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APPLICABILITY OF WOOL COVALENT BONDED *Bacillus circulans* 25 CELLS FOR MILK-CLOTTING ENZYME PRODUCTION BY BATCH, REPEATED BATCH AND CONTINUOUS PROCESS

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Abstract - The production of milk-clotting enzyme (MCE) by free and immobilized *Bacillus circulans* 25 cells was investigated. The investigation evaluates cell immobilization through entrapment and covalent binding to different carriers. *B. circulans* 25 cells immobilized covalently via glutaraldehyde (cross-linker) to natural wool exhibited higher effectiveness factor (0.76) compared to other carriers. Immobilized cells produce maximum level of MCE and highest ratio of milk-clotting activity/proteolytic activity (MCA/PA) after 24 h using 0.4g wet weight cells/g wool. In batch operation, decreasing biomass loading to 0.2 g enhanced the MCA/PA ratio and specific productivity by 5.94 and 59.88%, respectively. Immobilized cells on natural wool as an effective and suitable carrier were able to produce the same level of MCA, productivity and MCA/PA ratio for 9 repeated cycles (216 h). Under continuous operation immobilized cells in a packed-bed bioreactor were able to keep producing MCA with MCA/PA ratio for about 7 days at the same level. Moreover, continuous operation demonstrates a very good productivity of 0.385 KU/L/h, which is higher than the other production systems by 7.0-11.7 fold. Immobilized *B. circulans* 25 cells proved to be fully capable of continuous MCE production in a packed-bed bioreactor.

Keywords: Milk-clotting enzyme; Batch; Repeated batch; Continuous production; Packed-bed bioreactor.

INTRODUCTION

Traditionally used as a milk coagulant, calf rennet plays a critical role in the cheese production. However, decreasing number of slaughtered calves has led to an increased price of calf rennet versus the increasing demand of coagulant in the production of cheese necessitates the exploration for potential substitutes (Hang *et al*., 2016; Lemes *et al*., 2016). Candidate calf rennet substitutes has mainly been resourced from animals (Shamtsyan *et al*., 2014), plants (Shah *et al*., 2014), genetic engineering and microorganisms (Lemes *et al*., 2016) but their applications may be limited by factors such as cultivation and climatic variations, which can affect their production and supply. Thus, microbial enzymes (Microlant) are attractive and potential alternatives for milk coagulation (Lemes *et al*., 2016) due to their stability, availability, rapid growth, cheaper cost, greater biochemical diversity, easier genetic modification and offer a variety of properties and religious or dietary reasons (Ahmed *et al*., 2016; Hang *et al*., 2016; Lemes *et al*., 2016). Most proteolytic enzymes coagulate milk, but in most cases no stable cheese is formed, as the coagulum is

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further digested by continued proteolysis. Good MCE is characterized by a high specific caseinolytic activity and a low general proteolytic activity. Moreover, the proteolysis strongly affects the textural and sensory properties of cheese (Shah *et al*., 2014). Biocatalyst immobilization is one of the techniques based on the fixation of the biocatalyst into or onto various carriers, which can improve whole cell applications (Krasňan *et al*., 2016). Using immobilized cells for biochemical processes offers several advantages over the conventional free cell systems including increased robustness of the biocatalyst, higher cell density, higher productivity, possibility of cell recovery and reuse, lower downstream costs associated with cell separation, improved cell resistance to toxic and inhibitory compounds, better process, storage stability and facilitating continuous operation (Niknezhad *et al*., 2016; Siddiqui *et al*., 2016). Application of immobilization biocatalysts generally includes the production of chemicals, pharmaceuticals and food products (Saieb *et al*., 2015). Over the years, many immobilization techniques have been developed and the basic methods are adsorption, covalent binding, cross-linking, encapsulation, and entrapment. An industrial carrier for food fermentations should be cheap, nontoxic, stable to microbial degradation, with low diffusional limitation, reusable, high surface area, and appropriate density for the reactor type (Eş *et al*., 2015). Natural wool possesses rich reactive residues (such as lysine, serine, and glutamic acid), which might be used in surface activation in the immobilization process. Furthermore, its rigid structure and wide availability makes wool an attractive candidate as an immobilization carrier. The surface of untreated wool is quite hydrophobic, cell immobilization onto this surface can then be achieved by using glutaraldehyde (GA) as a cross-linking agent due to its fast reaction and low toxicity. The most widely accepted theory on the GA cross-linking mechanism is that the aldehyde groups in GA react with the lysine residues in the protein through a Schiff base reaction (An *et al*., 2014). Repeated batch fermentation is considered useful technology for enhancing the productivity due to the reduction in fermentation time and removal of the inoculum preparation step (Reddy *et al*., 2016). A packed- bed reactor is the most commonly used immobilized cell reactor in industry (Eş *et al*., 2015). The bioreactor consisted of a packed- bed of immobilized cells and its operation involved recycling of the broth through the bed (Sirisansaneeyakul *et al*., 2007). The present study focused on the production of MCE by the immobilized cells on different carriers. The productivity of MCE was evaluated using the immobilized cells of *B. circulans* 25 in batch, repeated batch, and continuous cultures in packed- bed bioreactors.

