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Enhancement of fibrinolytic enzyme production from *Bacillus subtilis* via immobilization process onto radiation synthesized starch/dimethylaminoethyl methacrylate hydrogel

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ABSTRACT

Hydrogel matrices based on starch and dimethylaminoethyl methacrylate (Starch/DMAEMA) were synthesized including γ-irradiation as a clean initiator. The prepared hydrogels were characterized in terms of their gel fraction yield, degree of equilibrium swelling. The prepared hydrogels were examined as carriers for immobilization of *Bacillus subtilis* that has the ability to secrete an extracellular fibrinolytic enzyme that degrades fibrin. Scanning electron microscope (SEM) analysis showed proliferation of the bacterial cells entrapped inside the polymeric matrix. The immobilization process increases the production time of fibrinolytic enzyme up to 120 h instead of 96 h for the free cells. The optimum temperature of activity broadened and a significant shift in the pH optima was observed upon immobilization. The reusability of immobilized cells under repeated batch fermentation conditions was also investigated. At the optimum production conditions, immobilization of *B. subtilis* cells onto Starch/DMAEMA resulted in a four fold increase in enzyme activity.

1. Introduction

The formation of blood clots is regarded as a protective phenomenon by the human body in order to prevent excess bleeding from wounds and injuries, but may sometimes block the blood flow causing myocardial infarction and stroke (Deepak et al., 2010). Fibrin is the main protein component of the blood clot, and it is normally formed from fibrinogen by the action of thrombin (Xiao, Zhang, Peng, & Zhang, 2004; Yong et al., 2001). Equilibrium in clot formation and its dissolution is necessary in order to maintain the blood flow correctly by forming the clot at the injured site and removal of fibrin from the unwanted site (Bajzar, Nesheim, & Tracy, 1996).

Fibrinolytic therapy is used to achieve the most important objective in patients with an acute myocardial infarction (MI), to open quickly the coronary flow after the occurrence of an acute coronary occlusion. At this time, the external supplement will be very much useful in saving an individual from mortality and morbidity. Indeed, fibrinolytic therapy leads to better survival recovery of left ventricular function (Joeng et al., 2007).

Microbial fibrinolytic enzymes are agents that dissolve fibrin clots and have considerable potential to be developed into thrombolytic agents (Peng, Yang, & Zhang, 2005). The fibrinolytic enzymes were successively discovered from different microorganisms, the most important among which is the genus *Bacillus* from traditional fermented foods (Batomunkueva & Egorov, 2001; Tough, 2005).

Bacillus subtilus produces a variety of extracellular and intracellular proteases including nattokinase (Nakamura, Yamagata, & Ichishima, 1992). The fibrinolytic enzymes from *Bacillus* sp. have attracted interest as thrombolytic agents because of their efficiency in the fibrinolytic process including plasmin activation.

A major challenge in cell immobilization technique is to provide an optimum environment that mimics natural conditions in order to maintain proper cellular functions. Cell entrapment within polymeric matrices is promising for improvement of the efficiency of bioprocess, especially in the production of metabolites (Chen & Lin, 1994). Advantages of the immobilized cells compared with free cells include (i) protection from harsh environmental conditions such as pH, temperature, organic solvent and toxic compounds; (ii) relative ease of product separation; (iii) reusability; (iv) increased cell density; and (v) reduced susceptibility to contamination by foreign microorganisms (Buyukgungor, 1992; Champagne, Lacroix, & Sodinigallot, 1994; Moslemy, Guiot, & Neufeld, 2002; Park & Chang, 2000; Takei et al., 2010; Uemura et al., 2000). Selection of a suitable

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matrix for cell immobilization is essential for successful application of the immobilized cells to bioprocess.

A hydrogel is a polymeric material that imbibes a considerable amount of water within a polymeric network without dissolution in water while keeping its three-dimensional stability. Both synthetic and naturally derived hydrogels provide the most tissue-like three-dimensional mesh or porous space that able to hold the cells in place (Ito et al., 2003; Woerly, Marchand, & Lavallee, 1990), while allowing the transport of nutrients, waste, and other essential molecules via the bulk fluid phase (King & Ashby, 1988). Cells entrapped in such a hydrogel can be utilized in vitro applications such as a bioreactor and in vivo studies, whereby the hydrogel matrix protects immobilized cells from mechanical and immunological damage (Hayashi et al., 1996; Jean-Francois, D'Urso, & Fortier, 1997).

