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# Biotransformation of Soybean Saponin to Soyasapogenol B by Immobilized *Aspergillus Parasiticus* Cells on Polyurethane Foam.

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**ABSTRACT**

Soyasapogenol B, aglycone of soybean saponin, is known to have hepatoprotective, antimutagenic, antivirus, and anti-inflammatory activities. Flexible polyurethane foam (PUF) was the best immobilization carrier among five porous carriers testedfor immobilization of *Aspergillus parasiticus* cells for soyasapogenol B production from soybean saponin. Best yield of 79.45 % soyasapogenol B was obtained using only 50% of the biotransformation medium contents supplemented with five polyurethane foam discs loaded with $10^5$ /ml spore suspension after 72h in the first cycle. This yield was higher thanthat produced by free cells (65 %) in full medium contents after 72h. Additionally, repeated batch biotransformation with immobilized fungal cells on flexible PUF could be successfully maintained for fifteen days and fermentation time could be reduced to 48h in the successive cycles (after the first one). The highest yield of soyasapogenol B (89.40 %)was reached on the third batch.

**Keywords:** Soybeansaponin, soyasapogenol B, immobilization, *Aspergillus parasiticus*, biotransformation, polyurethane foam.

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## INTRODUCTION

Saponins, which are glycosides of triterpenoids or steroids, are widely distributed in the plant kingdom. Among them, soyasaponins are triterpene glycosides with aglycones of oleanene skeleton like soyasapogenols. These molecules are found in various leguminous plants, *e.g.*, soybean, adzuki bean, peanut, and alfalfa [1]. SoyasapogenolB, obtained from soybean saponin, is known to have various physiological activities, such as anticomplementary platelet aggregation suppressing effect, preventive and therapeutic activity forimmune diseases and growth suppressing effect on cells derivedfrom human ovarian cancer, human breast cancer, hela and hepG2 cells [2]. This aglycone is produced by acid hydrolysis of soybean saponin, but there have been reports of aglycone production by microorganisms or enzymes.

*Aspergillus parasiticus* was screened and selected on the basis of its ability to hydrolyze soybean saponin to soyasapogenol B, while optimizing its production conditions [3].The morphology of fungi is playing a fundamental role in determining the overall process of productivity. In addition, the manipulation of fungal morphology resulted in an increased metabolite yield [4]. Immobilized microbial cell systems could provide additional advantages over freely suspended cells such as simple reuse of the biomass, easier liquid–solid separation and minimal clogging in continuous-flow systems [5].There are essentially four different procedures available for cell immobilization: adsorption, entrapment in gels or polymers, covalent coupling, and cross-linking to insoluble matrices [6].The nature of the cell to be immobilized, the nature of the substrates and products formed, and the culture conditions are major factors for the choice of the matrix and the immobilization procedure [7]. Adsorption techniques reduce problems associated with oxygen diffusion anddo not show drawbacks associated with scaling-up, which are evident with encapsulation matrices. These methods represent particular forms of cellular adhesion based on the ability of certain microorganisms to adhere to solid surfaces by means of polymucosaccharide secretion [7].

Hence, the objective of the present study was to investigate the capability of *A. parasiticus* cells to be adsorbed on the surface of different porous carriers and eventually produce soyasapogenol B from soybean saponins and comparing the product formation with that produced with free cells. Thus, capacity of cells to adhere to the carrier, as well as their capacity to produce soyasapogenol B was evaluated.

## MATERIALS AND METHODS

### Cultureand media

*Aspergillus parasiticus* used in this work was donated by the Center of Cultures of Chemistry of Natural and Microbial Products Department, National Research Center, Cairo, Egypt. It was maintained on potato dextrose agar (BiolifeItaliana, Italy).Unless otherwise stated,s oyasapogenol B production was carried out inmedium composed of (%, w/v): 4malt extract, 2yeast extract, 0.5galactose, 0.2$KH_2PO_4$, 0.2$(NH_4)_2SO_4$, 0.03$MgSO_4.7H_2O$, 0.03$CaCl_2.2H_2O$, and 3 soybean saponin (50%, Organic Technologies Co. USA), (pH 8) [3].

