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Co-cultivation of *Aspergillus nidulans* with *Actinoplanes utahensis* for the production of echinocandin B nucleus, a precursor of an antifungal agent anidulafungin

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ABSTRACT

Anidulafungin is a potent antifungal compound derived from echinocandin B, a cyclic hexapeptide with a linoleoyl side chain having antifungal activity. However, echinocandin B is known to exhibit red blood cell haemolysis which was a major concern. This was addressed through an enzymatic deacylation of the linoleoyl side chain with acylase derived from the fermentation broth of *Actinoplanes utahensis* (NRRL 12052). The acylase is a membrane bound enzyme which catalyses the cleavage of the linoleoyl group of echinocandin B, a key step in the anidulafungin production. Purification of echinocandin B produced by *Aspergillus nidulans* is a time consuming and also loss of product is more during recovery process. The bioconversion of echinocandin B to echinocandin B nucleus by *Actinoplanes utahensis* is the additional and limiting factor in the production of anidulafungin. An attempt is made in the present study to resolve this by using the concept of co-culture technique which has reduced the two-step purification process to single step process. *Aspergillus nidulans* and *Actinoplanes utahensis* are cultured separately in submerged medium and then pooled together to continue the fermentation process for a fixed time. This resulted in the formation of echinocandin B nucleus, thereby reducing the purification process for echinocandin B. The co-cultivation strategy of fungi and *Actinomycetes* has proved to be a novel method of producing natural products with various biological activities. This work focuses on the significant co-cultivation fermentation process which enhances the production of echinocandin B nucleus.

Keywords: *Aspergilllus nidulans, Actinoplanes utahensis*, Co-culture, Acylase, Bioconversion

INTRODUCTION

Fungal infections are a global health problem, where about 20% (325 species where 87 have been described since 1980) of human pathogens are fungi (Hasim and Coleman, 2019). The severity of fungal infections can range from superficial, affecting the skin or nails, to severe, causing life-threatening invasive or disseminated infections. The most severe infections occur primarily in patients with an underlying disease or the immunocompromised patients including cancer patients, hematopoietic stem cell or organ transplant recipients, those on a long-term corticosteroid treatment, and HIV patients (Hasim and Coleman, 2019). Despite the availability of several effective antifungal drugs, fungal infections cause considerable morbidity and mortality. Unlike animal cells, the fungal cell has a defined cell wall and therefore is the ideal target for the therapeutic treatment of fungal pathogens in humans (Georgopapadakou *et al.,* 1995; Denning, 2003). The discovery of an antifungal drug echinocandin is one of the major classes of antifungals to target the fungal cell wall (Sucher *et al.,* 2009; Wagner *et al.,* 2006). Three semisynthetic echinocandin derivatives have been developed for clinical use: caspofungin, micafungin, and anidulafungin (Cleary, 2009). The strength of these antifungals includes low toxicity and rapid fungicidal activity against most of the *Candida* spp. These antifungals also possess inhibitory spectrum on *Aspergillus* spp. and *Pneumocystis jirovecii*, but however, not on *Cryptococcus neoformans* (Denning, 2002; Fera *et al.,* 2009).

Echinocandin B (ECB) is obtained through the fermentation process of *Aspergillus nidulans*. It is one of the natural cyclic hexapeptides having linoleoyl side chain, which inhibits a crucial enzyme, β-(1,3)-D-glucan synthase involved in fungal cell wall biosynthesis (Nyfeler *et al.,* 1974). The linoleoyl side chain of ECB also shows hemolytic activity and therefore of much concern. This is overcome by modifying the molecule by enzymatic deacylation to a cyclic hexapeptide without a linoleoyl side chain and by subsequent chemical modification (a process called reacylation) to get anidulafungin (Debono *et al.,* 1988; Onishi *et al.,* 2000; Hasim *et al.,* 2019: Zou *et al.,* 2021). The deacylation of ECB to ECB nucleus is done with the help of an enzyme acylase produced by *Actinoplanes utahensis* (NRRL 12052) which catalyses the cleavage of the linoleoyl side chain from ECB (**Fig. 1**). This is an essential reaction for the subsequent synthetic steps in the formation of anidulafungin (Boek *et al.,* 1989). However, this enzymatic deacylation process is rate limiting when conducted with whole cells of *A. utahensis*. The low bioconversion yield is related to low expression levels and therefore inadequate production of acylase enzyme (Wang *et al.,* 2009). In the present study, we have worked on the concept of co-cultivation of *Aspergillus nidulans* with *Actinoplanes utahensis* strains for the production of echinocandin B nucleus and thereby developing a better bioconversion method for improving the yield of ECB nucleus.

