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Original Research

Effect of immobilisation on production of rapamycin by *Streptomyces hygroscopicus*

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Abstract: Numerous antibiotics as secondary metabolites have been isolated from microbes specifically *Streptomyces sp*. Rapamycin is one of these, which exhibits many roles as immunosuppressant, antifungal antibiotic, anti-tumour etc. Constant thrust has been put by many researchers to improve the production of rapamycin by *Streptomyces hygroscopicus*. Past research show that whole cell immobilisation is a useful strategy for antibiotic production with economical advantage. This study is another attempt in this direction which involves immobilisation of producer organism on various supports such as polyutherane foam, sintered beads, glass beads and foam peanuts. These support materials have been selected based on their availability and were pre-treated chemically to improve their adsorption capacity. Comparative profiles have been studied to identify the most suited support for the growth of *Streptomyces sp*. Variation of biomass immobilisation on various support materials with respect to time in batch culture was studied. Cell release study for the carriers was carried out in production medium to evaluate the performance of beads. Also, scanning electron microscopy (SEM) has been employed for visualisation and analysis of immobilised biomass and measurement of pore size. This study aims to determine a suitable support for immobilisation of *Streptomyces hygroscopicus* which could be used for rapamycin production in batch and continuous mode.

Key words: Rapamycin; Immobilisation; Polyutherane foam; Fermentation; Immunosuppressant.

Introduction

Rapamycin is produced by *Streptomyces hygroscopicus* NRRL 5491 which was first reported in 1975 (1). Rapamycin is an immunosuppressant which was approved by US-FDA for use in renal transplant (2). It is an antifungal antibiotic and has numerous roles other than immunosuppressant such as anti-proliferative agent and promising roles in treatment of Tuberous sclerosis, Alzheimer, Muscular dystrophy etc. It has also shown to extend lifespan in mice. Worldwide research has been progressively improvising industrial production of rapamycin (3).

Whole cell immobilization has been examined by many workers as strategy for secondary metabolite production technology. Immobilized cells have several economic and process related advantages for fermentative production of secondary metabolites. They can be employed for repeated batches or continuous mode of fermentation as biocatalysts are preserved through immobilisation (4). Other proposed benefits of immobilisation include increased cell densities, improved mass transfer and recycling of biomass for long runs (5). The basic methods for immobilisation of microbes are adsorption, entrapment, cell localisation behind a barrier and self-aggregation (4). Many workers have explored the immobilisation ability of different type of carriers of both organic and chemical origin. These include sawdust, crushed brick, polystyrene, activated carbon, firebrick etc and study was carried out to find out correlation between immobilisation capacity and zeta potential of different carriers. (6) Others have employed entrapment method to determine the effect of encapsulation on hyphae formation and antibiotic production (7). Adsorption is preferred by many workers as a means of immobilisation as the cells adhere to the surface and internal pores through physicochemical bonds. Materials with high porosity provide larger surface area for adsorption (8). Polyutherane foam (PUF) is one such support material which is inert in nature and has high porosity. Studies have been carried out to determine the effect of different pretreatments of carriers on their immobilisation ability. Hydrocarbon degrading bacteria have been immobilised on PUF and the degradation ability of free and immobilised bacteria have been compared (9). *Streptomyces sp.* have been immobilised by many workers on PUF for production of various metabolites. Some have employed different types of chemical pre-treatments of polyutherane foam which showed that positive cross linked PUF were able to improve the conversion rate (5). Others have immobilised Streptomyces *pristina espiralis* for pristinamycin production and optimised the factors affecting immobilisation throughresponse surface methodology (10). Sintered glass beads (Siran beads) were previously studied as support material for immobilisation of *Kluyveromyces marxianus* CCT 3172 (11). Glass beads hadbeen employed as support material for formation of biofilm of *Pseudomonas* C12B. Present study incorporates use of PUF, Siran beads and glass beads for immobilisation of *Streptomyces hygroscopicus* for production of rapamycin. Another support material which is commercially known as foam peanuts made up of corn starch having very low density and high porosity was also employed

for this study. This moisture resistant support material is widely used as packaging material. This study aims at development of efficient immobilised system which could be used for repeated batch fermentation as well as continuous mode of operation for rapamycin production.