**Spore inoculum preparation**

For spore production, the strain was grown on PDA plates at 30°C ± 2 for 7days. The agar plates containing sporulative fungi were washed with a sterilized solution of 0.1% (w/v) Tween 80 to obtain spore suspension, which was counted with a hemacytometer under a microscope and used for inoculation.

**Carriers' preparation and cell immobilization**

Five porous carriers were tested: wool, rigid PUF, flexible PUF, linen and jute. Prior to use, the carriers were pre-treated by boiling for 20 min and washed thoroughly twice with distilled water. Thereafter, these materials were dried overnight at room temperature [8]. Three wool squares measuring 2×2cm; three jute squares measuring 3×3 cm; three linen squares measuring 2×2 cm and four Polyurethane foam discs of 2×3 cm were used. Erlenmeyer flasks (250 ml) containing 100 ml growth medium and 0.388 g wool, 0.7327 g jute, 0.6513 g flexible PUF, 0.3825 rigid PUF or 0.2339g linenwere inoculated with a freshly prepared spore suspension of $10^7$ / ml. The inoculated flasks were incubated in a shaking incubator at 30$^o$C and 150 rpm for 72h. Cells were immobilized in situ in the flasks by natural adsorption on the immobilizing material. After incubation at 30$^o$C for 72h in a shaking incubator at 150 rpm, the matrices with immobilized cells were removed, washed and then transferred into new flasks each containing 100 ml of the biotransformation medium. Biotransformation was carried out at 30$^o$C and 150 rpm in a shaking incubator for 72h. For comparative purposes, transformation with free cells in flasks without the immobilization matrix was also carried out under the same culture condition. All batches of biotransformation were duplicated and averaged data were reported.

**Repeated batch fermentation using *A. parasiticus* cellsimmobilized onflexible PUF**

The biotransformation medium was decanted (at the end of each cycle) and the carrier discs were carefully squeezed and washed with distilled water. The discs were then aseptically transferred to freshly prepared and sterilized biotransformation medium. Biotransformation was carried out at 30$^o$C and 150 rpm in a shaking incubator.

**Quantitative estimation of the biomass**

Free cell mass concentration was determined by dry weight per vol. (g/100ml). At the end of fermentation, cells were collected by filtration of broth samples through pre-weighed filter papers. Washed with distilled water and dried at 80$^o$C to constant weight. The amount of cells attached to different carriers wasdetermined after washing the support material with distilled waterfor three times and drying at 80$^o$C to constant weight. Biomass dry weight was calculated as the difference between the weight of mycelium plus carrier and that of the carrier itself.

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**Quantitative analysis of soyasapogenolB**

At the end of incubation period, the reaction mixture was extracted twice with double its volume of ethyl acetate. Then, the organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The residue was taken up in chloroform - methanol mixture (1:1) and mounted on TLC plates. The plate was chromatographed for soyasapogenol B witha solvent system of benzene:ethyl acetate:acetic acid (12:4:0.5,v/v/v). Soyasapogenol B was detected on TLC plates by spraying with 10 % $H_2SO_4$, then heated for 10 min at 110°C and quantitatively analyzed with a TLC-scanner(Shimadzu CS-9000 dualwave-length flying spot, thin layer chromato-scanner,Japan) at λ = 530 nm[9].Calculation of obtained weight of soyasapogenol B was determined by calibration of the line obtained from authentic sample using the area under the curve of biotransformation products in each chromatogram.

Soyasapogenol B molar yield (%)

$$= \frac{[\text{weightof soyasapogenol B /MW of soyasapogenol B}]}{[\text{Weight of soyasaponin I /MWof soyasaponin I}]} \times 100$$

Where MW is the molecular weight; soyasaponin I represents soybean saponin.

