Jahangirnagar University J. Biol. Sci. **6**(2): 29-38, 2017 (December)

Repeated batch fermentation for protease production using biofilm fermentation of *Bacillus sp.*

Ripa Moni, Mohammed Salahuddin[1], Md. Abdullah Al Noman Khan, Umme Salma Zohora and Mohammad Shahedur Rahman*
Department of Biotechnology and Genetic Engineering, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

Abstract

Repeated batch fermentation is a stable, economically viable and environment friendly fermentation system. Biofilm of *Bacillus subtilis* was investigated for repeated batch fermentation for the production of protease enzyme. It was found that complete replacement of spent medium with fresh medium supplementation in biofilm produced similar amount of protease that is about 225 U/ml of enzyme in successive batches. Moreover, unlike some solid carriers the EPS layer does not entrap much enzyme inside it. Therefore, instead of using immobilized cells, biofilm of *B. subtilis* can be a good candidate for protease enzyme production using repeated batch fermentation.

Key words: *Bacillus subtilis*, biofilm, repeated batch, protease enzyme

INTRODUCTION

For production of various secondary metabolites, several fermentation processes are being used to run a stable, economically viable and environment friendly system. Repeated batch is one of the fermentation procedures that is reportedly useful and advantageous over other fermentation systems (Abdel-Naby *et al.*, 2000; Carmichael *et al.*, 2000). In repeated batch fermentation microbial cells are reused for subsequent fermentation runs. For the reuse of microbial cells, the cells usually need to be immobilized in solid carriers. Immobilized microbial cells are frequently applied for bioconversion, biotransformation and biosynthesis processes due to their better operational stability, easier separation from products for possible reuse, and satisfactory efficiency of catalysis as compared to free cells (Kroutil *et al.*, 1998; Pakula & Freeman, 1996; Gill & Ballesteros, 2000). Furthermore, cells and enzymes seem to be protected by matrices (Liu, 1998; Jirku, 1999; Junter *et al.*, 2002) and physiology of immobilized viable cells can be favorably modified in comparison to free cells (Abdel-Naby *et al.*, 2000; Angelova *et al.*, 2000; Junter *et al.*, 2002). A list of the immobilized microbial cells those are used for production of enzymes are summarized in Table 1. However, immobilization of microbial cells has some drawbacks. One of the biggest drawbacks of addition of solid carriers is their limited feasibility for industrial use. Carriers are usually expensive and require laborious pretreatment before application. Entrapment or

[1] Life Science Division, Overseas Marketing Corporation (Pvt.) Limited, Dhaka, Bangladesh
* Corresponding author. Email: rahmanms@juniv.edu

adsorption of the product with the solid carrier causes limited extraction of the target metabolite. Addition of carriers creates at least one or more step burdens during the purification process. Degradation of the carrier material also creates much complexity during product separation and purification (Xiu *et al.,* 2000).

Table 1. Enzyme production by different immobilized microbial cells

Immobilized cells	Enzyme	Support material for immobilization	References
Yeast	Peptidase	ENT-2000	(Dabdoub Paz *et al.*,1993)
Humicolalutea	Protease	Wort agar	(Grozeva *et al.*,1993)
Aspergillus niger	Pectinase	Wheat	(Fiedurek *et al.*,1995)
Clostridium sp.	Pullulanase	Ca-alginate	(Klingeberg *et al.*,1990)
E. coli	Penicillin acylase	Polyacrylamide agar	(Prabhune *et al.*,1992)
E. coli	α-amylase	k-carrageenan	(Ariga *et al.*,1991)
Bacillus megaterium	β-amylase	Acrylamide	(Ray *et al.*,1995)
Aureobasidium pullulans	Glucoamylase	Ca-alginate	(Federici *et al.*,1990)
Aspergillus niger	Glucose oxidase	Wheat, rye	(Fiedurek *et al.*,1994)
Aspergillus clavatus	Ribonuclease	PVA	(Manolov, 1992)
E. coli	Alkaline Phosphatase	k-carrageenan	(Manin *et al.*,1989)
Micromonospora chalcae	Chitinase	Ca-alginate	(O'Riordan *et al.*,1989)
Yeast	Invertase	Polymer	(Aykut *et al.*,1988)
Trichoderma reesei	Cellulase	Cellulosic fiber	(Kumakura *et al.*,1989)
Chrysosporium thermophile	Lipase	Alginate	(Johri *et al.*,1990)
Phanerochaete Chrysosporium	Lignin Peroxidase	Polystyrene divinylbenzene	(Ruckenstein and Wang, 1994)
Aspergillus sydowii	Xylanase	Ca-alginate	(Ghosh and Nanda, 1991)
Bacillus cereus, Bacillus circulans	CGTase	Ca-alginate	(Jamuna *et al.*,1993; Saswathi *et al.*,1995)
Caldariomyces fumago	Chloroperoxidase	k-carrageenan	(Carmichael *et al.*,1986)

