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Research Article

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Influence of Entrapment Technique and the Production of Xanthan on Repeat Batch Fermentation Process

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ABSTRACT

The purpose of this investigation was to study the effect of Xanthomonas campestris cells immobilized in various matrices, such as calcium alginate, k-Carrageenan and ployacrylamide, for the production of xanthan. Calcium alginate was found to be an effective and suitable matrix for higher xanthan productivity compared to the other matrices studied. Calcium alginate matrix was selected for repeated batch fermentation. The average specific volumetric productivity with calcium alginate was 69g/l, which was the higher production over the conventional free-cell fermentation. In the repeated batch fermentations of the shake flasks, an optimum level of xanthan production was maintained for 4 days using calcium alginate immobilized cells. From the results, it is concluded that the alginate immobilized cells of X. campestris can be proposed as an effective biocatalyst for repeated usage for maximum production of xanthan.

Keywords: *Xanthan production, X. campestris, immobilized cells, repeated batch fermentation.*

INTRODUCTION

Xanthan is the most commercially accepted microbial polysaccharide that is the subject of numerous studies. Xanthan exhibits three desirable properties: 1.high viscosity at low concentrations; 2.pseudoplasticity; and 3.insensitivity to a wide range of temperature, pH, and electrolyte concentrations. Because of its special rheological properties, xanthan is used in food, cosmetics, pharmaceuticals, paper, paint, textiles, and adhesives and otherwise in the oil and gas industry [1] as a stabilizing, viscosifying, emulsifying, thickening and suspending agent[2]. However, because of its high production costs, xanthan loses some markets to polymers from algae or plants [3,4]. Inorder to reduce production costs and to improve the competitive position of xanthan, its productivity should be increased [4]. Microbial products are usually produced either by free or immobilized cells. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process[5].

Whole cell immobilization by entrapment is a widely used and simple technique. Romo and Perezmartinez [6], reported the viability of microbial cells over a period of 18 months under entrapped conditions and it was considered as one of the potential applications. The success achieved with the entrapment technique prompted to study the production of xanthan with immobilized cells using this technique.

The purpose of the present investigation was to study the immobilization of *Xanthomonas campestris* cells for higher xanthan production using different entrapment techniques with matrices such as calcium

alginate, k-Carrageenan and polyacrylamide gel. The reusability of immobilized cells for xanthan production under repeated batch fermentation conditions was also investigated.

MATERIALS AND METHODS

Bacterial strains and host plants: Bacteria used in this investigation consisted to pathovars of *Xanthomonas campestris* which were pathogenic to most of the crucifer plants. The strain was isolated from infected leaves of *Brassica oleracea var. capitata* (cabbage). Isolated colonies were submitted to routine bacteriological tests and used as the parent strains. The stock cultures were maintained on Yeast Malt (YM) agar slants at 4^0c, and transferred every 14 days to maintain good viability and stability for xanthan production.

Inoculum Preparation: Five milliliters of sterile distilled water was added to a 24-hours old slant of *X. campestris*. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension at 10% level was transferred, asceptically into 250-mL Erlenmeyer flasks containing 45 mL of sterile YM broth (pH 7.0). The flask was kept in a shaker incubator at 220 rpm at 37ºC for 24 hours. The content of the flasks was centrifuged at 3000 rpm for 10 minutes and the supernatant was decanted. The cell pellet was washed thoroughly with sterile 20.0 g/L potassium chloride solution, followed by sodium chloride solution and sterile distilled water subsequently. Finally the cell mass was suspended in sterile sodium chloride solution (9.0 g/L). This cell suspension was used as inoculum for immobilization as well as for free-cell fermentations.

Whole Cell Immobilization by Entrapment in sodium alginate: The alginate entrapment of cells was performed according method of Johnsen and Flink (1986), Sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100 mL boiling water and autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension (equivalent to 0.03 g dry cell weight [DCW]) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added dropwise into 0.2 M $CaCl_2$ solution from 5-cm height and kept for curing at 4°C for 1 hour. The calcium ions react with alginate to form calcium alginate. The cured beads were washed with sterile distilled water 3 to 4 times. When the beads were not being used, they were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow unit.