MATERIALS AND METHODS

Microorganism

Bacillus circulans 25 was obtained from the Culture Collection of the National Research Centre, Dokki, Cairo, Egypt and was maintained on nutrient agar (Merck Co.) slants at 35ºC and transferred weekly.

Carriers for cell immobilization

Agar and sodium alginate were obtained from BDH Chemical Ltd., Poole, England.

Charcoal was from ADWIC Company, Egypt. Acrylamide, N,N-methylene bis-acrylamide and *k* -carragenan were obtained from Sigma Chemical Co., USA. Chicken bone, ceramic, glass wool and natural wool were collected from the local market in Egypt.

Culture media and growth conditions

The basal medium used for enzyme production had the following composition (g/L): lactose 20.0, yeast extract 1.0, peptone 1.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.25. The pH was adjusted to 6.0 prior to sterilization. One mL of cell suspension of 24 h-old slant (OD_{600}~0.3), was transferred to 250-mL Erlenmeyer flask each containing 50 mL production medium. The flasks were incubated at 35ºC on a rotary shaker at 180 rpm for 24 h.

Determination of the biomass

The optical density (OD) of the cell growth was measured at 600 nm using a spectrophotometer (Spectronic 2000, Busch & Lomb). The wet weight of cells was measured by centrifugation of a certain volume of the cultivated medium (6000 x *g*, 15 min at 4ºC. The cell pellets were washed with 0.09 M NaCl, collected by centrifugation and weighed. The cell dry weight was determined after heating the cell pellets at 50ºC to a constant weight.

Cell immobilization procedures

All the immobilization processes were performed under aseptic conditions. The viable cells of *B. circulans* 25 obtained in the logarithmic phase of growth were collected by centrifugation (6000 x *g*, 15

min at 4ºC. Then, the wet cells were suspended in 0.85 % sterile NaCl and used for cell immobilization. The same amount of the free cells was inoculated along with the immobilized cells, under identical conditions.

Immobilization by entrapment

In Ca-alginate

The viable cells of *B. circulans* 25 obtained from 50 ml culture (0.4 g wet weight cells) were mixed with 10 mL Na-alginate solution (3.0 % w/v). The mixture was then added dropwise to 3 % sterile solution of $CaCl_2$ with continuous gentle stirring. The produced gel beads were further hardened by allowing them to stand in 3 % $CaCl_2$ solution at room temperature for 2 h. The beads (~2.0 mm) were then recovered by screening and washed with sterile distilled H_2O. The obtained beads were used for inoculation of 50 mL of the production medium (Sirisansaneeyakul *et al*., 2007).

In agar and k-carrageenan

The same amount of the viable cells obtained from 50 ml culture (0.4 g) were mixed with 10 mL of 3 % (w/v) agar or *k*-carrageenan solution at 45ºC and was quickly cooled to 4ºC, cut into 2x2x2 mm fragments, and transferred to 50 mL of the production medium (Cheetham *et al*., 1985).