Immobilization of *Bacillus subtilis* for repeated production of various products have already been reported (Adinarayana *et al.,* 2004; Kokubu *et al.,* 1978; Konsoula and Liakopoulou-Kyriakides, 2006; Shih *et al.,* 2010). *B. subtilis* forms biofilm in static culture and can produce several secondary metabolites in this condition. The production of lipopeptide antibiotic iturin A by *B. subtilis* biofilm fermentation has been reported earlier (Rahman & Takashi, 2009; Rahman *et al.,* 2007; Zohora *et al.,* 2009).

However, repeated production of enzyme using *B. subtilis* biofilm is a novel technique. Biofilm is quite similar to immobilized cells. In biofilm, bacterium lives in a customized microniche, which is a complex microbial community that has primitive homeostasis, circulatory system and metabolic cooperation. Each of these cells reacts to its special environment so that it differs fundamentally from planktonic cell of the same species (Osterton *et al.,* 1995). The biofilm matrix is a highly complex array of micro-

environments. The different components within the biofilm like water, polysaccharides and other macromolecules offer a range of localized and constantly changing effects that generate osmotic and nutrient gradients. In any biofilm, these will contribute to the heterogeneous composition and the multi-cellular function of the matrix (Sutherland, 2001).

In this study, biofilm fermentation of *B. subtilis* was carried out in a production medium to produce extracellular protease. Biofilm fermentation offers easy microbial growth, production and purification steps. *B. subtilis* RB14 develops biofilm on the liquid medium in between the liquid – air interface. For this reason, separation of the spent liquid medium offers almost cell free enzyme removal. Following the withdrawal of the spent medium, biofilm was supplemented with freshly prepared medium and incubated for another batch for protease production. Production was observed without using any carrier for protease fermentation using biofilm. The aim of this study was to investigate the enzyme production using biofilm of *B. subtilis* in repeated batch fermentation.

MATERIALS AND METHODS

Inoculum preparation: *B. subtilis* RB14 strain was transferred from culture stock (kept at -20°C in 20% glycerol solution) into 5ml of TSB (Tryptic Soy Broth) medium (Hi-Media, India) and incubated overnight at 37°C, 120 strokes per minute (spm) in water bath shaker incubator.

Repeated batch fermentation: Three beakers (100 ml) each containing 20 ml No.3 medium (Zohora *et al.*, 2009) were used. The medium is composed of 3% Peptone (Uni-chem, China), 1% D-Glucose (Merck, Germany), 0.1% KH_2PO_4 (Scharlau, Spain), and 0.05% $MgSO_4.7H_2O$ (Merck, India). Initial pH of the medium was 6.8. One percent inoculum was used in this experiment. For this, 200 µl of freshly prepared inoculums was transferred in each of the 20 ml medium containing beakers. Then the beakers were kept in incubator at 37°C as standing culture for biofilm fermentation. Repeated batch fermentations were carried out in the above-mentioned three beakers as repeated batch fermenters as described in Figure 1.

Enzyme preparation: After 24 (day 1), 48 (day 2), 72 (day 3), 96 (day 4), 120 (day 5) and 144 hours (day 6), the pipetted out spent medium were centrifuged at 10000 × g for 20 minutes using bench top centrifuge (Eppendorf 5418, Germany). Then the supernatants were collected as crude enzymes from each batch *viz.* repeated batch 1, repeated batch 2, repeated batch 3, repeated batch 4 and repeated batch 5 respectively. After 144 hours, 10 ml spent medium and 10 ml spent medium with homogenized biofilm were taken and centrifuged at 10000 × g for 20 minutes and the supernatants were collected as crude enzymes of repeated batch 5 and repeated batch 5 homogenized sample respectively. These procedures of enzyme preparation were followed for all the three processes of repeated batch fermentations. After enzyme collection, protease activity was assayed.

