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Deacylation of Echinocandin B by Streptomyces Species: A Novel Method for the Production of Echinocandin B Nucleus

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ABSTRACT

Echinocandin B nucleus an intermediate used in the production of Anidulafungin a new class of antifungal agent used to treat chronic fungal infections. Echinocandin B gets converted to Echinocandin B Nucleus through an enzymatic bioconversion called deacylation by the enzyme Acylase also called as deacylase. A rapid bioconversion screening method was performed to select Actinomycete capable of producing Echinocandin B acylase amongst the group of Actinomycetes strains screened. A total of 140 strains of Actinomycete isolates were used for the preliminary qualitative plate assay with 53 strains selected positive for acylase production followed by two strains selected for quantitative assay. The selected strains were used for optimizing the bioconversion by varying parameters such as pH, temperature, feeding time temperature and substrate concentration in lab scale (50 ml medium) shake flask studies. The effect of temperature on conversion rate was also optimised. The optimised conditions were also tested in higher volume (500 mL medium) shake flasks which showed a 3 fold increase in conversion rate when compared to that of lab scale shake flask trials with *Actinoplanes utahensis* as the control.

Keywords: Antifungals, Echinocandin B, Anidulafungin, Acylase, *Aspergillus nidulans*

INTRODUCTION

The population of cancer patients, transplant recipients and other individuals receiving immunosuppressive treatment are at greater risk for fungal infections because of their weak immune system and the chronic nature of diseases. Therefore, treatment of such invasive fungal infections has created a major challenge for health care professionals. Antibiotics are known to target important cellular functions or growth processes of microbes. Echinocandins are synthetically modified non ribosomal cyclic hexapeptides conjugated with a fatty acid **(Figure 1)**. 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, an essential structural component of fungal cell wall [1-4]. They show fungistatic activity against *Aspergillus* spp. and fungicidal activity against several *Candida* spp. including strains that are fluconazole resistant. Caspofungin, Micafungin and Anidulafungin are the three important semisynthetic Echinochandins [5-7]. All have low oral bioavailability and therefore distribute well into tissues except in CNS and eye. Anidulafungin is a new echinocandin antifungal agent used extensively for the treatment of oesophageal candidiasis and candidemia. It is unique in that it undergoes chemical degradation in bile rather than via hepatic metabolism with longer half-life than Caspofungin or Micafungin. Anidulafungin is named after the fungus, *Aspergillus nidulans*, the first organism reported to produce Echinocandin B (ECB) [8]. Enzymatic deacylation of ECB to a cyclic hexapeptide without a linoleoyl side chain (Echinocandin B nucleus) and by subsequent chemical reacylation leads to the formation of anidulafungin [9-12]. Acyl peptides are generally unstable to chemical deacylation and therefore enzymatic deacylation is widely accepted. Deacylation of Echinochandin B to Echinochandin B nucleus is catalysed by the acylase (also named as deacylase). This enzyme is currently identified from *Actinoplanes utahensis* NRRL 12052. It is a membrane-associated heterodimer composed of 63 kDa and

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18-20 kDa subunits and the expression of its activity is not affected by any cofactors, metal ion chelators or reducing agents.

