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Deacylation of echinocandin B by immobilized *Actinoplanes utahensis* cells

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ABSTRACT

Immobilized cells of *Actinoplanes utahensis* (NRRL 12052) were used to selectively cleave the acyl group of echinocandin B yielding the deacylated form, echinocandin B nucleus. The effects of temperature, pH and substrate concentration on deacylation by immobilized cells were studied. In independent experimental runs, MOPS buffer with pH 6.8, substrate concentration of 3 g/L at growth temperature of 24°C, resulted in >90% bioconversion (deacylation). Recycle ability of immobilized cells were evaluated and found to retain >90% bioconversion up to 4 cycles, after which decrease in bioconversion rate was observed. Stability of immobilized cells were also checked at 4°C and found to retain bioconversion ability of >90% up to 5 days. Immobilization of whole cells can be a better option to process for bioconversion of echinocandin B to echinocandin B nucleus.

Keywords: Antifungals, Echinocandin B, Anidulafungin, Acylase, Immobilization, Sodium alginate

INTRODUCTION

Echinocandins, a new class of antifungals used for the treatment of invasive fungal infections has been a new hope for immune compromised patients. They are synthetically modified non ribosomal cyclic hexapeptides conjugated with a fatty acid. They have a unique mode of action against pathogenic fungi by non-competitively inhibiting the β-1,3 D- glucan synthase, a key enzyme involved in the synthesis of β-1,3 D- glucan. β-1,3 D- glucan is an essential structural component of fungal cell wall (Denning, 2003; Odds *et al.,* 2003; Wiederhold and Lewis, 2003; Zaas and Alexander, 2005).

Anidulafungin is a new echinocandin antifungal agent used extensively for the treatment of oesophageal candidiasis and candidemia. Anidulafungin is named after the fungus, *Aspergillus nidulans*, the first organism reported to produce echinochandin B (ECB) (Nyfeler and Keller, 1974). Enzymatic deacylation of ECB to a cyclic hexapeptide without a linoleoyl side chain (echinocandin B nucleus) and by subsequent chemical re-acylation leads to the formation of Anidulafungin (Uzun *et al.,* 1995; Debono *et al.,* 1998; Petraitiene *et al.,* 1999; Onishi *et al.,* 2000). Deacylation of echinochandin B to echinochandin B nucleus is catalysed by the enzyme acylase (also named as deacylase). This enzyme is currently identified from *Actinoplanes utahensis* NRRL 12052 and it is a membrane-associated heterodimer composed of 63-kDa and 20-kDa subunits. The expression of its activity is not affected by any co-factors, metal ion chelators, or reducing agents.

However, this enzymatic deacylation process is rate limiting when conducted with whole cells of *A. utahensis*. The low yield of echinocandin B nucleus is related to low expression levels of acylase enzyme and therefore inadequate production of anidulafungin. For deacylation process the culture producing acylase (*A. utahensis*) must be grown for each batch of deacylation cycle which is time consuming. To overcome this limitation, we have worked on immobilizing the whole cells of *A. utahensis* for improving the bioconversion efficiency of ECB to ECB nucleus and thereby enhancing the yield of ECB nucleus.

Concept of immobilization of microorganisms is a well-studied technique which has benefitted most of the enzyme dependent biotransformation process. The major advantage of immobilization is easy separation of biological material from the reaction medium containing the desired product (Martynenko and Gracheva, 2003). In addition, immobilized cell biomass can be re-used several times without the necessity of its re-proliferation and therefore cost effective. The selection of a carrier for immobilization is determined by the type of biological material and methods of immobilization (Survase *et al.,* 2010). Porous materials are used most frequently, to enable a free contact of substrate with immobilized biocatalyst, including carrageenan, polyacryl-amide gel and calcium alginate (Garbayo *et al.,* 2004).

In the present study, we immobilized actively growing cells of *Actinoplanes utahensis* using 4% sodium alginate with 0.1 M $CaCl_2$. The efficiency of the bioconversion of ECB to the ECB nucleus by immobilized cells was optimized by varying pH, temperature and substrate concentration. Stability and reusability of immobilised cells were also evaluated.