In polyacrylamide

The viable cells obtained from 50 mL culture (0.4 g) were mixed with 10 mL of 5 % (w/v) acrylamide solution with 3 % (w/w) cross-linker (N,N-methylene bisacrylamide). The gel obtained was cut into fragments (2x2x2 mm) and was used for inoculation of 50 mL of the production medium (Chibata *et al*., 1976).

Immobilization by covalent binding

In separate experiments, 1g of each carrier (charcoal, ceramic, glass wool, chicken bone, and natural wool) was treated with 10 mL of 0.025 % GA for 24 h. The carriers were collected by centrifugation (6000 x *g*, 15 min at 4ºC and washed with sterile distilled H_2O to remove the excess GA. Then the viable cells obtained from 50 mL culture (0.4 g) were incubated with 1 g of each treated carrier in 10 mL of saline. The carriers were collected by centrifugation, washed with sterile distilled H_2O and were used for inoculation of 50 mL of the production medium (Abdel-Naby *et al*., 2011).

Optimization of the immobilization process

Effect of glutaraldehyde concentration

The effect of GA concentration was tested by treating the natural wool with different concentrations of GA ranging from 0.025 to 0.3 %. In all cases, the viable cells obtained from 50 mL culture (0.4 g wet cells) were immobilized on 1g of wool treated with GA

Effect of biomass loading

In separate experiments, 1 g of 0.25 % GA treated wool was mixed with different wet cell concentrations (0.2 - 0.8 g).

Production of milk-clotting enzyme

By free cells in batch experiment

Unless otherwise stated, the batch experiments of free cells were performed in 250- mL Erlenmeyer flasks, each containing 50 mL production medium. The flasks were inoculated with 1 mL cell suspension (OD_{600}~0.3) and incubated at 35ºC and 180 rpm for 24 h. The cultivated culture medium was centrifuged at 4ºC, 6000 x *g*, for 15 min and the clear culture filtrate was taken for enzyme assay.

By immobilized cells

In batch experiment

The batch experiments of the immobilized cells were performed in 250-mL Erlenmeyer flasks, each containing 50 mL of sterile medium. The flasks were inoculated with the beads resulting from 1 g natural wool. The flasks were incubated at 35ºC and 180 rpm for 24 h. The cultivated medium was centrifuged at 6000 x *g*, 4ºC for 15 min and the supernatant was taken for enzyme assay.

In repeated batch experiment

This was done in 250-mL Erlenmeyer flasks, each containing 50 ml sterile medium. Each flask was inoculated with the calculated amount of immobilized cells on 1 g natural wool (containing 0.2 g wet cells). Fermentation was conducted at 35°C, 180 rpm for 24 h. At the end of each run, the beads before transferring

to the fresh medium were washed with tris-HCl buffer (0.01 M, pH 6.0) and distilled H_2O under sterile conditions.

In continuous fermentation using bioreactor

The packed- bed reactor is a glass column 1.8 cm in diameter and 32.0 cm in long (Figure 1). The reactor was filled with 173 g of freshly prepared immobilized beads comprising 5.25 g of wet weight of viable cells. The bioreactor volume was 325 mL. The bed volume was 93 mL, whereas the working volume (void volume) was about 232 mL. The fresh medium was introduced from the bottom of the bioreactor. Air was admitted in through a sterile air filter from the bottom of the bioreactor. The fermentation was conducted at 35°C and carried out in batch operation for 24 h, then the continuous operation was started at different dilution rates (0.05 - 0.7/h). The air flow was adjusted at 0.3 v/v/min. The system was considered to be in a steady state only after at least 5 residences times (replacement volumes) and the effluent from the reactor was collected in a holding tank.

Enzymes assays

Milk-clotting activity (MCA)

MCA was determined by the method reported by Berridge (1952). Enzyme solution (2.5 mL) was incubated with 10 mL of reconstituted skim milk from the Ministry of Agriculture, Giza. Egypt (12 % in 0.01 M $CaCl_2$) at 40°C and the clotting time was recorded. One unit of the enzyme activity (U) was equalized to 10 mL milk, clotted within 10 min.