The objective of this study is to immobilize *Bacillus subtilus* cells onto a polymeric hydrogel to enhance its fibrinolytic enzyme production. In this connection, starch, which is a cheap and biodegradable natural polymer and readily available will be copolymerized with dimethylaminoethyl methacrylate by gamma radiation. The parameters controlling the preparation of stable hydrogel will be optimized and the ability of the immobilized cell for the production of the fibirinolytic enzymes under different conditions will be evaluated. The reusability of immobilized cells for fibrinolytic enzyme production under repeated batch fermentation conditions will be investigated.

2. Materials and methods

Soluble starch (potato starch) with molecular weight $(162.14)_n$ was received from Hi Media Laboratories. Dimethylaminoethyl methacrylate, 98% (DMAEMA) were received from Sigma–Aldrich. Fibrinolytic enzyme producing strain of *Bacillus subtilis* used in this study was isolated in our laboratory from soil sample. The bacterial isolate was identified by investigating its morphological, physiological and biochemical properties. The isolate was finally classified according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The isolate was maintained on nutrient agar slants at 4 °C and was sub-cultured every 2 weeks.

2.1. Preparation of Starch/DMAEMA gels

Starch/DMAEMA hydrogels were obtained using γ-irradiation induced copolymerization of 20 wt% aqueous Starch/DMAEMA solutions of different compositions. The solutions were mixed well, transferred into small glass vials then irradiated at 30 and 40 kGy at room temperature using ^{60}Co gamma rays at fixed dose rate of 4 kGy/h. After copolymerization, the vials were broken; the formed hydrogel cylinders were removed and cut into disks of 2 mm thickness and 5 mm diameter. All samples were washed with excess water to remove the unreacted component, then air dried at room temperature up to constant weight.

2.2. Gel fraction

The gel content in the dried samples was estimated by measuring its insoluble part after extraction in distilled water for 48 h at 60 °C. Then, they were taken out and washed with hot water to remove the soluble part, dried, and weighed. The gel fraction was calculated according to Eq. (1).

$$\text{Gel content}\,(\%) = \frac{W_d}{W_o} \times 100 \quad (1)$$

where (W_o) is the initial weight of dried sample and (W_d) is the weight of the insoluble part after extraction with water and air dried.

2.3. Swelling studies

Pre-weighed Starch/DMAEMA hydrogel discs were allowed to swell in 50 mL distilled water up to equilibrium swelling at 37 °C, thereafter the discs were taken out from the water and gently pressed in-between two filter papers to remove excess water and finally weighed using a sensitive balance. The swelling degree (%) was determined from Eq. (2):

$$\text{Swelling degree}\,(\%) = \frac{W_s - W_o}{W_o} \times 100 \quad (2)$$

where W_s and W_o are the weights of the swollen and the dried hydrogel, respectively. The experiments were repeated three times, and the results were reported as average values.

2.4. Inoculum preparation

Five mL sterile distilled water was added to 24 h old culture of *B. subtilis*. The cells were scraped from the slant into sterile distilled water and the resulted cell suspension was transferred into 250 mL Erlenmeyer flask containing 45 mL of sterile inoculums medium. The composition of the growth medium (nutrient broth medium) is (g/L): Peptone 5.0; yeast extract 3.0; sodium chloride 5.0; lab lemco 1.0, pH 7.0. The flask was kept in a shaker incubator at 175 rpm at 37 °C. The microbial cells from 18 h old culture were used for both immobilization process and as free cells.

2.5. Immobilization of B. subtilis in Starch/DMAEMA hydrogel by Entrapment technique

A definite weight of Starch/DMAEMA hydrogel disc was sterilized and dropped into 50 mL of sterile production medium of composition (g/L) 2.0 g yeast extract; 10.0 g peptone; 2.0 g glucose; 0.8 g KH_2PO_4; 0.2 g K_2HPO_4; 0.05 g $CuSO_4{\cdot}5H_2O$; 0.07 g $FeSO_4{\cdot}7H_2O$; 0.7 g $MgSO_4{\cdot}7H_2O$, pH 6.6 and then were inoculated with 2 mL of the 18 h inoculums of the provided strain and the flasks were incubated at 37 °C under shaking condition at (150 rpm) for 24 h. After incubation for different periods (12, 24, 48, 72, 96 and 120 h), the culture broth in each was centrifuged at 6000 rpm in a cooling centrifuge at 4 °C in clear supernatant fibrinolytic activity was determined. Simultaneous experiments with free cells equivalent to those used in immobilized cultures were also conducted.

