\text{CH}_3\text{CO}_2\text{H}$),

Preparation of cell suspension and laccase assay. *Pseudomonas aeruginosa* NR.22 (Ps.NR.22); a Gram negative-rod shaped bacterium was isolated from a lake in Shah Alam, Selangor, Malaysia [5]. Lyophilized cells were inoculated in growth medium containing 1.25% (w/v) glucose, 1.25% (w/v) yeast extract and 0.80% (w/v) NaCl [6] for 24 h under aerobic condition at 37°C, in 1200ppm Bisphenol A (BPA) and biomasses were harvested by centrifuging at 4000 rpm for 10 min at 4°C. The cultures were washed twice by sterile saline solution (0.9%) and used in the microencapsulation process. The laccase enzyme was assayed by using method of *Niku-Paavola et al.* [7]. Laccase activity in the BPA oxidation was determined spectrophotometrically at 465 nm with 5ppm BPA as a substrate in a reaction mixture containing 50mM phosphate buffer (pH = 6.5) ($\epsilon_{465} = 48,000\text{M}^{-1}\text{cm}^{-1}$). One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.001 units per min at 37°C; the activities were expressed in U/ml. All assays are repeated thrice to gain the mean \pm sd of three applications.

Microencapsulation and chitosan coating. The microencapsulation procedure was modified from *Donthidi et al.* [8] and *Sultana et al.* [9]. Alginate beads were prepared by adding 200mL calcium chloride 0.1M into a mixture and were allowed to stand for 30 min. Calcium alginate beads were harvested and kept in 0.1% peptone solution at 4°C. Initial cell concentrations of the *Pseudomonas aeruginosa* NR.22 was study from 4% to 10% (v/v). Size and morphology of the microcapsules were studied using Scanning Electron Microscope (SEM) using method by *Nik Raikhan* [6]. For chitosan coating, aqueous solution of chitosan was prepared using modification of method by *Krasaekoopt et al.* [10]. The chitosan powder was dissolved in 100mL of distilled water (acidified with 1% glacial acetic acid) to prepare a final chitosan concentration of 0.40% (w/v). This was followed by pH adjusting to 6.5 using 1.0M NaOH. The solution was then filtered through a filter paper (Whatman No. 41) before autoclaving at 121°C for 15 min. An amount of 15g of washed microcapsules of alginate beads containing bacterial cells were immersed in 100mL of sterile chitosan solution and shaken at 100rpm for 40min on an orbital shaker for coating process to take place. The chitosan-coated

microcapsules were washed and kept in 0.10% peptone solution at 4°C.

Viability of entrapped bacteria test and encapsulation yield. The solution was stirred on a shaker for 15min vigorously. The counts (CFU/g) were determined by plating the solution on *Pseudomonas* agar plates and incubating for 24h at 37°C. The viability of microencapsulated cells in sterile sodium chloride solution (0.5%, w/v) was also measured at 4°C for three months. The microcapsules were dissolved in the phosphate buffer solution and were used to determine the total number of viable cells [11]. Encapsulation yield (EY) is defined as the number of bacterial cells that survived the process and encapsulated inside the microcapsules. It was calculated using the following equation.

$$EY = (N/N_0) \times 100 \quad (1)$$

Where N_0 is the number of viable bacteria in CFU/mL of culture and N is the number of viable bacteria in CFU/g of microcapsules. For Ca-alginate mechanical strength, studies on maximal deformation, deformed beads size, percentage of maximal deformation and effect of shaking rates to time of maximal deformation to achieve has been performed.

Results and Discussion

Shape and size of microcapsules and encapsulation yield. In this study, the Scanning Electron Microscopy (SEM) has showed that the shape of all chitosan coated alginate bead microcapsules was generally almost spherical and uniformly produced. The non-coated calcium alginate shared the same shapes, but the surface structure was viewed to show lots of holes and pits (Fig. 1B). Chitosan coating has significantly changed and modified the surface morphology of alginate beads (Fig. 1A). The size of calcium alginate beads was maintained at 0.1mm for all the non-coated species but coating has increased the diameter of the beads (Table 1). Significant results were reported by study of beads surface by *Mokarram et al.* [11] and has proved that coating will increase microencapsulation yield and sizes.