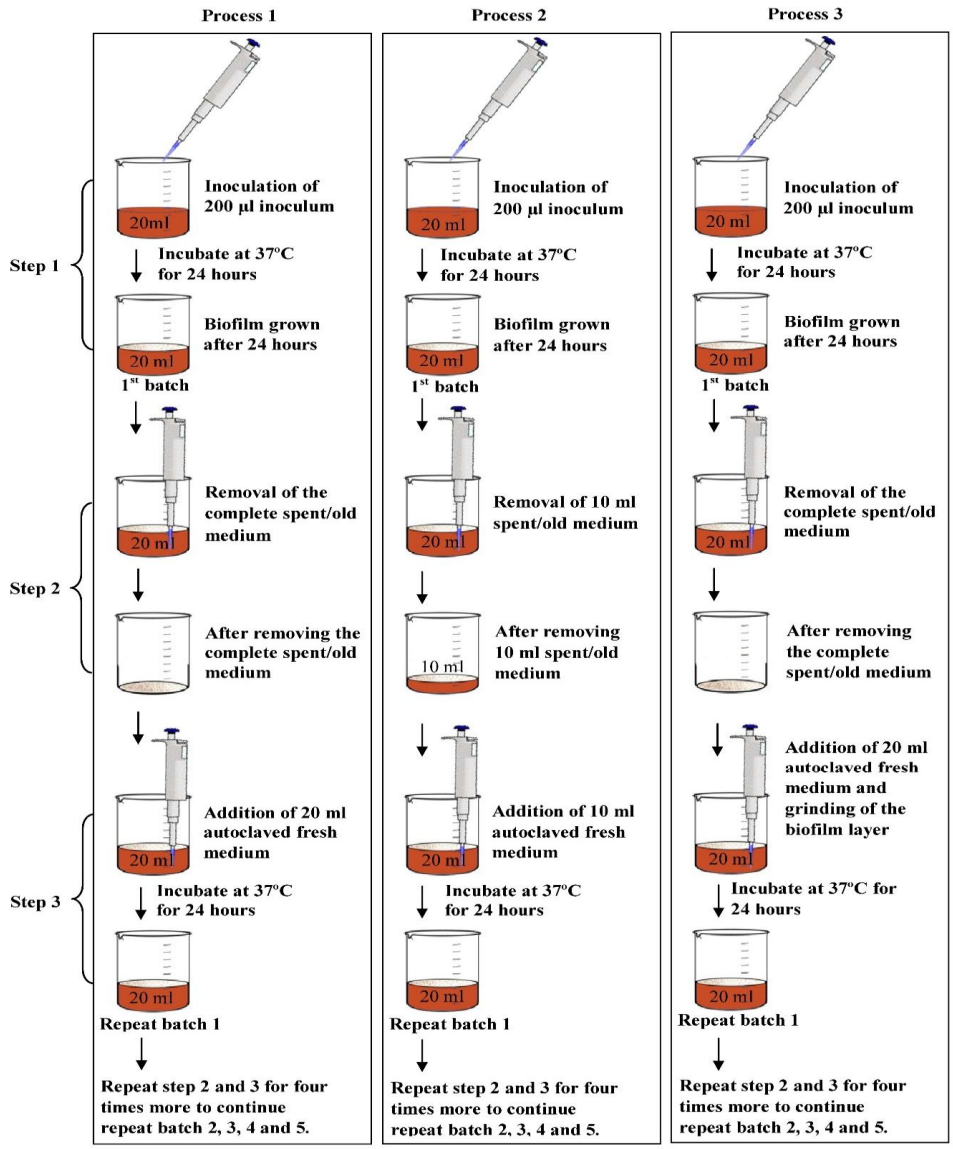

Fig. 1. Illustrated representation of repeated batch biofilm fermentation processes. Process 1: complete removal of the spent medium (20 ml) with fresh autoclaved No.3 medium after 24 hours every time. Process 2: replacement of half of the spent medium (10 ml) with fresh autoclaved No.3 medium after 24 hours every time. Process3: complete removal of the spent medium (20 ml) with fresh autoclavedNo.3 medium after 24 hours and grinding the biofilm layer every time

Protease activity assay: To measure protease activity of the crude enzymes, 100 µl of 1% azocasein [0.1 g azocasein (Sigma, Germany) dissolved in 10 ml phosphate buffer solution] was added to 200 µl of crude enzyme, mixed well by vortexing and incubated at 37 °C for 30 minutes. Then 700 µl of trichloroacetic acid (TCA) (BDH, London) was added and kept in ice for 20 minutes. After centrifugation at 15000 × g for 5 minutes, 600 µl supernatant was added to 600 µl 0.5 M sodium hydroxide (Merck, Germany) and measured optical density (OD) at 440 nm using spectrophotometer (Mecasys Optizen Pop UV/Vis Spectrophotometer, Korea). Blanks were prepared by following the same procedure except crude enzyme was added after adding TCA. From the $OD_{440,}$ enzyme activity (U/ml) was measured. One unit (U) of enzyme is defined as change in absorbance at 440 nm of 0.01 /h (Kohlmann *et al.,* 1991).