Immobilization of Whole Cells in k-Carrageenan: k-Carrageenan (4%) was weighed and added to 18 mL of 0.9% sodium chloride. It was dissolved by gentle heating and sterilized by autoclave. The cell suspension (2 mL equivalent to 0.03 g DCW) was added to the molten k-Carrageenan solution maintained at 40°C, mixed well, and poured into sterile flat bottom 4-inch-diameter petriplates. After solidification, the k-Carrageenan blocks were cut into equal size cubes (4 mm^3) and added to sterile 2% potassium chloride solution and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water [9].

Immobilization of Whole Cells in Polyacrylamide: A cell suspension was prepared by adding 0.03 g cells to 10 mL chilled sterile distilled water. To another 10 mL of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: 2.85 g acrylamide, 0.15 g bisacrylamide, 10 mg ammonium persulphate, and 1 mL TEMED (NNN^1N^1 tetra methyl ethylene diamine). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter petriplates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm^3), transferred to 0.2 M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until use.

Production of xanthan by Batch Process With Immobilized Cells: The immobilized beads/blocks (cells equivalent to 0.03 g DCW) were transferred into 50 mL of production medium g/l (Glucose 20.0,Yeast extract 3.0, $MgSO_4$ 0.2, K_2HPO_4 5.0, pH 7.2) in 250-mL Erlenmeyer flasks. The flasks were incubated at 37°C for 96 hours. Samples were withdrawn every 24 hour and analyzed for concentrations of biomass and xanthan.

Production of xanthan by Repeated Batch Process: One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously. Therefore, the reusability of *X. campestris* cells immobilized in matrix was examined. After attaining the maximum production of xanthan (72 hours), the spent medium was replaced with fresh production medium (50 mL) and the process was repeated for several batches untill the beads/blocks started disintegrating.

Analytical Methods

Biomass estimation: Growth in the medium was estimated by measuring the dry weight of washed cell mass. 5ml broth was separated in a centrifuge at 10,000 rpm for 15 minutes. The supernatant was decanted, pellet washed in deionized water and dried to a constant weight at 80-100^{0}c.

Xanthan estimation: The polymer was recovered from the fermentation medium by centrifugation of 5ml broth at 10,000 rpm for 15 minutes. The pellet was decanted and the supernatant was precipitated with 2 to 3 volumes of isopropyl alcohol with shaking to precipitate out the polysaccharide. The precipitate was separated by centrifugation at 6000 rpm for 15 minutes. The residue was transferred to pre-weighed aluminium foil cups and dried for 18 hours in hot air oven at 60^{0}C. The cups were cooled to 30^{0}c for 1 hour and the dry weight gave the xanthan concentration of the fermented broth.

RESULTS AND DISCUSSION

Production of xanthan with Immobilized Cells in Various Matrices by Entrapment Techniques: Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not least, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed [7].

Production of xanthan by Immobilized Cells in Calcium Alginate: The amount of cell mass entrapped in calcium alginate matrix increased gradually up to 72 hours of incubation after which there was no appreciable change (Figure 1), whereas with free cell fermentation gradual cell growth was observed up to 72 hours followed by decline phase (Figure 2).

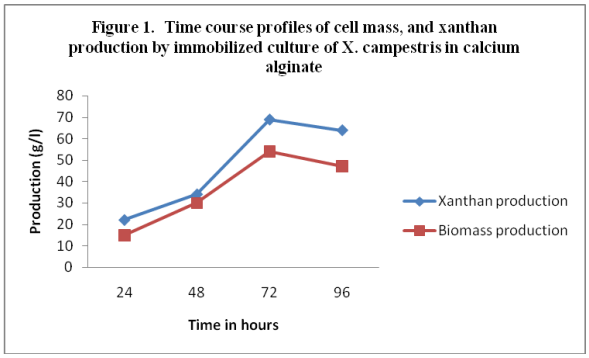


Figure 1. Time course profiles of cell mass, and xanthan production by immobilized culture of X. campestris in calcium alginate