## RESULTS AND DISCUSSION

### Biotransformation of soyasaponin to soyasapogenol B using *A. parasiticus* cells immobilized on different carriers

**Table 1: Soyasapogenol B production by *A. parasiticus* cells immobilized using different carriers and concentration of immobilized cells.**

| Immobilization carrier | Soyasapogenol B (mg/100ml) | Molar yield (%) | Immobilized cells (g/g carrier) |
|---|---|---|---|
| Wool | 289.68 | 40.56 | 1.433 |
| Jute | 267.47 | 37.45 | 1.211 |
| Flexible PUF | 299.82 | 41.98 | 1.676 |
| Rigid PUF | 255.40 | 35.76 | 0.029 |
| Linen | 173.40 | 24.27 | 0.020 |
| Free cells (No carrier) | 464.24 | 65.00 | ..... |

*A. parasiticus* was immobilized separately on 0.388 g wool, 0.7327 g jute, 0.6513 g flexible PUF, 0.3825 rigid PUF and 0.2339g linen. Transformation culture medium consisted of (%, w/v): malt extract, 4; yeast extract, 2; galactose, 0.5; $KH_2PO_4$, 0.2; $(NH_4)_2SO_4$, 0.2; $MgSO_4.7H_2O$, 0.03; $CaCl_2.2H_2O$, 0.030; and soybean saponin 3 (pH 8). Initial spore concentration: $10^7$/ml; 30°C; agitation rate: 150 rpm; fermentation period: 72h. Values are the average of three independent experiments and the maximal mean deviation is ±5%.

As shown in Table 1, *A. parasiticus* cells were able to adhere to the different porous materials,but with different capacities.Relatively high quantity of cells adhered to jute

(1.211g/g carrier) and wool (1.433g/g carrier), but the highest cell masses adhered to flexible PUF (1.676g/g carrier). However, linen and rigid PUF showed the low immobilized cells concentration.Cellsare adhered to the solid support surfaces by vanderwaals forces, and ionic or covalent interactions [10]. Moreover, when the pore size of the matrix is small, bearing in mind the dimensions of the cell, adsorption occurs only at the surface, as in the case of clays, and other related materials. However, when the carrier has pores that are large relative to the dimensions of the cell, it is possible to find adhesion within the pores [8]. This situation occurs in materials such as PUF, which is commonly employed for the attachment procedure [11].

With regard to soyasapogenol B production, flexible PUF resulted in the highest production (299.6 mg/100mL) of soyasapogenol B, corresponding to 41.98 % molar yield, which represented about 64.58 % of amount produced by free cells (65 %). However, jute achieved the lowest results (37.45%).It could be visualized that the fungus was immobilized internally and externally to the flexible PUF, and gradually grew as a dense layer on the surface, so that at the end of biotransformation, the entire carrier was covered with cells.This may be due to the fact that PUFhas high porosity (near 97%) and therefore has a large adsorption surface [12].Another important advantages of this immobilization support is that oxygen diffusion problems can be reduced as a result of its large pore size, which a particularly relevant factor for aerobic microorganisms [12]. This explains why the medium containing flexible PUF as a carrier gave the highest biotransformation parameters. Hence it was chosen for the following experiments.

**Effect of biotransformation medium concentration on soyasapogenol B production**

Soyasapogenol B molar yield using the total concentration of the biotransformation medium inoculated with immobilized *A. parasiticus* cells (41.98%) was lower than that produced by fungal free cells in total medium (65%) content by about 16%.The decrease in activity of immobilized compared to the free cells was attributed to diffusion problems caused by excessive mycelial growth [13].In an attempt to minimize the contents of the biotransformation medium to overcome the excessive mycelial growth, three different concentrations of the biotransformation medium were examined;quarter, half and total medium content.As shown in Figure 1, the maximum soyasapogenol B molar yield (54.26%) isachieved using half medium content which was higher than that produced by fungal free cells (36.76%)in half medium content. This yield represented about 83.47 % of the amount produced by free cells in full medium content.Further increase in the medium concentration (total medium concentration) resulted in a decrease in soyasapogenolB molar yield. This may be due to excessive mycelial growth obtained using full medium contents resulted in thick biofilm growth on PUF discs which led to diffusion problems. Many authors reported that the problems arising from continued cell growth were: increasedbiofilm thickness and reduced mass transfer [2, 8, 14]. On the other hand, lower medium concentration (quarter medium concentration) gave lower yield of soyasapogenolB (12.72 %). This could be attributed to the reduced contents in the latter biotransformation medium.