MATERIALS AND METHODS

All the chemicals and reagents used were of analytical grade and were purchased from Merck. Echinocandin B was prepared in-house by the fermentation of *Aspergillus nidulans* (US Patent 4322338, Abbott *et al.,* 1982)

Microorganism and culture conditions

The actinomycete, *Actinoplanes utahensis* used in this study was obtained from NRRL (NRRL 12052). This was grown on ISP-4 (from Difco) agar medium plates for 7 days at 28°C for sporulation and the spores were stored as glycerol stocks.

Immobilization and biotransformation process

Spores from the glycerol stock vial were inoculated into Streptomyces seed medium (4 % glucose, 1 % yeast extract, 0.1% $CaCO_3$, pH adjusted to 6.5 and 25 ml of the media dispensed in 250 ml flask) and incubated at 28°C for 3 days in an orbital shaker at 230 rpm. Three ml of the inoculum was transferred into production medium (4% glucose, 1% soya peptone, 1% yeast extract, 1% KH_2PO_4, 0.5% K_2HPO_4, 0.1% KCl, 0.01% calcium carbonate, pH adjusted to 7.0 and 30 ml

of the media dispensed in 250 ml flask) and incubated at 30°C for 2 days in an orbital shaker at 230 rpm. After 2 days of growth, 2.5 ml of the broth was mixed with 5 ml filter sterilized 4% sodium alginate and mixed well.

Culture beads were made by dropping this mixture into ice cold 0.1 M calcium chloride with the help of automatic pipette. The formed sodium alginate beads were washed repeatedly with sterile water to remove traces of calcium chloride.

These beads were taken in sterile conical flasks with 10 ml of 0.001 M MOPS buffer (pH 6.8), 3 g/L echinocandin B substrate feed was added and incubated at 24°C shaker for 48 hrs. Samples were withdrawn at an interval of 24 hours from the time of inoculation, centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of echinocandin B nucleus by HPLC.

Analytical methods for the detection of ECB and the ECB nucleus

After the samples were withdrawn from the flasks, the bioconversion reaction was stopped by the addition of methanol, centrifuged at 3000 rpm for15 minutes to remove precipitated proteins and mycelia. The activity of the acylase was determined by monitoring the formation of the cyclic hexapeptide (the ECB nucleus) by HPLC. Agilent Poroshell EC18 (150*4.6mm, 2.7µm pore size) column with KH_2PO_4 buffer and HPLC grade acetonitrile mobile phases were used for HPLC analysis. ECB and ECB nucleus from standard commercial sources were used as the standard to quantify the ECB nucleus formed and unconverted ECB (Ueda *et al.,* 2011). The identity of the compounds was confirmed by LC-MS.

Optimisation studies for deacylation of echinocandin B to echinocandin B nucleus

The parameters (temperature, buffers and substrate concentration) affecting the deacylation of echinocandin B to echinocandin B nucleus by immobilised culture beads of *Actinoplanes utahensis* were studied. The end-product echinocandin B nucleus was analysed by HPLC (Ueda *et al.,* 2011).

Effect of buffers

The immobilized culture beads were suspended in different buffers (MOPS, tris HCl, tris base, phosphate buffer, citrate buffer and milli Q water as control) at 0.001 M concentration. The culture beads in these buffers were supplemented with the substrate echinocandin B (3g/L) and were incubated at 24°C for 48 hrs. in an orbital shaker. Samples were withdrawn at an interval of 24 hrs. from the time of inoculation, centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of echinocandin B nucleus by HPLC.

Effect of temperature

The immobilized culture beads were suspended in 0.001 M MOPS buffer. The culture beads in this buffer were added with the substrate echinocandin B (3g/ L) and were incubated at 20, 24, 28 and 30°Cin an orbital shaker for 48 hrs. Samples were withdrawn at an interval of 24 hrs. from the time of inoculation, centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of echinocandin B nucleus by HPLC.

Effect of substrate concentration

The immobilized culture beads were suspended in MOPS buffer at 0.001 M concentration. The culture beads in these buffers were supplemented with different concentrations of the substrate echinocandin B (3, 3.5, 4, 4.5 and 5 g/ L) and were incubated at 24°Cin an orbital shaker for 48 hrs. Samples were withdrawn at an interval of 24 hrs. from the time of inoculation, centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of echinocandin B nucleus by HPLC.