Materials and Methods

Materials and reagents

Four types of support materials were used namely Siran beads (borosilicate based), Polyutherane Foam (PUF, made up of polyol, isocyante and water), Glass Beads (consisting sodium aluminium silicate) and Foam Peanuts (made up of corn starch). PUF and glass beads were purchased from local market, siran beads were obtained from Bioengineering AG, Switzerland and foam peanut was obtained as packaging material by Eco Plus Uni. Rapamycin was purchased from Apex Biotech LLC. HPLC grade acetonitrile and methanol were purchased from Merck (Mumbai, India) whereas other chemicals were obtained from Himedia (Mumbai, India).

Strain and sub-culturing

Strain of *Streptomyces hygroscopicus* NRRL 5491 was obtained from NRRL, USA which was routinely sub-cultured.

Seed culture and fermentation medium

The seed culture medium for*Streptomyces hygroscopicus* consisted of : 3g/L malt extract, 3 g/L yeast extract, 5 g/L peptone and 10 g/L glucose. Fermentation medium consisted of (g/L): Soya peptone 15, Mannose 25, Lysine 17, K_2HPO_4 5 and MES Buffer 200 mM.

Pre-treatment of carriers

The PUF and foam peanuts were cut into 1 cm x 1cm x 1 cm cubes and were washed with autoclaved distilled water.

Cubes of PUF were submerged in glutaraldehyde (4% w/v) as described by Zhu et. al. [4] . Then they were rinsed with distilled water and soaked in 4% HCl for 10h. Subsequently, all the cubes were washed several timed with distilled water and dried at 50 °C. PUF cubes were pre-treated based on study carried out by Zhu *et. al*. (4)

Glass beads diameter was approximately 0.5 cm. Glass beads were submerged in concentrated HNO_3 for 3 hours and later with distilled water. Siran beads (particle size ranging from 600-1000 µm) were washed twice with distilled water and were then heated in muffle furnace at 600°C for 16 hours. Foam peanuts were washed with distilled water and then dried in oven at 50°C.

Cell immobilization and cell release

Following pre-treatment all carriers were immobilized by *Streptomyces hygroscopicus* NRRL 5491. This was done by adding 2% inoculum from freshly prepared seed culture to autoclaved (at 121°C and 15 psi pressure for 15 minutes) carriers containing 50 mL of seed culture media, under sterile conditions. It was cultured for 48 hours at 200 rpm, 28°C andimmobilised carrierswere regularly withdrawn from theculture. These were rinsed twice with sterile water and were lyophilized for 10 h. The weight of immobilized cells were determined by calculating the difference in weight of lyophilised support and pre-determinedweight of support material.

Cell release study was carried out by withdrawing 2 mL of fermentation broth at regular intervals and concentrations of dry cell mass in the samples were determined. Samples were then filtered through pre-weighed microfilters and were washed with distilled water. Microfilters were then dried by deep freezing and subsequent lyophilisation. The weight of immobilised cells were then determined by calculating the difference in weight of lyophilised microfilters and their initial weight.

Production of rapamycin

Four different carriers were allowed to immobilize in the seed culture medium as discussed in the previous section. After two days of culture the immobilised supports were withdrawn from the media and were rinsed with autoclaved distilled water twice. These were then transferred to the production medium. The weight of each carrier transferred varies (which was recorded) as their volume, density and porosity varies. Therefore, for the sake of uniformity the data showed in result and discussion section were represented in terms of per unit weight of carriers. Fermentation of rapamycin was carried out for four days at 28°C and 200 rpm.

Methanolic extraction of rapamycin

2 mL of fermentation broth were withdrawn at regular intervals and were centrifuged. The pellets so obtained were extracted twice by shaking with 2 mL of methanol for 30 minutes. The methanolic extracts so obtained were added to supernatant obtained after centrifugation which were subjected to HPLC analysis.