Proteolytic activity (PA)

PA was determined according to the casein digestion method described by Deane *et al*. (1986). The reaction mixture contained 1.0 mL of enzyme preparation and 1.0 mL of 1.5 % casein solution in tris-HCl buffer (0.01 M, pH 6.0). The reaction mixture was incubated for 30 min at 40°C. Then 2.0 mL of trichloroacetic acid (15 %) were added and the mixture was centrifuged at 6000 x *g* for 10 min. The solubilized proteins in the supernatant were measured using the method of Lowry *et al*. (1951). One unit of the enzyme activity (U) is defined as the amount of the enzyme that releases one µ mole of tyrosine/mL.

Protein determination

The protein content was determined by the method of Lowry *et al*. (1951).

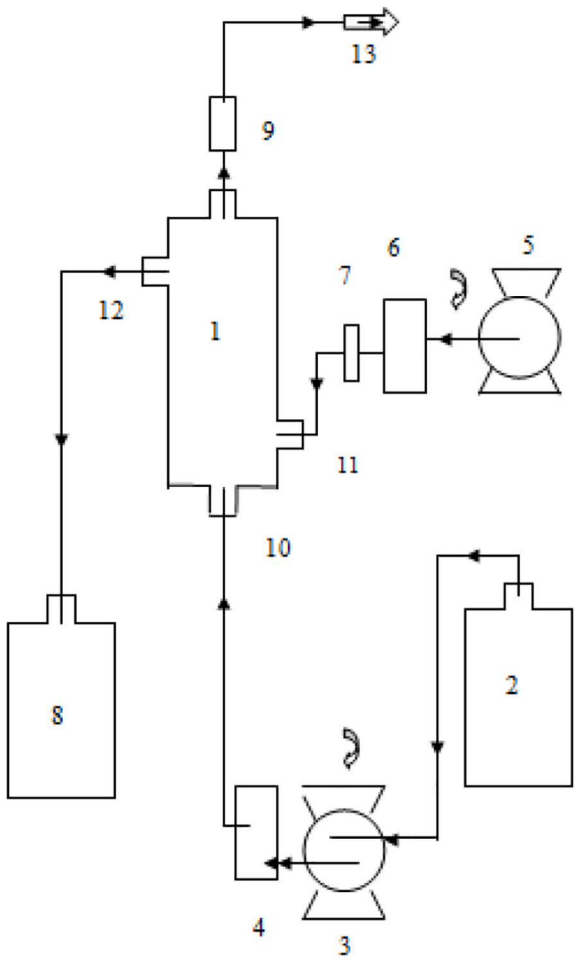


Figure 1. Schematic diagram of the continuous fermentation system. (1) The column bioreactor, (2) medium feed reservoir, (3) peristaltic pump, (4) flow rate regulator, (5) air pump, (6) rotameter, (7) air filter, (8) product collection vessel, (9) outlet air filter (10) medium inlet, (11) air inlet, (12) product outlet, (13) air outlet.

RESULTS AND DISCUSSION

Fermentation course for the production of MCE by *B. circulans* 25 free cells

The results illustrated in Figure 2 showed that maximal MCA (1.33 U/mL) with maximal ratio of MCA/PA (15.1) was reached after 24 h, increasing the time to 48 h decreased the MCA by 80 %. This result is superior to that obtained by Wu *et al*. (2013), who produced crude enzyme from *B. subtilis* natto with a MCA/PA ratio of 6.4. On the other hand, maximal PA (0.14 U/mL) was reached after 48 h and remained stable up to 60 h incubation period. The ratio of MCA/PA is an important factor for the enzyme for industrial use in cheese production and it should be considered to obtain stable curd with good quality. The viable cells

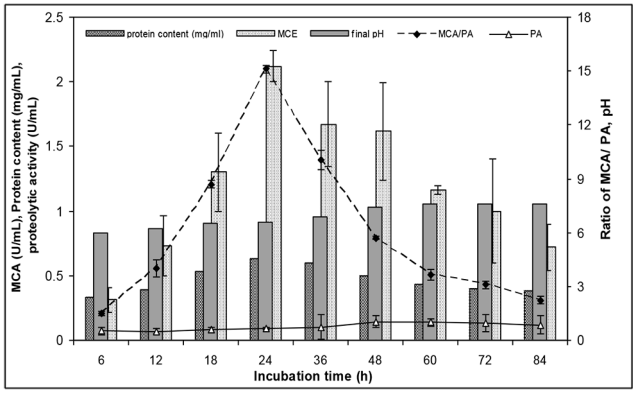


Figure 2. Fermentation course for the production of MCE by free *B. circulans* 25 cells.

obtained in the logarithmic phase of growth (24 h) were used for the immobilization process.