Cultivating two types of microbes (unrelated strains) in the same medium is co-cultivation (also called "mixed fermentation"). In the natural ecological system, microbes always coexist within complex microbial communities. Competition between these microbes is deliberately provoked in the hope that biosynthetic genes that remain silent under luxurious culture conditions will be activated and transcribed under stressful conditions. Several co-cultivation studies that have been conducted in recent years prove the feasibility of this strategy and suggest that co-cultivation is a viable experimental approach for enhancing the chemical diversity of microorganisms when grown in vitro (Marmann *et al.,* 2014). Co-cultivation is also known to be effective in

the production of enzymes that activate the metabolite precursor produced by the producer strain, yielding the active metabolite, or that the inducer strain may induce epigenetic modifications in the producer strain (Abdelmohsen *et al.,* 2015).

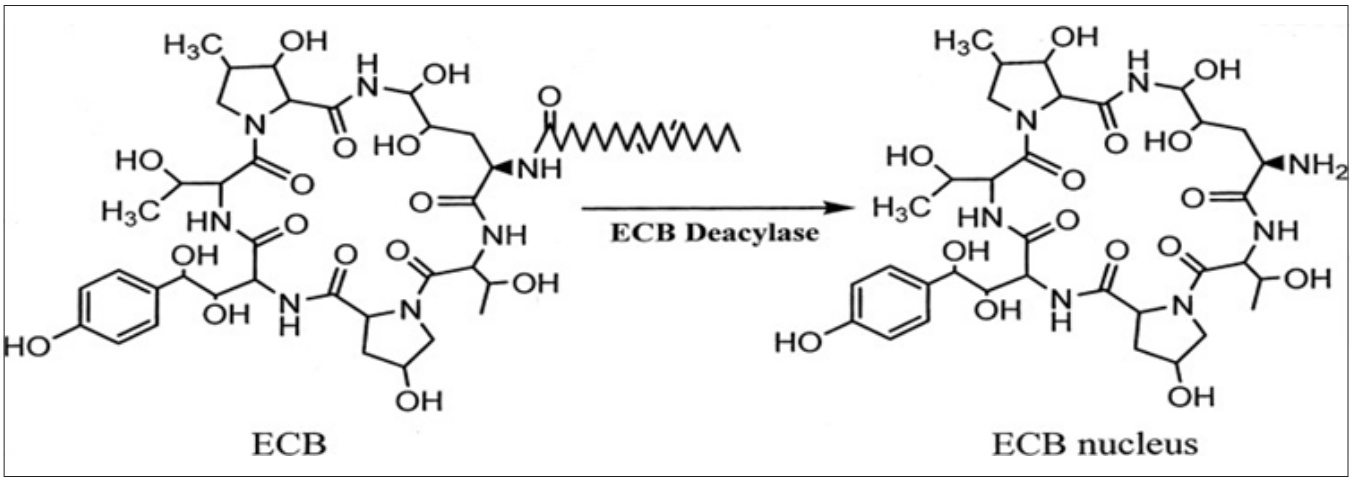

Fig. 1: Deacylation of ECB to ECB nucleus

MATERIALS AND METHODS

Chemicals and reagents

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).

Microorganisms and culture conditions

The organisms *Aspergillus nidulans* (NRRL 11440) and *Actinoplanes utahensis* (NRRL 12052) were obtained from Agricultural research service culture collection (NRRL), Peoria, Illinois. Both organisms are stored as glycerol stocks at the Biocon India Culture Collection (BICC).