HPLC analysis

For HPLC analysis samples were filtered through 0.2 micron syringe filters (nylon microfilters, Axiva). Analysis was carried out by Waters HPLC and 20 µL samples were passed through C18 column (SunFire™ C18, 5µm) and analysed by PDA detector (Waters 2998 Photodiode Array Detector) and computed using Empower Pro software. Solvent system comprised of methanol and acetonitrile in ratio 80:20 and flow rate was kept 1 mL/min.

Scanning Electron Microscopy (SEM) imaging

All the immobilised carriers were washed with distilled water and kept at -80°C overnight. They were then lyophilised and visualised under Evo 18 Research, Zeiss SEM after coating with gold particles by Q 150R ES Qourum.

Results and discussion

Effect of carriers on immobilisation of *Streptomyces hygroscopicus*

Four different carriers were used for immobilisation study and depending on their properties such as porosity, charge, composition and particle size (3), different amount of biomass were immobilised as shown by the Fig 1. Immobilised biomass was calculated in terms of weight of *S. hygroscopicus* immobilised per unit

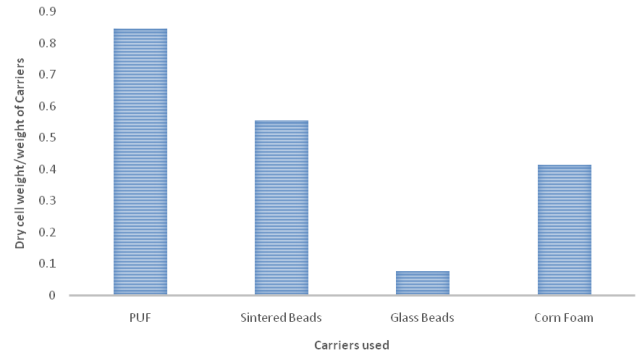

Figure 1. Variation of dry cell weight per unit weight of carriers.

weight of carriers. It is clearly observed that maximum immobilisation is observed in PUF as they have very high porosity approximately 94% and least was shown by glass beads with minimum porosity out of the for carriers used.Hence, maximum immobilization (cells per unit weight of carrier) was observed in case of PUF and minimum in case of glass beads as shown in Fig. 1. Sintered beads and foam peanuts exhibited intermittent immobilisation capacity.

SEM imaging of different carriers

Immobilised carriers were visualised under SEM at different magnification. SEM images (shown in Fig. 2) also verify the results obtained in previous section. SEM images of PUF cubes showed uniform distribution of film of *Streptomyces* mycelia. Microscopic view of siran beads and peanut foam cubes showed dense immobilisation whereas most sparse growth was observed in glass beads.

Cell release study

Cell release study was carried out to determine the concentration of cells which were released in media from immobilised support during fermentation. Cell release was calculated on percentage basis which is given by:

$$\%age\ of\ cell\ release = \frac{\text{weight of dry cell mass released per unit weight of carriers}}{\text{weight of dry cell mass immobilised by unit weight of carriers}} \text{-(1)}$$

As shown by Fig 3, maximum cell release was observed in case of immobilised glass beadsprobably due to its lower porosity so most the surface adsorbed cells were released during shaking conditions at as high as 200 rpm. While, minimum cell release was exhibited by Siran beads due to its smaller pore size. Foam peanuts and PUF showed intermittent features as their pore size were larger than siran beads.