Table 1. Microcapsule size and microencapsulation yields of coated and non-coated microcapsules

Alginate Beads	Microcapsule yield (EY, %)	Microcapsule size (μm)
Non-coated calcium alginate beads without bacterial cells	-	92 \pm 0.01
Chitosan-coated calcium alginate beads without bacterial cells	-	111 \pm 0.01
Non-coated calcium alginate beads with bacterial cells	90 \pm 0.02	97 \pm 0.03
Chitosan-coated calcium alginate beads with bacterial cells	94 \pm 0.01	116 \pm 0.01

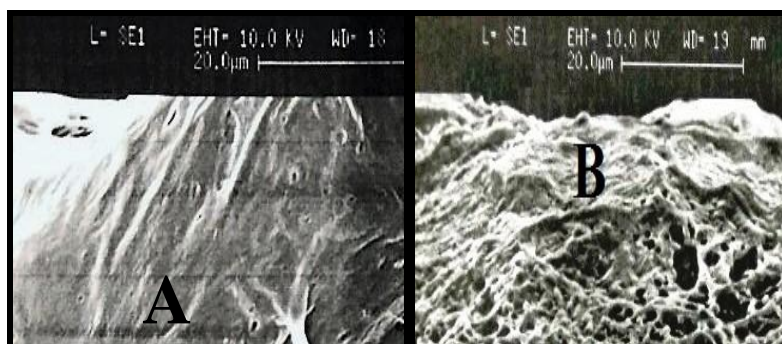


Fig. 1. Chitosan-coated calcium alginate surface focus with well covered area (A). The non-coated calcium alginate surface focused with lots of holes and pits (B).

Study on cell survival in alginate beads. Figure 2 shows the percentage of cell survival among free cells, microencapsulated cells in alginate beads and microencapsulated cells in alginate beads coated with 0.40% (w/v) chitosan using initial cell concentration of 5% (v/v). On the first 30 min, the 100% cell survived excess of BPA in the chitosan coated beads, which was not recorded with free cells or non-coated alginate. In all the time length studied, chitosan coating has increased cell survival rate. The specific growth (μ) of the *Pseudomonas aeruginosa* NR.22 was $0.19h^{-1}$. BPA degradation was recorded increasing with time, with maximum degradation achieved at 180 min.

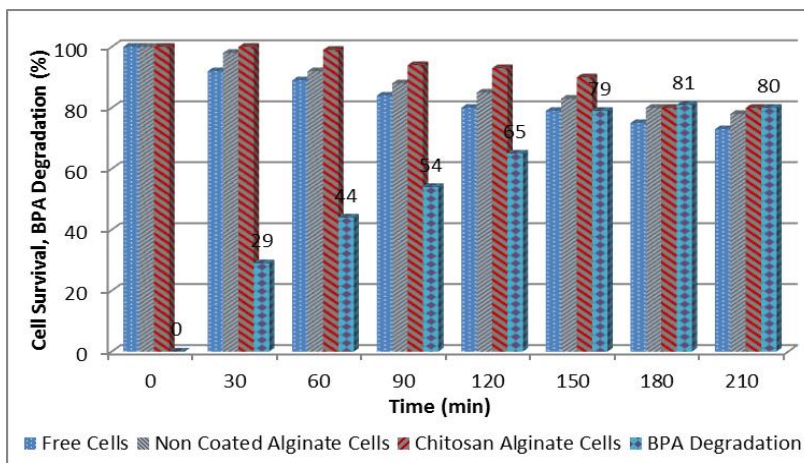


Fig. 2. Percentage of cell survival of free cells, microencapsulated cells in alginate beads and microencapsulated cells in alginate beads coated with 0.40% (w/v) chitosan using initial cell concentration of 5% (v/v), with percentage of BPA degradation.