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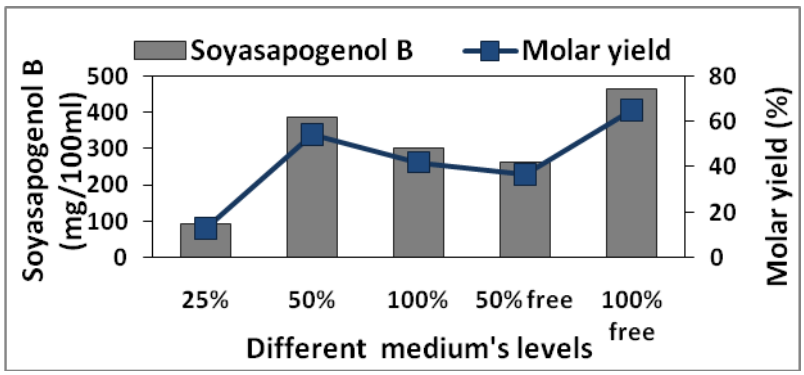


**Figure 1: Effect of biotransformation medium concentration on soyasapogenol B production by *A. parasiticus* cells immobilized on PUF.**

Fermentation medium: three different concentrations were examined; quarter, half and total biotransformation medium content; initial spore concentration: $10^7$/ml; number of PUF discs: 4 discs; 30 ◦C; agitation rate: 150 rpm; fermentation period: 72 h. Values are the average of three independent experiments and the maximal mean deviation is ±5%.

## Effect of PUF foam discs count on soyasapogenol B production

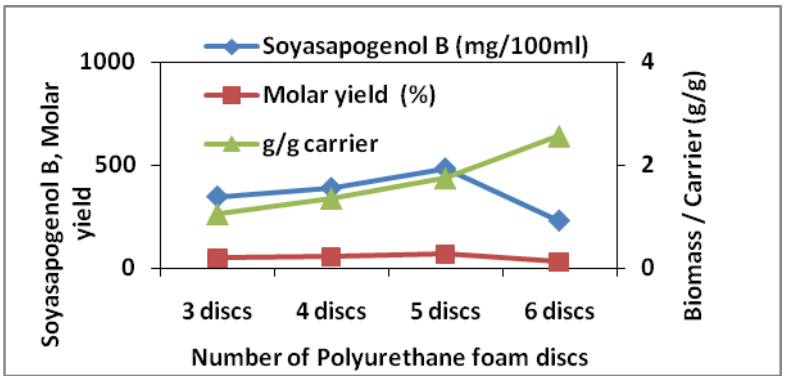


**Figure 2: Effect of PUF discs count on soyasapogenol B and biomass.**

Fermentation medium: 50% of transformation culture medium; 30°C; initial spore concentration: $10^7$/mL; agitation rate: 150 rpm; fermentation period: 72 h. Values are the average of three independent experiments and the maximal mean deviation is ±6%.

The growth medium was supplemented with different number of PUF discs (3, 4, 5 and 6). SoyasapogenolB production was positively responded to the increase of PUF discs number and the maximum soyasapogenolB molar yield (67.49%) was achieved using five PUF discs (Figure2).Increasing soyasapogenol B yield with increasing the number of the discs may be referred to the increase of exposed surface area of the fungal mycelia, which consequently reduces mass transfer limitations and allowing easy access to substrate [15]. On the other hand, further increase in the number of the carrier discs was accompanied by the production of lower yields of soyasapogenol B. With three discs, there was a limited surface area for biofilm growth and the mycelia grew into a large clump, which severely limited oxygen and decreased substrate transfer into the inner cell biomass. Reduced oxygen availability as well as substrate

diffusional resistance in the large mycelial clump, resulted in lower yield of soyasapogenol B in case of fermentation with lower disc count (3 discs)[15].