RESULTS AND DISCUSSION

Repeated batch fermentation is possible using the same biofilm, which was produced in the very first batch. Repeated batch biofilm fermentation was conducted to achieve a repeated supply of protease enzyme by repeatedly supplementing fresh medium. This investigation showed that complete replacement of old medium continuously produced approximately same amount of protease enzyme in five batches as shown in figure 2a. Partial replacement of old medium produced lower amount of protease enzyme with increased repeated batch number (Fig. 2b). When old medium was completely replaced and biofilm was homogenized after each batch, protease production was successively decreased in every batch (Fig. 2c). So complete replacement of old medium without homogenizing the biofilm layer was found to be the better way of biofilm based repeated batch fermentation to achieve a continuous supply of approximately constant amount of protease enzyme (Table 2).

Table 2. Results of three repeated batch biofilm fermentations

Type	Biofilm thickness	Enzyme production	Remarks
Full replacement of old medium	Increased with time	Stable in all batches	Can be continued for large number of repetition
Half replacement of old medium	Increased with time	Slightly reduced in each successive batches	Can be continued for small number of batch repetitions
Full replacement of old medium and homogenization	Decreased with time	Gradually reduced in each successive batches	Can not be continued for batch repetitions

When the process was repeated without involving any further inoculation or earlier steps, it took only 6 days for producing six consecutive batches, and the same amount of product was produced as it can be seen from their activity profile of the produced enzymes (Figure 2a). When biofilm was homogenized at the end of the repeated enzyme production, higher amount of protease enzyme was obtained than that in non-homogenized biofilm sample (Fig. 2a and 2b). In repeated batch fermentation process 3,

where old medium was completely replaced with fresh medium and old biofilm was disintegrated by homogenization, it was observed that the disintegrated biofilm particles could not reform (Fig. 2c). For this reason in every repeated batch, a new very thin biofilm was found. But in process 1 and 2, with increased repeat batch number, the biofilms gradually got thicker, increased their rigidity and was with reduced wrinkles (Fig. 3).

Repeated batch fermentation for protease enzyme production has been successfully observed using biofilm of *B. subtilis* RB14. It was observed that complete replacement of old medium with fresh medium supplementation every time, produces similar amount of protease enzyme. No additional carrier was required to use because biofilm itself performed the action of a carrier to retend the cells within its' matrix. Effective application of biofilm is promising with repeated batch fermentation. In biofilm structure, cells are abundant and remain in active metabolic stage for a longer period of time (Lindsay *et al.,* 2005). Consecutive supply of nutrients keeps the cells at their metabolic stage for production of protease enzyme. It can be expected that optimization of the culture conditions and effective use of suitable medium components will offer higher number of batch repetitions and higher production rate of protease enzyme. Therefore, repeated batch fermentation using biofilm can be a new and attractive technique for large-scale enzyme production.

Complete replacement of old medium without homogenizing the biofilm layer is the best way of biofilm based repeated batch fermentation to achieve a continuous supply of approximately constant amount of protease enzyme because it produces similar amount of protease up to repeated batch 6 and will produce equal amount of enzyme in successive batches. Moreover, this is a time saving procedure. In batch fermentation, it takes 2 days for each batch those include the first day for inoculum preparation and the second day for fermentation for protease production. Therefore, for 6 consecutive batches it will take 12 days, but in this study the best repeated batch fermentation process only takes 6 days in addition to a single inoculation leaving the inoculum preparation requirement for production in every steps, as a result, this process offers higher productivity. As productivity is related with time, it can be said that, in this process, productivity is higher than that in normal batch fermentation process, and with increased repeated batch number productivity will increase exponentially.

Disintegrated biofilm particles cannot reform. In biofilm state, bacterial cells are surrounded by a matrix composed of extracellular polymeric substances (EPS), secreted by the bacterial cells. EPS are mainly polysaccharides, proteins, nucleic acids and lipids; they provide the mechanical stability of biofilm, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells (Flemming *et al.,* 2007). When biofilm layer is homogenized, the EPS layer is disintegrated. Later the disintegrated EPS cannot integrate itself but the bacterial cells make new EPS and form new biofilm layer. That is possibly the reason why, in repeated biofilm fermentation process 3, in every batch new thin biofilm layer was seen (Fig. 3). At the end of the each fermentation, increased amount of protease enzyme was observed when biofilm layer was homogenized and assayed (Fig. 2a, 2b and 2c).