Production of MCE by immobilized *B. circulans* 25 cells

The efficiency of the immobilization process is evaluated by different parameters including MCA, the effectiveness factor of the immobilization (EF=MCA of the immobilized cells/ MCA of the same amount of free cells under identical conditions), and the rate of product synthesis. The specific productivity (U/g wet cells/h) is the key parameter. Due to the special application of MCE, the ratio of MCA/PA of the enzyme produced by immobilized cells should be considered. In all cases of our study, the MCA obtained from the immobilized cells was lower than that obtained from the same amount of free cells, and the EF of the immobilized cells was lower than one. This is because the immobilized cells represent a heterogeneous catalysis fermentation, in which the synthesis of primary or secondary metabolites is dependent upon the external and internal mass transported (Abdel-Naby *et al*., 2011).

Immobilized cells by entrapment

As shown in Figure 3A, *B. circulans* 25 cells were entrapped in different gel carriers (agar, Ca-alginate, κ-carrageenan and polyacrylamide). *B. circulans* 25 cells entrapped in κ-carrageenan gel showed the highest ratio of MCA/PA (4.6), highest specific productivity (4.17 U/ g wet cells/ h) and highest EF (0.613). This result is in line with that reported by Konti *et al*. (2016), who entrapped *Pseudomonas putida* DSM 437 cells in Ca-alginate with EF<1 (0.73). On contrary, Saieb *et al*. (2015) found that alginate immobilized *B.licheniformis* cells recorded higher enzyme production than free cells.

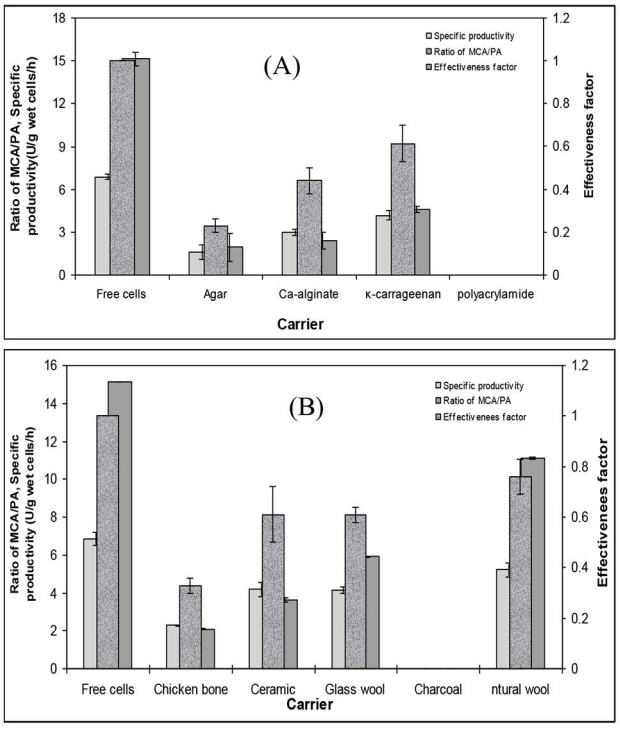


Figure 3. Immobilization of *B. circulans* 25 cells (A) by entrapment, (B) by covalent binding.