2.6. Preparation of human fibrin

Fibrin clots were prepared from thawed fresh frozen plasma collected in citrate anticoagulant from normal volunteer donor plasma. One mL was mixed with 0.8 mL of clotting reagent in 60 mm Petri dishes. The preparation was mixed and then plasma was allowed to clot for a few minutes at room temperature. The fibrin matrix was then incubated at 37 °C for 30 min and washed three times with 5 mL of 0.15 M NaCl and allowed to dry at 50 °C (Pearl, Lai, Thomas, & Sande, 1985).

2.7. Assay of fibrinolytic enzyme activity

The reaction mixture contained 10 mg of human fibrin, 15 μmol phosphate buffer 0.2 M, pH 7.0 and 0.5 mL enzyme source (culture filtrate of the provided strain) in a total volume 1 mL. The reaction mixture was incubated for 60 min at 37 °C in shaking water bath at 50 rpm. The reaction then stopped by the addition of 15% (w/v) trichloro acetic acid (TCA). This was followed by centrifugation at 4000 rpm and assay of the soluble proteins in the supernatant using the phenol color method was estimated (Greenberg, 1955). A fibrinolytic unit was defined as the amount of enzyme that affords an

increase in absorbance at 280 nm equivalents to 1 μg of tyrosine per minute at 37 °C.

2.8. Production of fibrinolytic enzyme by repeated batch fermentation process

The reusability of *B. subtilis* cells immobilized in Starch/DMAEMA was examined. After attaining the maximum production of fibrinolytic enzyme after 48 h the hydrogel discs were washed several times with saline and transferred to 50 mL of fresh sterile production medium (50 mL) and the fermentation process repeated several times until the activity decrease.

2.9. Effect of incubation temperature on the produced fibirinolytic enzyme

50 mL of sterile production medium containing a definite weight of Starch/DMAEMA hydrogel were inoculated by 2.5 mL of 18 h old culture of *B. subtilis* and incubated at different temperatures with shaking at 150 rpm. After 48 h, the cells were removed by centrifugation (6000 rpm) at 4 °C and the supernatants were measured for fibrinolytic activity. Simultaneous experiments with free cells equivalent to those used in immobilized cultures were also conducted.

2.10. Effect of production medium pH on the produced fibirinolytic enzyme

50 mL of sterile production medium containing a definite weight of Starch/DMAEMA hydrogel adjusted to different pH values and then inoculated by 2.5 mL of 18 h old culture of *B. subtilis* with shaking at 150 rpm at 37 °C. After 48 h the cells were removed by centrifugation (6000 rpm) at 4 °C and the supernatants were determined for fibrinolytic activity. Simultaneous experiments with free cells equivalent to those used in immobilized cultures were also estimated.

2.11. Scanning electron microscopy

Free Starch/DMAEMA hydrogel and immobilized hydrogel discs with *B. subtilis* cells were freeze dried, then coated with gold to be finally observed with a JEOL JSM 6360 LV scanning electron microscope at an acceleration voltage of 10 kV.

3. Results and discussion

3.1. Characterization of the Starch/DMAEMA hydrogel

It is well known that radiation induced copolymerization and crosslinking process has some advantages over chemical crosslinking and it is widely used in recent years for the synthesis of various hydrogels for biomedical applications (Hattery & Mc Ginniss, 1983, chap. 22).

Generally, grafted starch copolymers have higher swelling than starch itself (Al-Karawi & Al-Daraji, 2010). Theses copolymeric materials swell more in water and contain large amount of water, which is considered to be better for their biocompatibility for living tissues because the interfacial free energy between water-swollen gel and the aqueous biological environment is very small and the inner water provides good permeability to oxygen, metal ions, and other metabolites.

3.1.1. Gel fraction

When an aqueous mixture of Starch/DMAEMA is irradiated with gamma rays, simultaneous crosslinking of DMAEMA, degradation of starch and grafting of the crosslinked polymer chains onto starch fragments will take place simultaneously resulting in the formation of insoluble copolymer network (gel). The overall rate of these graft copolymerization processes could influence the gel fraction and swelling of the hydrogel. The stability of the prepared hydrogels against dissolution in hot water is simply known as gelation degree (El-Hag Ali & AlArifi, 2009).