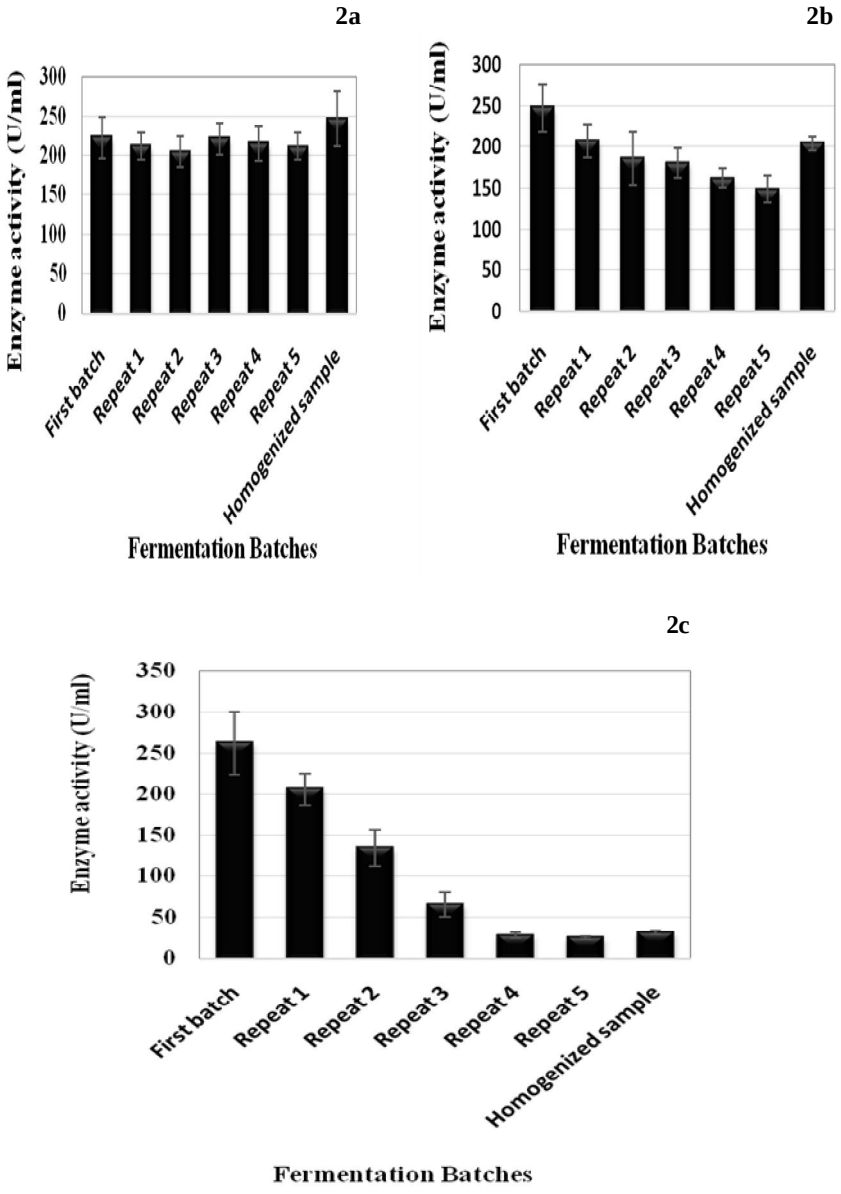

Fig. 2. Production of protease enzyme in repeated batch fermentation (solid bars) using *B. subtilis* biofilm in three processes. Repeated batches were carried out by removing spent medium in complete (2a), removing partial spent medium (2b) and complete spent medium removal with homogenization (2c) and supplemented with fresh medium respectively after 24 h in each batches (n ≥3)