**Effect of spore concentration on soyasapogenol B production**

The inoculum size or the initial spore number in suspension can affect the fungal morphology and subsequent fermentation performance.It was reported that the spores did not adhere on the carrier and did not aggregate with each other. They remained isolated in suspension during swelling and germination. They became immobilized only when their germ tube was long enough to be mechanically retained by the carrier[16].The appropriate spore inoculum size and its effect on biotransformation can be highly specific to the used immobilization matrix [17].The effect of initial spore concentration on fermentation wasinvestigated in flasks with fivePUF discs at a spore concentrationrange of $10^5$–$10^7$/ml(Table 2).The highest yield of soyasapogenol B (79.45 %) was obtained at the inoculation level of $10^5$/ml. Thereafter, biotransformation values decreased gradually with the increase of initial spore concentration. This may be due to formation of a thick mycelia mat over the PUF discs, which reduces the availability of the fungal cells to the fermentation medium containing soyasaponin and consequently lowers fungal cells productivity.This fact was agreed with Rahman*et al.*[18], who stated that high inoculum sizes may not necessarily give higher production yield as they could result in the lack of oxygen and nutrient depletion in the culture media.

**Table 2: Effect of inoculum size loaded on PUF discs on soyasapogenol B production and biomass.**

| Inoculm size $10^n$/ ml | Soyasapogenol B (mg/100 ml) | Molar yield (%) | Immobilized cells (g/100 ml) | Biomass/carrier (g/g) |
|---|---|---|---|---|
| $10^3$ | 298.60 | 41.81 | 0.9835 | 1.207 |
| $10^4$ | 359.17 | 50.29 | 1.3332 | 1.6374 |
| $10^5$ | 567.42 | 79.45 | 1.7608 | 2.1625 |
| $10^6$ | 511.43 | 71.61 | 1.497 | 1.8385 |
| *$10^7$ | 482.01 | 67.49 | 1.4117 | 1.7338 |

Fermentation medium: 50% of transformation culture medium; number of PUF discs: 5 discs; 30°C; agitation rate: 150 rpm; fermentation period: 72h. Values are the average of three independent experiments and the maximal mean deviation is ±5%.

**Repeated batch soyasapogenol B production by immobilized cells onPUF**

To find out the suitable time at which the maximum activities of the biotransformation process could be achieved by immobilized *A. parasiticus* cells in repeated batch fermentation, soyasapogenol B yield was determined at different time intervals 24, 48, 36, 96 and120 h in two successive repeated batches.During first use, soyasapogenol B production increases with theincrease of the incubation period(Figure 3). The maximum yield of soyasapogenol B (79.45%) was obtained after 72h of incubation, with anobvious decline thereafter.However, the highestyield of soyasapogenol B was reached after 48h on the second batch, *i.e.*the transformation period reduced from 3 days on the first batch to 2 days on the second one. The

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fungal growth on the support material was observed to increase during batch operation, especially on the first one. On the second batch, it is likely that the fungus had reached optimum growth, thus being more adapted to transformation medium. Because of this adaptation, the immobilized mycelia exhibited rapid transformation, thereby reducing the transformation time. Therefore, further batches were conducted for 2 days each. Moreover, a decline of soyasapogenol B yield after 48hwas most probably due to a further metabolism of the product[2]. After incubation period for three days, the aeration rate cannot keep up with the culture oxygen consumption rate. So, the concentration of dissolved oxygen drops, the carbon source, or any other nutrient, may be consumed to the point where its availability limits growth.In addition, toxins may accumulate and inhibit growth or metabolic waste products accumulate [19].