Co-cultivation process

Aspergillus nidulans is grown on malt extract agar (MEA) and *Actinoplanes utahensis* is grown on ISP-4 (Difco) agar medium plates at 28°C for one week for sporulation. After the growth, these strains were inoculated (an agar disc ~ 1 cm^2) into echinocandin seed medium (2.5% Sucrose, 3.6% molasses, 1% malt extract powder, 1% casein and 0.2% KH_2PO_4, pH 6.5 and 25 mL of the medium was dispensed in 250 mL conical flask) and *Actinoplanes* seed medium (4% glucose, 1% yeast extract, 0.1% $CaCO_3$, pH adjusted to 6.5 and 25 mL of the media was dispensed in 250 mL flask) and incubated at 28°C for 3 days in an orbital shaker at 230 rpm (Shivakumar and Savitha, 2021). From both the seed flasks three mL of the inoculum was transferred into echinocandin production medium (Glucose 2.5%, starch 1%, soya peptone 1%, molasses 1%, casein 0.4%, $CaCO_3$ 0.2%, $MgSO_4.7H_2O$ 0.05%, pH 6.8 and 30 ml media dispensed into 250 mL conical flasks) and acylase 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 was dispensed in 250 mL flask) respectively. Production flasks were incubated at 30°C for 3 days in an orbital shaker at 230 rpm (Shivakumar and Savitha, 2021). After 3 days 15 ml inoculum from both the production flasks were transferred aseptically into a sterile 250 mL conical flask, mixed well and incubated at 30°C for 3 days in an orbital shaker at 230 rpm. 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 (Shivakumar and Savitha, 2019).

Analytical method for the detection of ECB and 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 analysis of ECB nucleus and ECB by HPLC (Shivakumar and Savitha, 2019). Echinocandin B and ECB nucleus from standard commercial sources were used as the standard to quantify the ECB nucleus formed and unconverted ECB (Shivakumar and Savitha, 2019). The identities of the compounds were confirmed by LCMS.

Optimisation studies for deacylation of echinocandin B to echinocandin B nucleus

The physico-chemical parameters (incubation temperature, inoculum ratio and incubation time) affecting the production of echinocandin B nucleus were studied. The end-product echinocandin B nucleus was analysed by HPLC (Shivakumar and Savitha, 2019).

Effect of incubation temperature

The co-cultivation flasks were incubated at 20, 24, 28 and 30°C in an orbital shaker for 3 days. 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 (Shivakumar and Savitha, 2019).

Effect of inoculum ratio

The inoculum concentrations of *Aspergillus nidulans* v/s *Actinoplanes utahensis* at different ratios were checked. Ratios of 1:1, 2:1, 3:1 and 4:1 were inoculated and the resultant co-cultivation flasks were incubated at 24°C in an orbital shaker at 230 rpm for 3 days. 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 (Shivakumar and Savitha, 2019).

Effect of incubation time

The co-cultivation flasks were incubated for 5 days at 24°C in an orbital shaker. 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.

is limiting when performed with whole cells of *A. utahensis*, which is related to low levels of acylase enzyme expression. Furthermore, low purification efficiency of ECB is also a contributing factor to the lower yield of ECB nucleus. In the present study, we explored the concept of co-cultivation of *A. nidulans* and *A. utahensis* strains for the production of echinocandin B nucleus, which reduced the ECB purification step and thus developed a better bioconversion method to improve the yield of the ECB nucleus.

Optimisation studies for the production of echinocandin B nucleus

The physico-chemical parameters/factors (incubation temperature, inoculum ratio and incubation time) affecting the bio-conversion of the echinocandin B to echinocandin B nucleus through co-cultivation were studied under submerged fermentation conditions. The end-product echinocandin B nucleus was analysed by HPLC **(Fig. 2)** (Shivakumar and Savitha, 2021).

Effect of incubation temperature

Temperature plays an important role on the growth of microbes and also on enzymatic activity. The deacylation process also depends on the temperature which affects the yield of the ECB nucleus. Temperatures between 20°C and