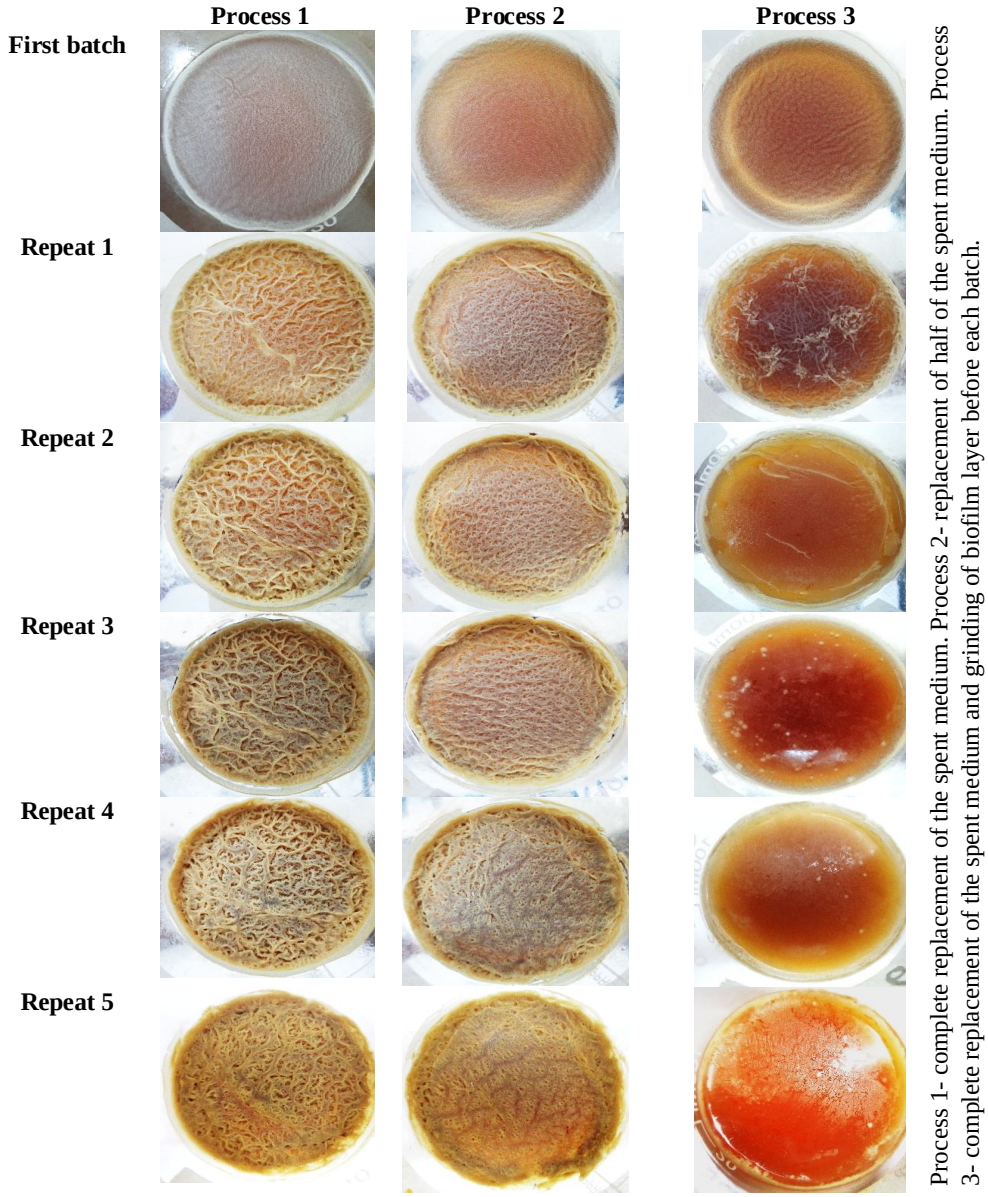

Fig. 3. Biofilm formation in repeated batch fermentations. With increased batch repetition, biofilm layer got thicker in process 1 and 2 but in process 3, biofilm layer got thinner

In biofilm, bacterial colonies are surrounded by EPS. Bacterial cells produce protease enzymes inside this EPS and from this, protease enzymes diffuses to the medium below the biofilm layer. Thus, protease is produced in the medium below the biofilm layer.

However, some protease enzymes remain entrapped inside the EPS layer. When biofilm was homogenized, the EPS layer disrupted, and the entrapped protease enzyme was released. For this reason, when biofilm layer was homogenized, increased amount of protease was produced. The amount of entrapped enzyme was found to be low and can be extracted at the end of the fermentation, so the use of biofilm for repeated batch fermentation for protease production might be a worthy and useful idea.

Acknowledgement: This research is supported by the grant (No. 39.00.0000.012.002.01.03.004.16-154/838) sponsored by Ministry of Science and Technology, Government of Bangladesh.

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