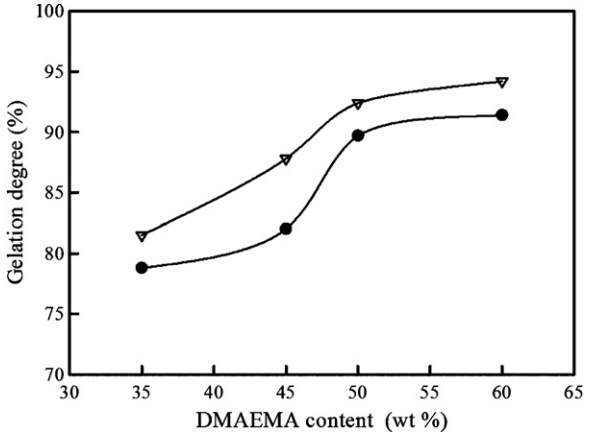

Fig. 1. Effect of DMAEMA content on the gelation degree at different irradiation doses; (●) 30 and (▽) 40 kGy.

The dependence of gelation degree of the prepared hydrogel on the DMAEMA content and irradiation dose is given in Fig. 1. It can be seen that the gel fraction increases with increasing DMAEMA content and the irradiation dose and it seems never to reach 100% of gel. This certainly indicates that in the Starch/DMAEMA system chain scission also accompanies by chain crosslinking.

3.1.2. Swelling behavior of Starch/DMAEMA hydrogel

The basic feature of the hydrogel is to absorb and hold huge amount of water in its network structure. The high water content of the hydrogels provide them important characteristics that support cell immobilization such as good permeability toward nutrients that ease cell growth. When a hydrogel is brought into contact with water the water diffuses into the network, resulting in the expansion of the hydrogel. The swelling equilibrium occurs when the values of the osmotic force driving the water into the network and of the elastic force of the stretched sub-chains become equal. Equilibrium swelling ratio is defined as the ratio of the solvent-swollen weight of the hydrogel equilibrated at a given temperature to the weight of the hydrogel in its dry state.

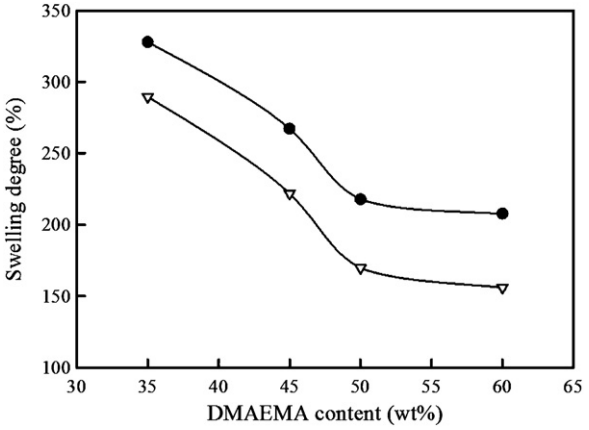

Fig. 2. Effect of DMAEMA content on the swelling degree at different irradiation doses; (●) 30 and (○) 40 kGy.

Fig. 2 shows the equilibrium swelling profile of the Starch/DMAEMA hydrogel as a function of DMAEMA content in the feed solution and preparation dose. It is clear that with increasing DMAEMA content in the feed solution as well as irradiation dose, the swelling degree of the prepared hydrogels decreases obviously. Such decrement in the swelling degree would attribute to the nature of DMAEMA and the increment in the crosslinking density, respectively. DMAEMA is a relatively hydrophobic vinyl monomer; the increase in its content would lead to tight crosslinked structure that reduces the swelling ability of the produced hydrogels. On the other hand, the increment in the irradiation dose leads to formation of higher number of free radical which in consequence leads to higher degree of crosslinking. The swelling property of the hydrogel will assure a maximum contact of the immobilized bacteria with the fibrinolytic enzyme production media.

3.2. Cell immobilization on Starch/DMAEMA matrix

A wide variety of parameters play an important role in the immobilization process, such as matrix porosity and water content of the polymer matrix. Photo 1(a, b and c) shows SEM microphotographs of the Starch/DMAEMA matrix before and after 48 h of bacterial incubation. Photo 1(a and b) shows Starch/DMAEMA swollen hydrogel with different DMAEMA content before immobilization, it is clear that the swollen hydrogel possess a highly porous structure and the porosity decrease by increasing the DMAEMA content in the prepared hydrogel. Such porosity favored diffusion of substrates and metabolites.