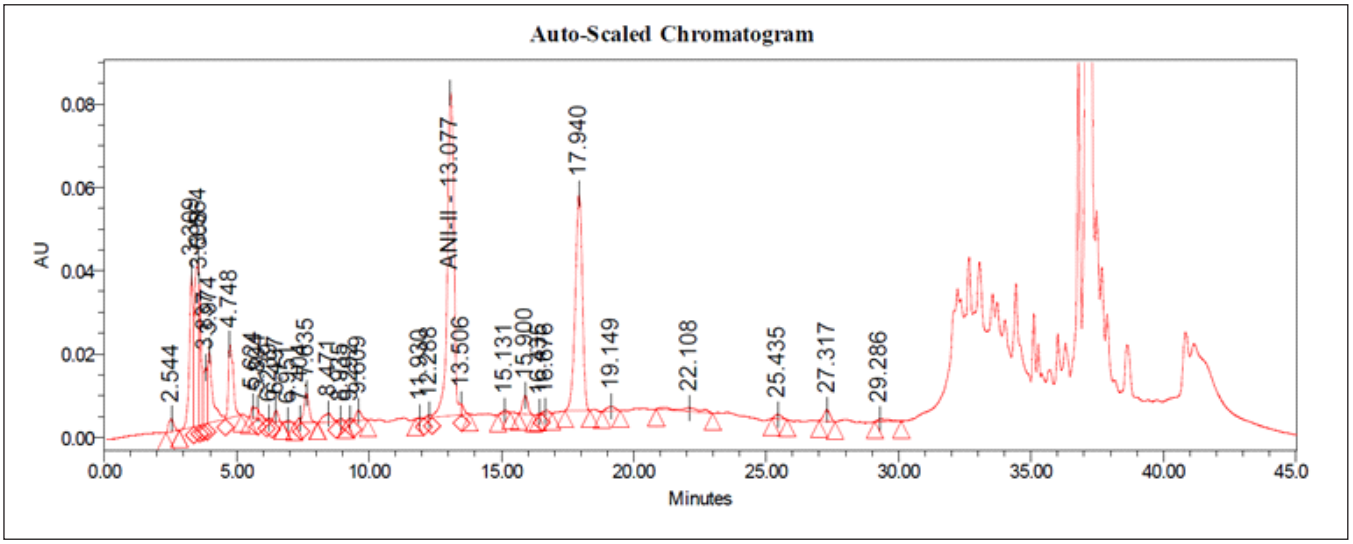

Fig. 2: HPLC chromatogram showing echinocandin B nucleus and echinocandin B

RESULTS AND DISCUSSION

Cultivating two types of cells (unrelated strains) in the same medium is known as co-cultivation (also called "mixed fermentation"). In the natural ecological system, microbes always coexist within complex microbial communities. Competition for limited resources and antagonism are characteristics of these microhabitats, which favour various defence mechanisms that are mainly based on the production of bioactive secondary metabolites (Ola *et al.,* 2013).

The process of enzymatic deacylation of ECB to ECB nucleus

30°C were tested for ECB nucleus formation by co-cultivating *A. nidulans* with *A. utahensis*. Among the four temperatures tested, 24°C was found to be optimum as it showed maximum conversion of ECB to ECB nucleus (**Fig. 3**). At 20° and 30°Cthe conversion rate was observed to be least, whereas 28°C showed less conversion rate than 24°C.

Effect of inoculum ratio

The inoculum concentrations of *A. nidulans* v/s *A. utahensis* were checked at different ratios for the production of echinocandin B nucleus. Ratios of 1:1, 2:1, 3:1 and 4:1 were

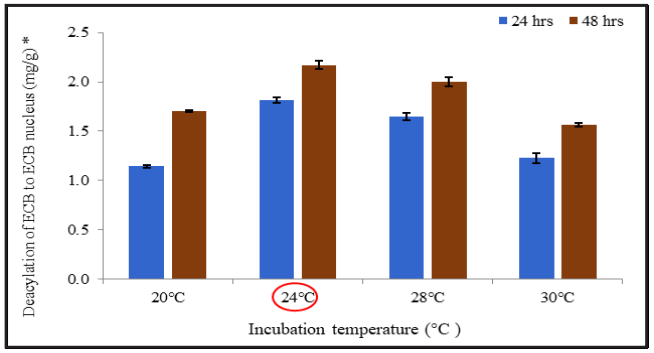

Fig. 3: Effect of incubation temperature on the deacylation of echinocandin B to echinocandin B nucleus by co-cultivation of *Aspergillus nidulans* with *Actinoplanes utahensis* (NRRL 12052). *Deacylation of ECB by the action of acylase.

inoculated and the resulting co-cultivation flasks were incubated at 24°C on an orbital shaker at 230 rpm for 3 days. Among the four, 1:1, 2:1 and 3:1 showed higher conversion of ECB to ECB nucleus (>90%) (**Fig. 4**) and 4:1 showed the least. Although the conversion rate is uniform in the above mentioned 3 ratios, the yield of ECB nucleus is comparatively higher in 3:1 than the other two ratios (1:1 & 2:1) (**Fig. 4**).