Effect of initial cell concentrations to Ca-alginate bead strength. Cell survival in non-coated Ca-alginate beads and chitosan coated using 4-10% (v/v) initial cell concentrations is depicted in Figure 3. The study was done in 60 min, using 1200 ppm BPA. Results showed the BPA degradation to take place excellently. Laccase activity was recorded high and was very significant to the growth and balance of BPA in ppm. Table 2 summarized the findings. In Fig. 3, all cell concentration has recorded very high survival rate with values from 90% to 99%. Cell concentration between 4-8% (v/v) was suitable for chitosan coated Ca-alginate with maximum recorded at 5% (v/v). The non-coated Ca-alginate has 12% lower cell survival rate (Fig. 3). The cell survival rate was documented as significantly low with 9% and 10% (v/v) cells ($65\pm0.01\%$ and $40\pm0.001\%$, respectively), which was totally contrasting in the coated beads ($93\pm0.001\%$ and $90\pm0.001\%$, respectively).

Ca-alginate mechanical strength. The results of destruction characteristics of alginate beads are summarized in Table 2. The time to maximal deformation of beads was recorded as 120 min for the non-coated beads and 360 min when coating. This is suggesting that destruction of the material is depending on the coating as well as the shear load applied using the cells concentrations and the shaking rates. Chitosan coated beads has been proved to give better mechanical strength and long-lasting gel structure even at high cell loading. Maximal deformation has been recorded significant to the deformed beads diameter (see Table 2). This result has followed Stress Relaxation Model for beads deformation by *Kostov et al.* [12].

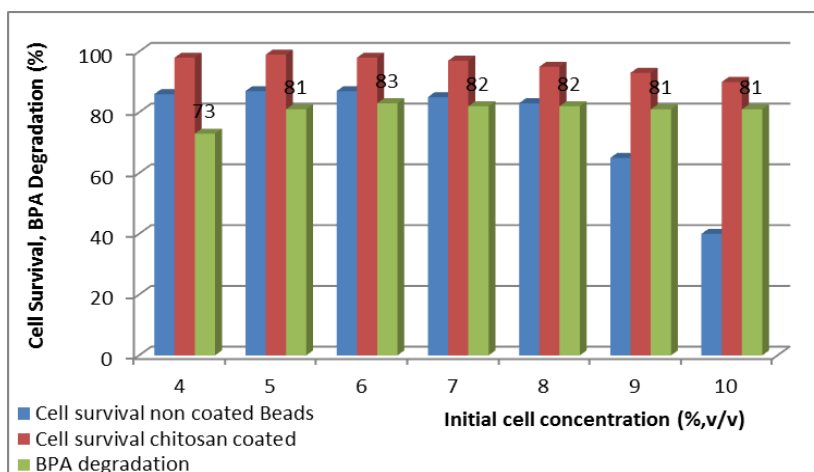


Fig. 3. Cell survival (%) in alginate beads of non-coated and 0.40% (w/v) of chitosan coated using 4-10% (v/v) initial cell concentrations in 60 min of BPA biodegradation, reported in percentages (%).

Table 2. Destruction characteristics of Ca- alginate beads containing 8% (v/v) cells

Ca-alginate	Time to maximal deformation	Deformed beads diameter/percentage of maximal deformation	Effect of shaking rates to time of maximal deformation
Non coated	120 min	45 μm/50%	200 rpm/90 min, 250 rpm/80 min
Chitosan coated	360 min	88μm/79%	200rpm/300 min, 250 rpm/250 min

Conclusion

Microencapsulation of *Pseudomonas aeruginosa* NR.22 in calcium alginate with chitosan coating resulted in better survival of cells in the harsh condition of very high BPA concentration introduced to the biodegradation system with high rate of BPA degradation. Chitosan coating has enhanced the mechanical strength and elastic properties of the alginate beads even at with very high initial cell concentrations suggesting that the applied approach may have proved chitosan stabilized the beads from deformation and residual stress.

Acknowledgment

The authors gratefully acknowledged the Acculturation Grant Scheme from Ministry of Education, Malaysia and FKK, UiTM for the funding of research. Humongous thanks go to the team who has been supporting this work.

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Received: September 19, 2017

Accepted: June 09, 2018