Photo 1(c) shows the hydrogel after the cell entrapment process, the good adhesion of the immobilized bacterial cells within the matrix due to the favorable balance of the micro environmental conditions that achieved by the chosen matrix.

3.3. Factors affecting fibrinolytic enzyme production from cell-entrapped Starch/DMAEMA matrix

Different factors affecting the production of the fibrinolytic enzyme from the cell-entrapped Starch/DMAEMA matrix such as, nature of the immobilized matrix, the fermentation time, temperature and pH of the media, were investigated.

3.3.1. Effect of the matrix composition

The porosity of the hydrogel network is mainly governed by the hydrogel composition and the preparation irradiation dose. Highly porous matrix provides a good permeability toward nutrients that ease cell growth. Fig. 3 shows the effect of the Starch/DMAEMA composition and irradiation dose on the yield of fibrinolytic enzyme production expressed by its activity. It is clear that as the DMAEMA content increases, the enzyme activity decreases. Also it can be seen that by increasing the irradiation dose that used for hydrogel preparation, the activity decreases. Such decrease is mainly due to the increase in the crosslinking density by increasing both the DMAEMA content and irradiation dose. The increase in the crosslinking density leads to less porous network structure which is not favorable for good immobilization process.

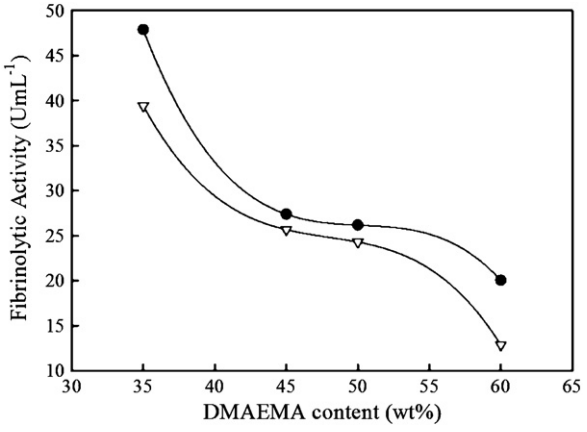

Fig. 3. Effect of matrix composition and preparation dose on the fibrinolytic enzyme activity.

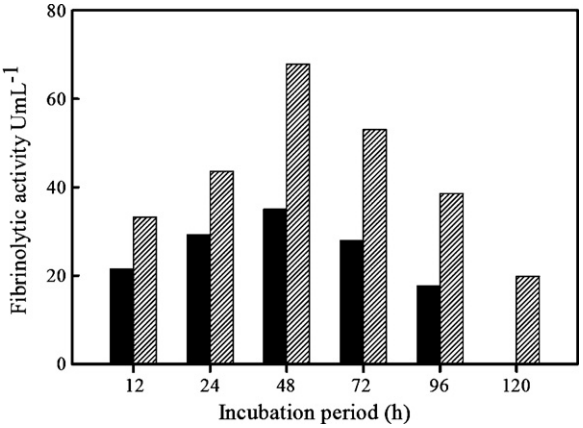

Fig. 4. Effect of incubation period on the fibrinolytic enzyme activity of (■) free and (▨) immobilized cells.

3.3.2. Effect of incubation period

Cell immobilization is one of the common techniques for increasing the over cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. A comparison of time dependant fibrinolytic enzyme activity by immobilized and free cells of *B. subtilis* was done by running the fermentation batch technique till 120 h. From Fig. 4, it can be seen that the cell-entrapped technique prolonged the fibrinolytic enzyme production time up to (120 h) if compared by free cells (96 h). In addition, the production yield (expressed by the activity) from the cell-entrapped Starch/DMAEMA is two times greater than that obtained from the free cells. Thereafter, the decrease in enzyme production might be due to the depletion of nutrients. Moreover, the accumulation of metabolites in the fermentation medium leads to an increase in medium pH that decreased the stability of the enzyme. This was in congruence with other pervious results (Anisha & Prema, 2006) who conclude that, the time of maximum enzyme production is significant because it is considered to be the cycle time for reusability transfer in repeated batch fermentation and in this case a period of 24 h could be saved in each fermentation cycle. Adinarayana, Jyothi, and Ellaiah (2005) reported that the immobilization of *B. subtilis* PE-II in calcium alginate by entrapment technique was more efficient, the alkaline protease production by *B. subtilis* PE-II was higher with immobilized cells than that of free cells.