Stability and reusability of immobilised culture beads

The immobilized culture beads were suspended in MOPS buffer at 0.001 M concentration and were stored at 4°C for 0, 2, 5 and 7 days. The culture beads were then supplemented with substrate echinocandin B (3g/ L) and incubated at 24°C in an orbital shaker for 48 hrs. Samples were withdrawn at an interval of 24 hrs. from the time of inoculation, centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of echinocandin B nucleus by HPLC.

The immobilized culture beads were also checked for reusability up to 4 cycles. The culture beads at each cycle were supplemented with the substrate echinocandin B (3g/ L) and were incubated at 24°C in an orbital shaker for 48 hrs. Samples were withdrawn at an interval of 24 hrs. from the time of inoculation, centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of echinocandin B nucleus by HPLC.

RESULTS AND DISCUSSION

In the production of anidulafungin, deacylation of ECB to ECB nucleus is a crucial step. The enzyme used in deacylation is membrane bound and therefore purification is challenging. To overcome this limitation, immobilization of whole cells of *A. utahensis* was taken up in the present study. In addition, immobilized cells can be stored and reused several times without the necessity of its re-proliferation for each batch of deacylation and therefore it is time saving and cost effective.

The immobilized cells were fed with the substrate and bioconversion ability was found satisfactory as we achieved >90% conversion in each cycle. Therefore, we tried optimizing the factors responsible for the deacylation of ECB to ECB nucleus by immobilized cells.

MOPS, tris HCl, tris base, phosphate and citrate buffers were tested for deacylation by immobilized cells and their integrity. Among them MOPS buffer was found to be effective in bio conversion as well as binding the cells intact during the process (Fig.1). The pH plays a crucial role in the integrity of the immobilized cells as the MOPS buffer was found to be an optimum buffer (pH 6.8) when compared to that of the other buffers studied. Hence, MOPS buffer was considered as the

effective buffer for further process.

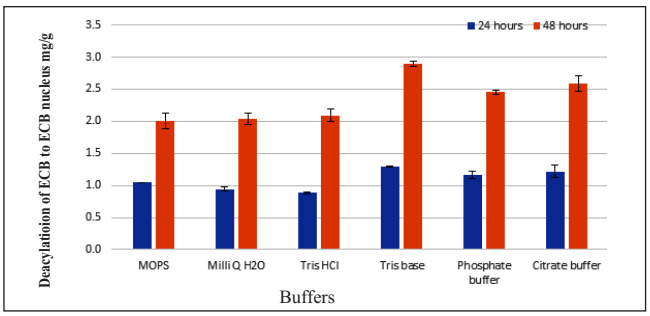

Fig. 1: Effect of buffers on deacylation of ECB to ECB nucleus by immobilized cells of *A. utahensis*

Enzyme activity is mainly dependent on temperature which affects the deacylation process. Therefore, temperature ranging between 20°C to 30°C tested for the deacylation of ECB to ECB nucleus. The optimum temperature was found to be 24°C which showed >90% of conversion in all the cycles tested (Fig.2).

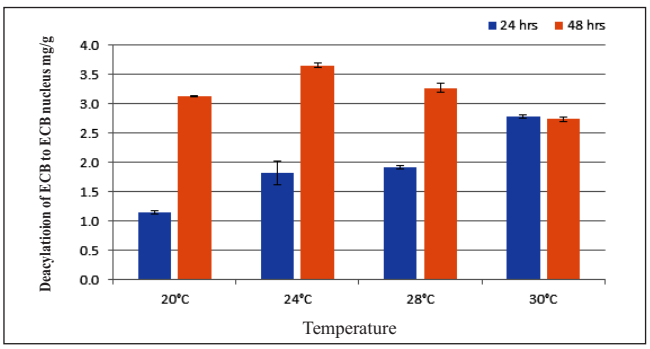

Fig. 2: Effect of temperature on deacylation of ECB to ECB nucleus by immobilized cells of *A. utahensis*

Any substrate beyond a concentration can be toxic to cells or can reduce the activity of the enzyme. Echinocandin B was fed at different concentration i.e., 3, 3.5, 4, 4.5 and 5g/L to the immobilized culture beads for deacylation and we could observe higher conversion rate (>90%) at 3 g/L ECB, but the conversion rate was decreased when the concentration of the substrate (ECB) was increased (Fig. 3). Similar such observation was made in our previous study (Shivakumar *et al.,* 2019) where increased substrate concentration beyond 4 g/L decreased deacylation.