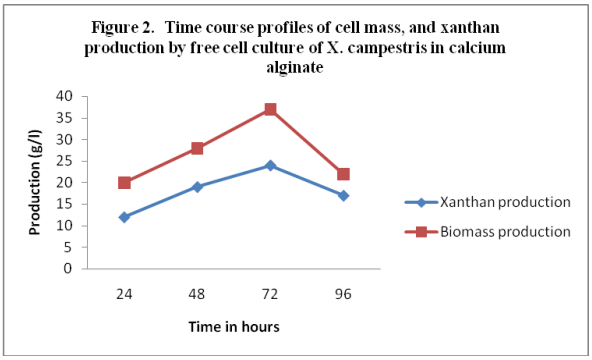


Figure 2. Time course profiles of cell mass, and xanthan production by free cell culture of X. campestris in calcium alginate

The xanthan production was started at 24 hours with immobilized cells and reached a maximum level (69g/l) by 72 hours. On further incubation, xanthan production was gradually decreased, whereas maximum xanthan production was also observed by 72 hours in the case of free cells. It is evident that the xanthan production was higher with immobilized cells (69g/l) than that of free cells (24g/l). Ramakrishna *et al*., [8] reported the immobilization of *Bacillus cereus* in calcium alginate and employed packed-bed and fluidized-bed reactors to continuously synthesize thermostable α-amylase. They spun alginate fibres by pultrusion technique to reduce the diffussional resistances in the gel matrix, and thereby a 24-fold increase in the productivity compared to batch fermentation with free-cells was attained.

Production of xanthan by Immobilized Cells in k-Carrageenan: A few reports on immobilization of *Streptomyces fradiae* [9] and *Penicillium chrysogenum* [10] cells for the production of tylosin and penicillin respectively were available in the literature about the use of

k-Carrageenan as an entrapment matrix. The immobilization procedure is similar to alginate, and several other groups have used this polysaccharide as a preferred gel matrix either alone or in combination with other gums because of the mild conditions required and good gel stability. Using k-Carrageenan, immobilized Brevibacterium flavum attained high stability against several denaturing chemicals 11. The rate of cell leakage could be lowered by hardening the gel with potassium cations.

The pattern of results is shown in Figure 3. The maximum xanthan production (42g/l) was attained at 72 hours. The xanthan obtained with this carrier was less than that of free and immobilized cells with the other carrier (calcium alginate).

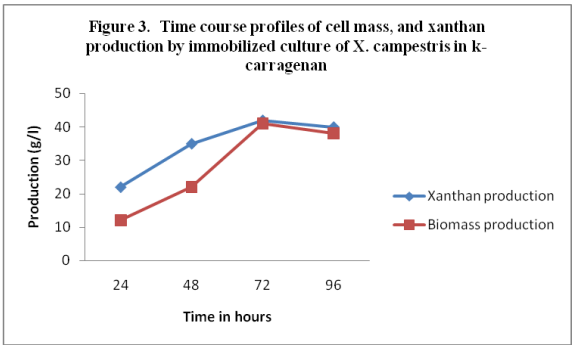


Figure 3. Time course profiles of cell mass, and xanthan production by immobilized culture of X. campestris in k-carragenan

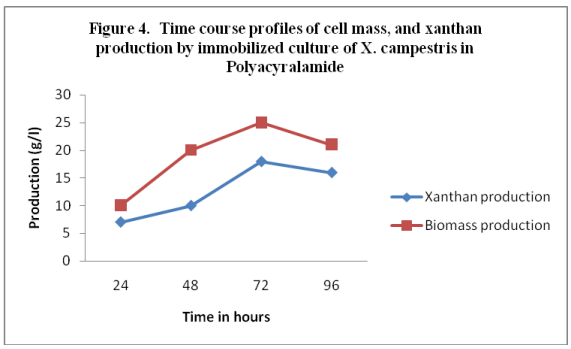
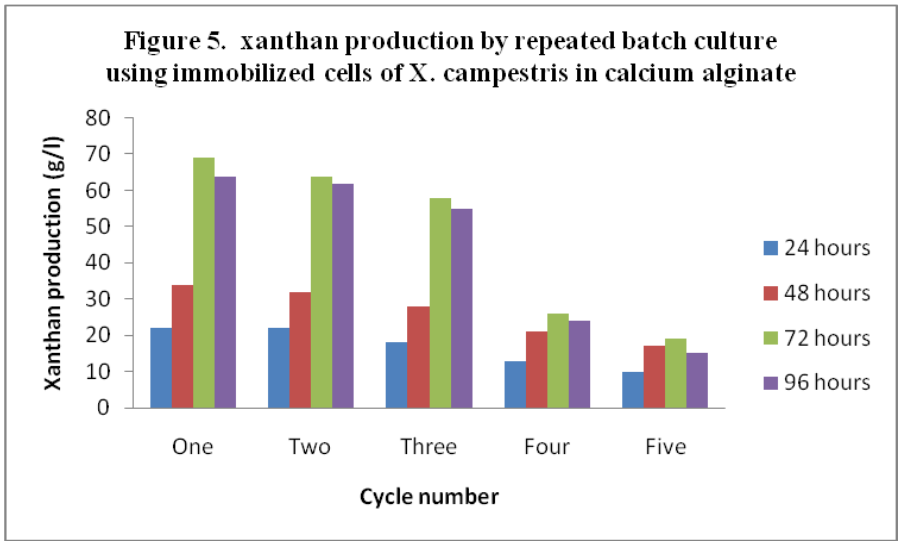