3.3.3. Effect of incubation temperature

Thermostability of the produced enzyme is a bench marker for determining the efficiency of the immobilization process (Gianfreda & Scarfi, 1991). Fig. 5 shows the effect of incubation temperature on the fibrinolytic enzyme activity from the cell-entrapped Starch/DMAEMA matrices prepared at 30 and 40 kGy in comparison with that obtained by free cells. It is clear that, the immobilization process increases the thermal stability of the produced enzyme. Also, it can be seen that the maximum activity of the produced fibrinolytic enzyme from the free cells is attained

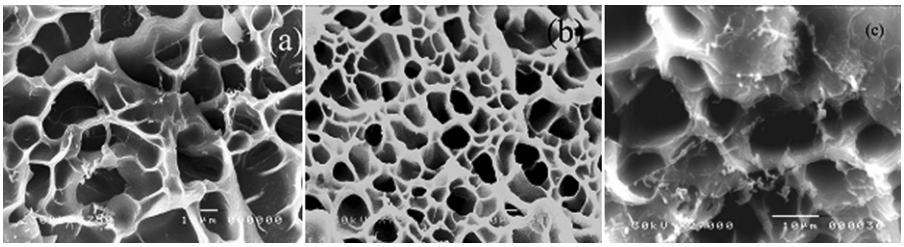

Photo 1. SEM micrographs of swollen Starch/DMAEMA hydrogel with different DMAEMA (wt%) before immobilization (a) 50 and (b) 65 and (c) after immobilization 50 (wt%).

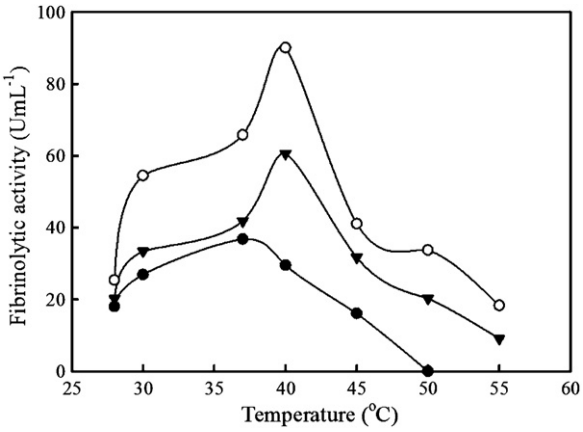

Fig. 5. Effect of incubation temperature on the fibirinolytic enzyme activity of (●) free cells, (○) 30 and (▼) 40 kGy.

at 37 °C, beyond which it starts to decrease and it completely lost above 45 °C.

While in case of cell-entrapped Starch/DMAEMA matrix, it can be seen that the immobilization process shifts the maximum temperature for the enzyme activity up to 40 °C. Also it can be seen that the enzyme activity decreases by increasing temperature but still stable up to 55 °C, the decrease in the enzyme activity can be explained by the inactivation of the enzyme at temperatures above 40 °C. From this figure we can concluded that the hydrogel prepared at 30 kGy is more suitable for the immobilization process.

3.3.4. Effect of pH

The fibrinolytic enzymes belong to proteases are generally active at neutral and alkaline pH, with an optimum between pH 8.0 and 10 (Kim & Choi, 2000; Ko, Yan, Zhu, & Qi, 2004). Fig. 6 shows the effect of pH in the range from 5 to 10 on the fibrinolytic enzyme activity for both cell-entrapped Starch/DMAEMA matrices prepared at 30 and 40 kGy in comparison with that obtained by free cells. It is clear that at any pH value under investigation the fibrinolytic enzyme activity obtained from the immobilized cells was higher than that obtained from the free cells. The stability of the fibrinolytic enzyme activity from the immobilized cells toward different pHs was much higher than that obtained from the free cells. The maximum activity of the fibrinolytic enzyme from the free cells was at pH 6.5 and it decreases by increasing the pH value and the activity was completely lost at pH 9.0. The maximum activity from the cell-entrapped Starch/DMAEMA was achieved at pH 8 after which the stability dramatically decreases. Also, it can be noticed that, the hydrogel prepared at 30 kGy is more suitable for the immobilization process. The obtained results indicate a broadening of the pH optima as a function of immobilization, a characteristic which is highly desirable for bioconversion (Fokina, Arinbasarova, Zubov, Lozinsky, & Koshcheenko, 1995). (Deepak, Pandian, Kalishwaralal, & Gurunathan, 2009) concluded that the immobilized nattokinase enzyme showed 25% increase in the activity at pH 10.0 after which the activity decreases, the purified free enzyme was stable up to 50 °C after that a dip in the activity is observed while, the immobilized enzyme was stable up to 70 °C without loss in the activity.