Immobilized cells by covalent binding

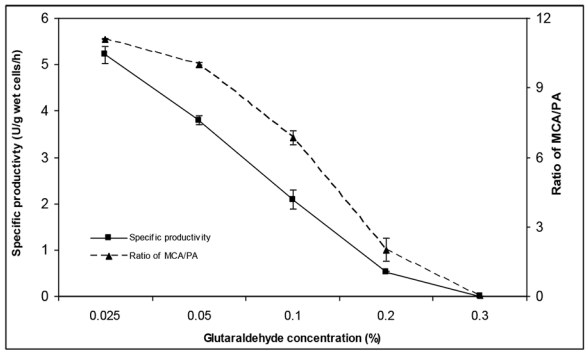
B. circulans 25 cells were covalently bounded to different treated carriers with GA (chicken bone, ceramic, glass wool, charcoal and natural wool) for MCE production and were compared with the same amount of the free cells. Data illustrated in Figure 3B showed that *B. circulans* 25 cells were successfully immobilized on natural wool with highest ratio of MCA/PA (11.11), highest specific productivity (5.21 U/ g wet cells/ h) and highest EF (0.76). Protocols for covalent biocatalyst immobilization using GA as spacer group often begin with a surface modification or activation step showing good loading efficiency and prevent the cells from leaking out. This good loading efficiency might be due to the formation of stable cross linking between functional groups present on the surface of the carrier and functional groups belonging to amino acid residues on the surface of the biocatalyst. In addition, covalent binding through a spacer group probably increased the local surface area of the carrier and consequently the biocatalyst molecule (Chang *et al*., 2008; AbdEl-Ghaffar and Hashem, 2010). The suitable carrier must have mechanical resistance against the conditions of applications, zero toxicity, size-flexible matrix, allowing internal cell growth for different microorganisms, and it must be non-biodegradable (Krasňan *et al*., 2016). Unlike

the materials for cells immobilization described in previous publications, natural wool not only has more compact structure and is easily manipulated, but also more reliable in the fabrication of various reactor geometries for continuous use. The immobilized *B. circulans* 25 cells on natural wool are highly recommended for dairy production.

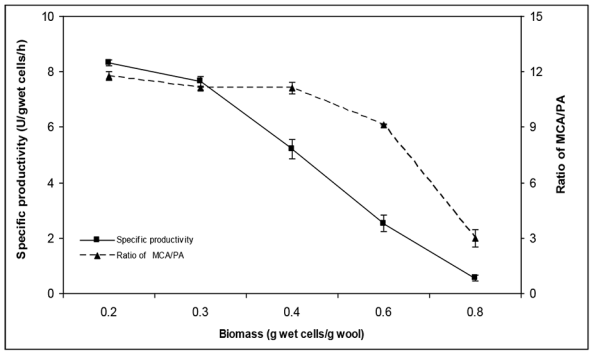
Optimization of immobilization process

Effect of glutaraldehyde concentration

The results (Figure 4a) showed that the highest MCA/PA ratio (11.11) with highest specific productivity (5.21 U/g wet cells/h) was obtained from treated wool with 0.025 % GA. Higher concentrations of GA led to a gradual decrease in MCA and MCA/PA ratio. On the other hand, the immobilized *B. circulans* 25 cells on treated wool with 0.3 % GA showed no MCA. Bagherinejad*et al*. (2012) reported that the optimum GA concentration for immobilization of *E. coli* cells was 5 %.



(a)



(b)

Figure 4. Effect of (a) glutaraldehyde concentration, (b) biomass loading on immobilization of *B. circulans* 25 cells on natural wool.

Effect of biomass loading

The apparent specific productivity of the immobilized cells depends on the amount of the biomass loaded on the immobilization carrier. As shown in Figure 4b the highest specific productivity (8.33 U/g wet cells/h) and highest MCA/PA ratio (11.77) were obtained with a cell load of 0.2 g wet cells/g wool. Gradual increase of the cell load up to 0.4 g decreased the specific productivity to about 62.5 % compared with that obtained at a cell loading of 0.2 g. The higher cell concentrations did not show any further improvement in enzyme production, which might be attributed to substrate limitations or product inhibition (Panesar *et al*., 2007).