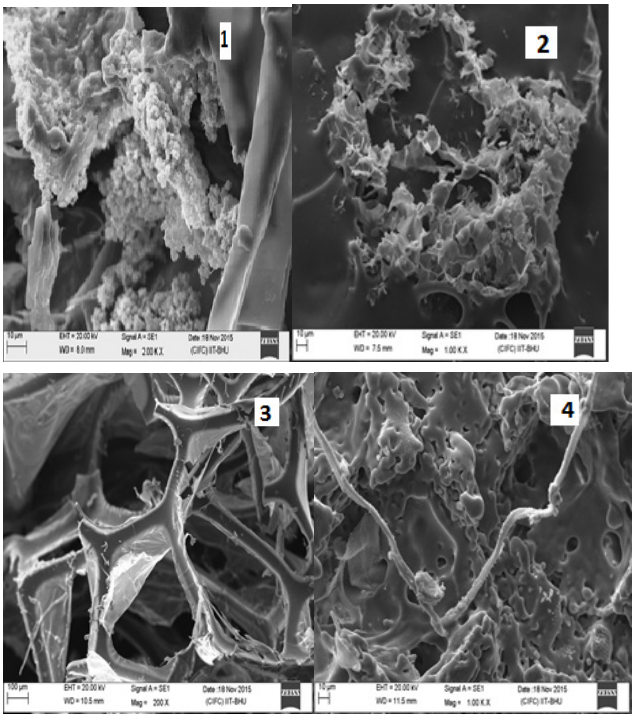

Figure 2. SEM image of different immobilised carriers **1:** Foam peanuts, **2:** Glass Beads, **3:** PUF, **4:** SiranBeads.

Production of rapamycin using different carriers for immobilisation

Rapamycin production was determined using HPLC. Production was studied for four days as previous study showed maximum production on the fourth day of fermentation (12). Calibration curve was plotted (not shown) using analytical grade pure rapamycin. This curve was then used to determine the concentration of rapamycin produced by immobilisation on different carriers as shown in Fig 4. It was observed that PUF cubes showed maximum rapamycin production.

Production was calculated in terms of weight of rapamycin produced per unit weight of carriers as shown in Fig 4. It was determined that though maximum production per unit weight was observed in PUF carriers but minimum was observed in glass beads. This could be correlated to Fig 1 where PUF cubes maximum immobilisation while glass beads exhibited minimum cell growth.

Other workers have also reported PUF as one of the suitable carriers for immobilisation owing to their high porosity and inert nature (13).

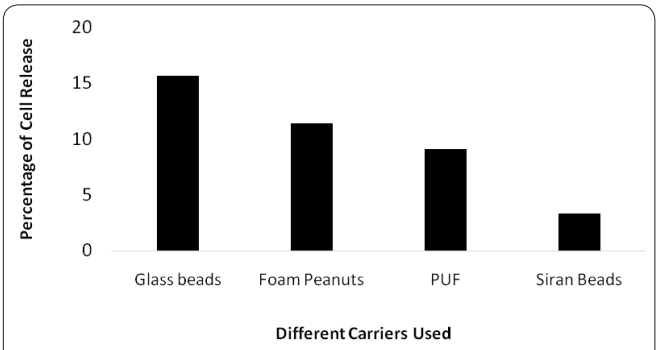

Figure 3. Cell Release Study using different immobilised systems.

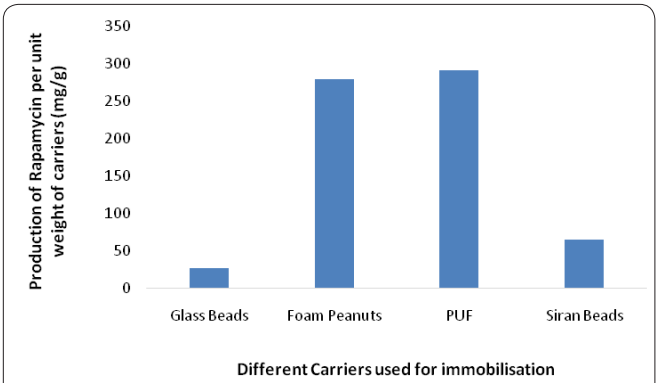

Figure 4. Production of rapamycin (weight of rapamycin produced per unit weight of carriers).

Conclusion

Present study shows that PUF cubes are a suitable carrier for immobilisation of rapamycin. Future application of this study could be validation of suitability of PUF cubes as carriers through repeated batch and continuous modes of fermentation. The limitation of immobilization strategy to be applied at industrial scale have been described elsewhere (4). Those issues could be resolved by further scaling up of process. This work is the first report of immobilisation of *Streptomyces hygroscopicus* NRRL 5491for rapamycin production as far as authors' knowledge.

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