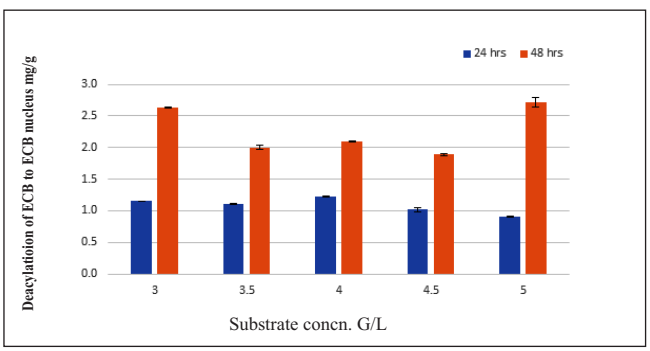

Fig. 3: Effect of substrate concentration on deacylation of ECB to ECB nucleus by immobilized cells of *A. utahensis*

Stability of immobilized culture beads stored at 4°C was checked at regular time intervals for bioconversion and found to lose their deacylation ability by 25% after 7 days, however, retained the deacylation ability up to 5 days. Immobilized culture beads were reused for several cycles and found to retain their activity up to 4 cycles, which reduced beyond 5 cycles (Figs.4 & 5).

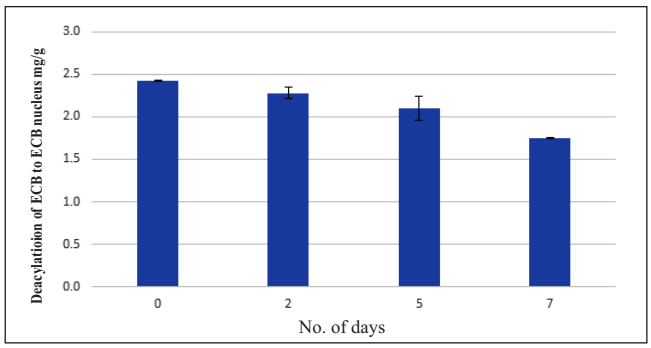

Fig. 4: Stability of immobilized cells of *A. utahensis* on deacylation of ECB to ECB nucleus

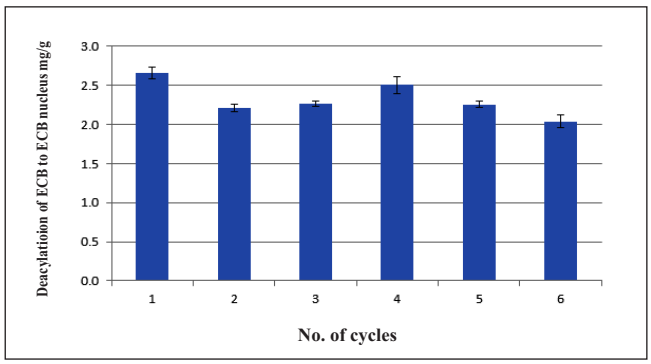

Fig. 5: Reusability of immobilized cells for deacylation of ECB to ECB nucleus

There are very few reports available with respect to deacylation of ECB to ECB nucleus by immobilized cells. However, recent publications by Afzali *et al.* (2021) reported immobilization of *Streptomyces griseobrunneus* for the production of cyclodextrin glucanotransferase enzyme, where immobilization was effective in improving enzyme activity. Hassan (2016) reported the enhanced stability and re-usability of penicillin G acylase by immobilizing the enzyme which has increased its stability without significantly altering activity.

In the present study, the limitation of using the actively growing cells for deacylation is overcome by immobilization of *A. utahensis* cells which has shown >90 % bioconversion efficiency. The significant findings from our present study has revealed that immobilization of whole cells can be an alternative option to complex fermentation process of bioconversion of echinocandin B to echinocandin B nucleus. This also accelerates the commercial purification process of the deacylated echinocandin B.

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