Production of MCE by immobilized cells under optimum conditions

In batch

From the above experiments it can be concluded that immobilized *B. circulans* 25 cells on natural wool produced the highest ratio of MCA/PA (11.77) with the highest specific productivity (8.33 U/g wet cells/h) using 0.2 g wet cells/ g wool treated with 0.025 % GA after 24 h incubation at 35ºC and 180 rpm.

In repeated batch

In batch culture, both fermentation time and the production cycle led to a reduction in overall productivity and add to the production costs. Therefore, the possibility of reuse of immobilized cells was investigated. The optimized conditions for the immobilization and the optimized fermentation medium were used. Repeated batch fermentations (Figure 5) demonstrated the feasibility of using immobilized cells for multiple fermentation cycles. The *B. circulans* 25 cells immobilized on natural wool were reused to produce MCE successfully for 9 cycles (216 h) with productivity (1.5-1.66 U/h), MCA (0.71-0.80 U/mL) and the MCA/PA ratio remaining between 11.72 and 12.49. The loss in activity after that might be due to destruction of cells as well as autolysis during the centrifugation and washing process. The stability of the immobilized *B. circuulans* 25 cells is lower than that obtained by Ahmed and Abdel-Fattah (2010) in the production of protease by immobilized *B. licheniformis* ATCC 21415 cells on wool (480 h). Our result is higher than that obtained by Costa *et al*. (2015), who found that the productivity of immobilized *B. circulans* DF 9R cells was considered only for 6 cycles.

In continuous production

Continuous production of MCE by the immobilized cells of *B. circulans* 25 was accomplished in a

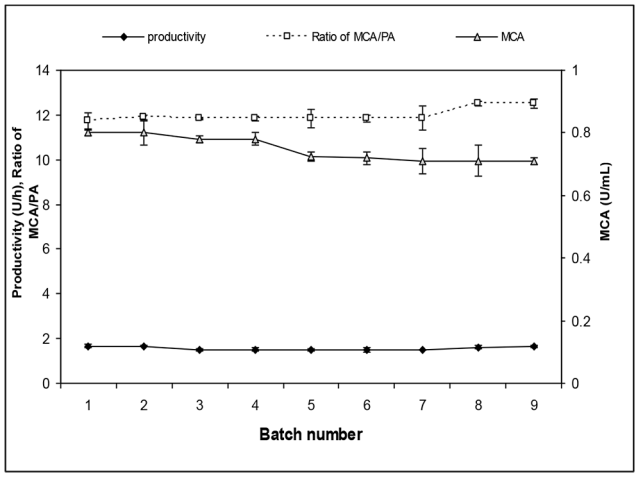


Figure 5. Production of MCE by immobilized cells in repeated batch operation.

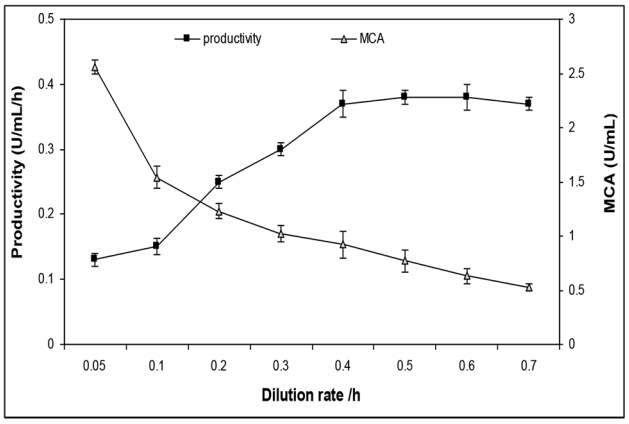


Figure 6. Effect of dilution rate on continuous production of MCE by immobilized cells.