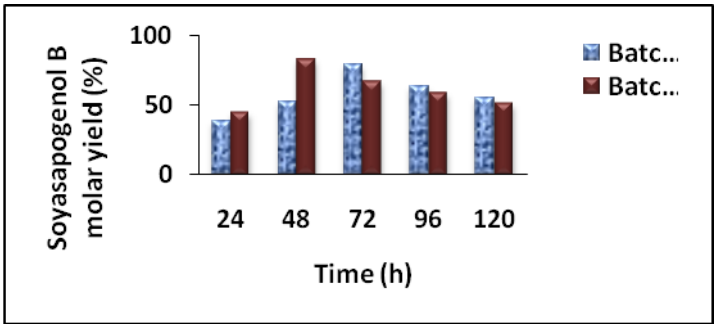


**Figure 3: Effect of incubation period on soyasapogenol B production by *A. parasiticus* cells immobilized on PUF in two repeated batches.**

Fermentation medium: 50% of transformation culture medium; 30◦C; initial spore concentration: $10^5$/ml; number of PUF discs: 5 discs; agitation rate: 150 rpm; two repeated batches were conducted each batch lasting five days. Values are the average of three independent experiments and the maximal mean deviation is ±4%.

Therefore, feasibility and performance of immobilized *A. parasiticus*cells on PUF for soyasapogenol B productionin repeated batch operations were performed in half medium contents and each cycle lasting for 48h except the first one (Figure 4).Soyasapogenol B production was studied up to eight reuse cycles. Soyasapogenol B production increased continuously in the firstthree batches, reaching the highest soyasapogenol B concentrationof 638.48 mg/100ml on the third cycle, which correspondedto 89.40 % (w/w) soyasapogenol B yield from consumed soybeansaponin. This yield is higher than that obtained by free cell fermentation (65 %) in full medium contents after 72h. Then soyasapogenol B production decreasedgradually over the next five batches. Out of the eight batch fermentations,the first five batches had over 70% soyasapogenolB yield, which translates into an overall soyasapogenol B yield of 77.3 % during eleven days of successive five cycles. Therefore the fungus maintained a relatively stable biotransformation process for a long period. After the first six batches, there was a sharp decline in soyasapogenol B production (Figure 4).This is may be due to reduced oxygen availability as well as substrate diffusional resistance in the large mycelial clump [2]. Consequently,soyasapogenol B production in repeated batch fermentation with *A. parasiticus* cells adsorbed on PUF could be successfully maintained for fifteen days.

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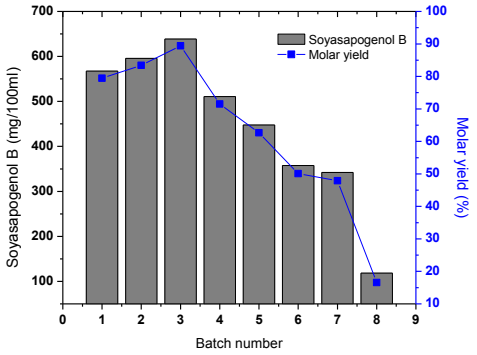


**Figure 4: Soyasapogenol B production in repeated batches by *A. parasiticus* cells immobilized on PUF.**

Fermentation medium: 50% of transformation culture medium; 30◦C; initial spore concentration: $10^5$/ml; number of PUF discs: 5 discs; agitation rate: 150 rpm, fermentation time: 48h for each batch except the first batch 72 h. Values are the average of three independent experiments and the maximal mean deviation is ±4%.

## CONCLUSION

Flexible PUF have been used as the mostsuitable carrier for*A. parasiticus* cells for soyasapogenol B production from soybean saponin.Effective soyasapogenol B yield was also observed using immobilized PUF in successive fermentations up to 6 batches and the highest yield (89.4 %) was achieved on thethird batch.Compared to free cell fermentation, this yield(89.4%) was obtained using half medium contents after 48h and it was of higher magnitude than that obtained by the free cells (65 %) in full medium contents after 72h.PUF have anadequate pore size which provides a satisfactory environmentfor fungal growth. Moreover,PUF is an inert material with good mechanical properties (high resistance and elasticity) and very low commercial cost.The current results couldbe the groundwork for industrial application of this immobilizedcellfermentation process for soyasapogenol B production.

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