3.4. Reusability of B. subtilis-entrapped Starch/DMAEMA matrix

One of the advantages of using immobilized whole cells is that they can be used repeatedly and continuously. Repeated batch cultivation experiment was used to investigate the operational stability (reusability) of immobilized cells. The *B. subtilis*-entrapped Starch/DMAEMA matrix obtained under the optimal conditions described (at 40 °C and pH 8) was used to produce fibrinolytic enzyme repeatedly 5 times. Fig. 7 shows a gradual decrease in the enzyme activity with the cycle number.

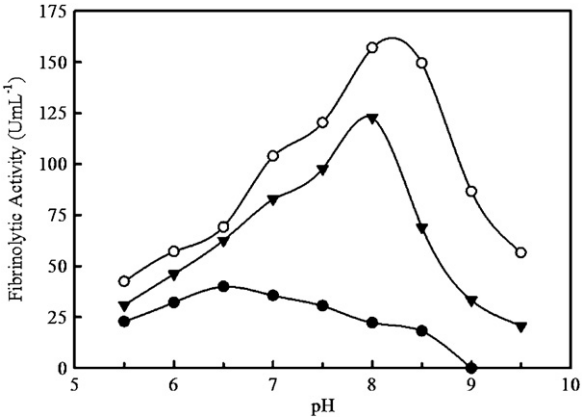

Fig. 6. Effect of pH on the fibirinolytic enzyme activity of (●) free cells, (○) 30 and (▼) 40 kGy.

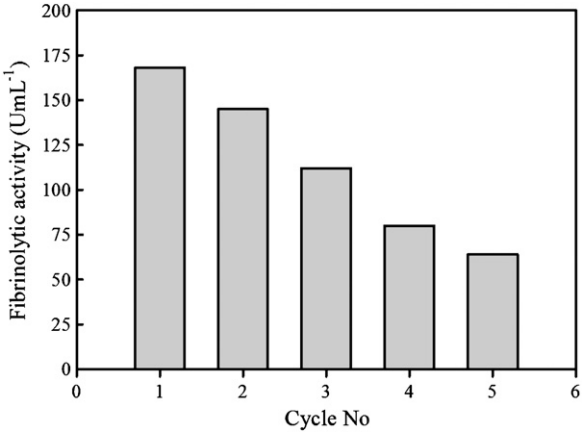

Fig. 7. Reusability of the *B. subtilis*-entrapped Starch/DMAEMA matrix.

These findings were in accordance with those obtained previously for the protease production by immobilized *Serratia marcescens* and *Myxococcus Xanthus* in calcium alginate beads. It was found that protease production by immobilized *S. marcescens* increased with repeated growth cycles and reached a maximum after 5 cycles (Vuillemard, Terre, & Benoit, 1988). Also Adinarayana et al. (2005) reported that alkaline protease production by immobilized *B. subtilis* PEII increased with repeated growth cycles. The repeated batch fermentation with calcium alginate beads was successfully run for 9 batches (9 days). The decrease in enzyme yield with successive fermentation might be due to the loss of cell viability (Anisha & Prema, 2008).

4. Conclusion

Cell-entrapped technique was used for the immobilization of *B. subtilis* within a series of Starch/DMAEMA hydrogels prepared by γ-ray as clean initiator. Different factors affecting the fibrinolytic enzyme activity (as indicator for the enzyme production yield) from the immobilized cells were investigated and compared with that obtained from the free cells. It was found that, the cells have been successfully immobilized onto the hydrogel discs, which appears to not affect microbial activity. Upon immobilization, the maximum activity was achieved at pH 8 and 40 °C and the production yield is four times greater than that obtained from the free cells. The operational stability of the immobilized cells reflects the capability of the Starch/DMAEMA matrix to retain the activity of the immobilized cells and to form a stable system that can be used for several production processes.

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