packed-bed reactor. The fermentation was carried out in batch operation for 24 h, and then continuous operation was started. The flow rate varied between 11.6 -162 mL/h. The results (Figure 6) showed that maximal protein content (0.64 mg/mL) with highest MCA (2.56 U/mL) occurred at low dilution rate (0.05 /h) and gradually decreased as the dilution rate (decrease of residence time) increased. Similarly, Sirisansaneeyakul *et al*. (2007) suggested that increasing the dilution rate reduces the concentration of the product in the effluent. This trend was expected since the contact time between the medium and the immobilized cells was decreased as the dilution rate increased. The kinetic parameters of the bioreactor with different dilution rates (Table 1) indicated that the bioreactor productivity (dilution rate x enzyme activity) increased with the increase in the dilution rate up to 0.5 /h (residence time of 2 h), whereby maximal bioreactor productivity was attained of 0.385 KU/L/h. The performance of the operational stability of the bioreactor at different dilution rates indicted that, at the optimum dilution rate (0.5 /h), the bioreactor was able to keep producing MCA of about 0.7 U/mL with MCA/PA activity ratio of 11.0 for about 7 days at the same level. Then, the activity started to decline gradually to the level of 0.28 U/mL after 20 days of operation (data not shown). The long term viability and continuous metabolic activity is one of the most important advantages when working with continuous fermentation using immobilized biocatalyst

Comparison of MCA and productivity in various systems

Comparison of MCA and productivity of the free and immobilized cells of *B. circulans* 25 in various systems is presented in Table 2. The highest MCA was achieved by free cells (1.33 U/mL) in batch fermentation which is 33 %, 66 %, and 73 % higher than the immobilized cells in batch, repeated batch and continuous systems, respectively. On the other hand, the productivity of the immobilized cells under continuous operation in the bioreactor resulted in 0.385 KU/L/h, which is higher than the free cells, immobilized cells in batch and

Table 1. Kinetic parameters for the production of MCA by immobilized *B. circulans* 25 cells on wool in continuous culture.

Dilution rate (/h) (A)	Residence time (h)	Protein content (mg/mL)	MCA (U/mL) (B)	Total units of MCA (U/h)	productivity (U/mL/h) (A x B)	Specific productivities (U/g wet cells/h)
0.05	20.00	0.64	2.56	11.90	0.13	2.27
0.1	10.00	0.63	1.54	14.30	0.15	2.72
0.2	5.00	0.58	1.23	22.90	0.25	4.36
0.3	3.33	0.57	1.00	27.90	0.30	5.31
0.4	2.50	0.57	0.92	34.34	0.37	6.54
0.5	2.00	0.56	0.77	35.76	0.38	6.81
0.6	1.66	0.54	0.63	35.15	0.38	6.69
0.7	1.43	0.52	0.53	34.76	0.37	6.62

Table 2. Comparison of MCA and productivity in various systems.

Production systems	MCA (U/mL)	MCA / PA ratio	Rate of enzyme production (U/h)	Specific productivity (U/g wet cells/h)	Productivity (KU/L/h)	Relative productivity (- fold)
Free cells Batch	1.33	15.10	2.77	13.88	0.055	1.00
Immobilized cells						
1. Batch	1.00	11.11	2.08	10.40	0.042	0.76
2. Repeated batch (Average of 8 cycles)	0.80	11.08	1.66	8.33	0.033	0.61
3. Continuous (dilution rate 0.5/h)	0.77	17.70	89.32	17.01	0.385	7.00

in repeated batch operations by 7, 9.2 and 11.7-fold, respectively. Gungormusler *et al*. (2011) reported that the production in a packed-bed bioreactor designed with an immobilization system achieved 2.5-fold higher productivity compared to the free system.

CONCLUSIONS

Cell immobilization is a technique that restricts the free movement of microorganisms in the process, and it has many advantages. Natural wool is an attractive and suitable as an immobilization carrier because it is cheap, available and non-toxic. Immobilization of *B. circulans* 25 cells on natural wool by covalent binding is promising for milk-clotting enzyme production by batch, repeated batch and continuous fermentation. In repeated batch, immobilized cells on natural wool were able to produce the same level of MCA, productivity and MCA/PA for 9 cycles (216 h). Under continuous operation, immobilized cells in a packed-bed bioreactor were able to keep producing MCA with MCA/PA ratio for about 7 days at the same level. Moreover, continuous operation produces productivity of 0.385 KU/L/h which is higher than the other systems by 7.0-11.7 fold. The long term viability and continuous metabolic activity is one of the most important advantages when working with continuous fermentation using an immobilized biocatalyst.

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