Effect of incubation time

The effect of incubation time on co-cultivation was carried out by mixing the 3 days grown cultures of *A. nidulans* with *A. utahensis* and incubating the flasks for 5 days at 24°C in an

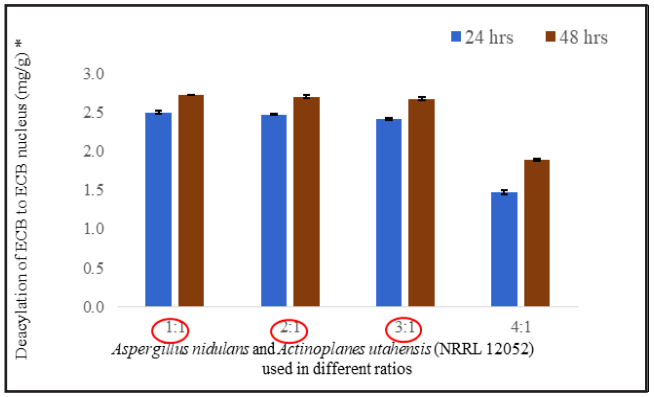

Fig. 4: Effect of inoculum ratio on echinocandin B nucleus production by co-cultivation of *Aspergillus nidulans* with *Actinoplanes utahensis* (NRRL 12052). *Deacylation of ECB by the action of acylase

orbital shaker. In general *A. nidulans* when cultured in ECB production medium shows highly viscous growth. Samples were collected at 24 hour intervals for estimation of ECB nucleus. Consequently there was a significant change in broth viscosity after the addition of *A. utahensis* broth. The titre values of ECB nucleus showed increasing trend till 48 hours, after which it remained constant (**Fig. 5**). The correlation of viscosity reduction in the culture broth after the addition of *A. utahensis* might have an influence on the bioconversion of ECB which needs to be studied further.

Co-cultivation of *A. nidulans* with *A. utahensis* has resulted in the formation of echinocandin B nucleus. These results indicate that co-cultivation of both the organisms has led to a new concept of production of echinocandin B nucleus thereby, reducing two major steps in anidulafungin production and therefore cost effective.

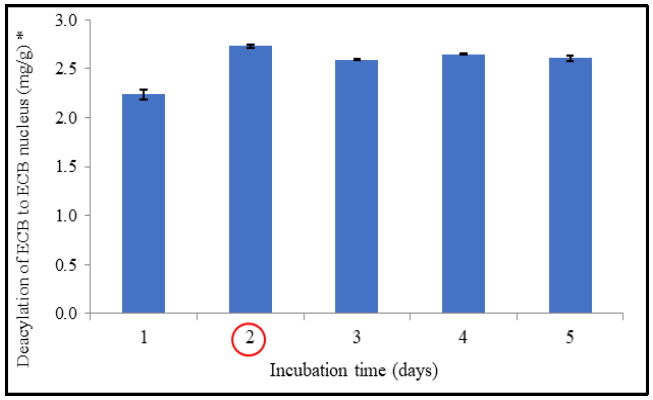

Fig. 5: Effect of incubation time on echinocandin B nucleus production by co-cultivation of *Aspergillus nidulans* with *Actinoplanes utahensis* (NRRL 12052). *Deacylation of ECB by the action of acylase

There are no reports available so far on the concept of co-cultivation for the production of echinocandin B nucleus. However, a few related studies reported have been discussed here. Kreuzman *et al.* (2000) reported optimum temperature of 25°C and optimum pH of 6.0 for maximum recovery of ECB nucleus by membrane bound ECB acylase. Ola *et al.* (2013) reported a 78-fold increase in accumulation of secondary metabolites (lateropyrone and fusaristatin A) when *Fusarium tricinctum* was co-cultured with *Bacillus subtilis* (168 trpC2) on solid rice medium incubated at 23°C. Wakefield *et al.* (2017) reported the dual induction of newly detected bacterial and fungal metabolites by the co-cultivation of the fungal isolate *Aspergillus fumigatus* (MR2012) and two *Streptomyces leeuwenhoekii* strains (C34 and C58). Co-cultivation of these strains at 30°C led to the production of *luteoride D* and *pseurotin G* which were not traced before from these strains under different fermentation conditions.

CONCLUSION

The findings of co-cultivation for echinocandin B production and formation of echinocandin B nucleus is a milestone in the development of anidulafungin production. This has a direct impact on the rapidly increasing demand of anidulafungin. Synthesis of the ECB nucleus by chemical deacylation is extremely difficult, and the bio-transformation method by microbes overcomes this problem. In the present study, we have devised a powerful bio-transformation strategy, with the help of co-cultivation to produce ECB nucleus, which in turn used for the synthesis of anidulafungin.

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