Figure 4. Time course profiles of cell mass, and xanthan production by immobilized culture of X. campestris in Polyacyralamide

Production of xanthan by Immobilized Cells in Polyacrylamide: Polyacrylamide was successfully used for immobilization of many enzyme systems [12]. It was also used for the immobilization of cells for the production of other primary metabolites [13]. A gradual increase in xanthan production was noticed from 24 hours onwards to 72 hours; on further incubation decline in xanthan production was observed (Figure 4). The maximum xanthan production was 18g/l was observed at 72 hours and it was found to be a lower titer compared to alginate matrix.

Comparison of xanthan Production by Immobilized Cells in Various Matrices by Entrapment Technique: The xanthan production with immobilized cells in alginate matrix was found to higher followed by k-Carrageenan. Low level of xanthan production was observed with polyacrylamide. Probably polyacrylamide monomers were toxic for the cells.

Repeated Batch Fermentation With Free Cells and Immobilized Cells: The semi-continuous fermentation was terminated to investigate the stability of the biocatalysts and their ability to produce xanthan under repeated batch cultivation conditions. Figure 5 shows the possibility for reuse of the calcium alginate biocatalysts to produce xanthan gum in semicontinuous mode. The results revealed that the amount of xanthan production with types of immobilized cells gradual decrease in xanthan production from the first batch onward was observed. The beads were disintegrated during the fifth batch operation. Thus the repeated batch fermentation with calcium alginate beads was successfully run for 4 batches. These findings were in accordance with those obtained previously for the protease production by immobilized *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads [14]. It was found that protease production by immobilized *S marcescens* increased with repeated growth cycles, and reached a maximum after 5 cycles. Bandyopadhyay *et al*., [15] studied erythromycin production by *Streptomyces erythreus* entrapped in calcium alginate beads and obtained efficient productivity of erythromycin. They could conduct repeated batch fermentation successfully (each batch 48 hours) for 12 batches (30 days). Similarly, Farid and Enshasy, [16] reported that a good level of oxytetracycline was produced for a period of 28 days (7 batches) using *Streptomyces rimosus* cells immobilized in 4% calcium alginate.



The alginate matrix was found to be superior to the other matrices studied in this paper. In addition, the alginate matrix is less expensive, nontoxic, and preparation of biocatalyst involves mild conditions, which is an added advantage. In contrast, free cells showed lower xanthan production than the calcium alginate–immobilized ones and their activity decreased markedly with repeated batch cycles (data not shown). After the immobilized biocatalyst had been in use for about 5 days, it still possessed significant xanthan production. It has been shown that immobilized cells were able to produce xanthan consistently and that they might be used for continuous xanthan production.

CONCLUSIONS

The results show that calcium alginate is a promising method of *X. campestris* immobilization for xanthan production. xanthan production by immobilized cells is superior to that of free cells because it leads to higher volumetric activities within the same time of fermentation. Specific advantages of this technique such as long life-term stability, reusablility, and possibility of regeneration to be adaptable also to scale-up the obtained data. In addition, the experiments with repeated batches of alginate immobilized bacterial growth by introducing fresh nutrients every 24 hours leads to a specific volumetric productivity that is higher than that obtained with free cells.

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