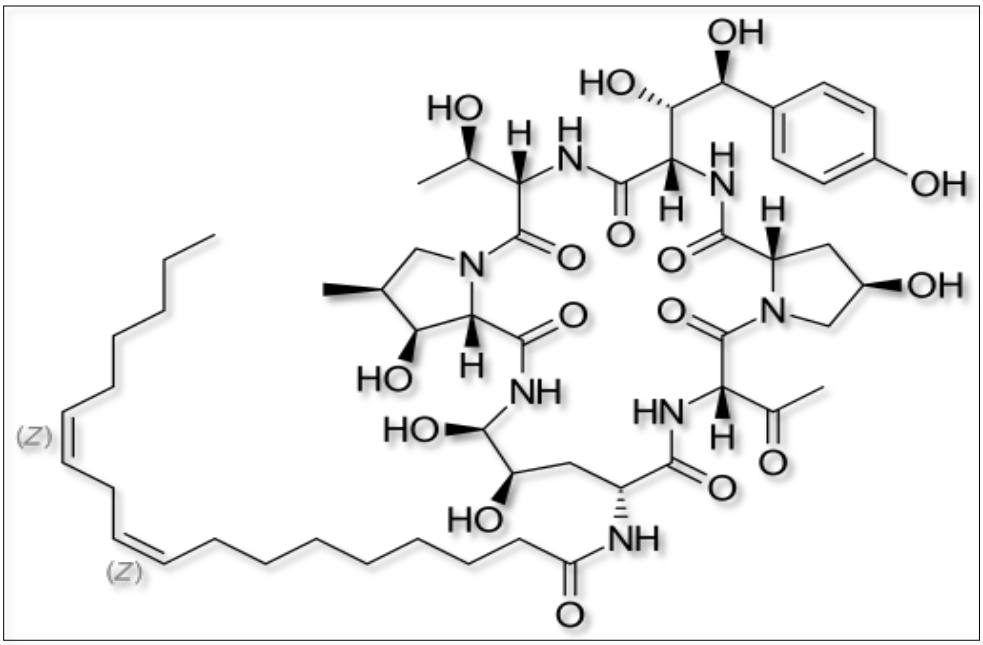

Figure 1. Echinocandin B.

The present study was initiated with an aim to identify an organism having a lipopeptide, acylase, with high specificity towards Echinocandin B. Therefore, a rapid screening protocol was undertaken with bacteria belonging to Actinomycetes, isolated from tropical soil. The selected species was evaluated for its efficiency to bio convert ECB to ECB nucleus and the cultural and environmental parameters for the bioconversion was optimized.

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* [13].

Microorganisms and culture conditions

All the cultures used for screening were isolated from natural sources (Soil, water or endophytes) across Karnataka, India. The cultures thus isolated were stored as glycerol stocks at the Biocon India Culture Collection (BICC). Totally, 140 Actinomycetes were cultured on ISP-4 (from Difco medium) plates at 28°C for one week. The control organism *Actinoplanes utahensis* (NRRL 12052) a type strain obtained from Agricultural research service culture collection (NRRL), Peoria, Illinois.

Enzyme screening studies

Qualitative plate assay for screening of Acylase producing strains: In the first round of screening, all the 140 Actinomycetes were inoculated onto fresh ISP-4 plates containing 1 mg/ml of Echinocandin B. The plates were incubated at 30°C for 7 days and at the end of the incubation, the plates were overlaid with 5 mL of soft agar containing 10^3 CFU/mL of *Candida albicans*. [14]. The overlaid plates were further incubated at 30°C for 24 h and the plates with the growth of *C. albicans* were considered as positive for acylase.

Quantitative assay for production of acylase under submerged fermentation (SMF): Fifty-three strains positive for acylase were selected from quantitative plate assay. These strains were further inoculated into Streptomyces seed media (4% glucose, 1% yeast extract, 0.1% $CaCO_3$, pH adjusted to 6.5 and 25 mL of the media was dispensed into 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 was dispensed into 250 mL flask) containing filter sterilized Echinocandin B (dissolved in methanol) at a concentration of 3.0 g/L and incubated at 30°C for 3 days in an orbital shaker at 230 rpm. Samples were withdrawn at an interval of 24 h from the time of inoculation, centrifuged at 3000 rpm for 15 min and the supernatant was analysed for the formation of Echinocandin B nucleus by HPLC. Agilent Poroshell EC18 (150*4.6 mm, 2.7 µm pore size) column with KH_2PO_4 buffer and HPLC grade Acetonitrile mobile phases used for HPLC analysis

using Echinocandin B and Echinocandin B nucleus from standard commercial sources [15].

The production of Acylase in Streptomyces seed and production media (devoid of the substrate – Echinocandin B) were analysed by separating the cells by centrifugation at 3000 rpm for 15 min. 1 g/L of Echinocandin B (in 50 mM phosphate buffer at pH 7) was added to the cell free supernatant and incubated for 24 h at 30°C. The reaction mixture was centrifuged at 3000 rpm for 15 min and the supernatant was analysed in HPLC (as described above) to detect Echinocandin B nucleus.

Optimisation studies for bioconversion of Echinocandin B to Echinocandin B nucleus

For the optimisation studies different parameters which affect the bioconversion of Echinocandin B to Echinocandin B nucleus were studied which include pH, temperature, substrate concentration, feeding time and media volume. Two strains positive for acylase production coded as BICC-8848, BICC-8547 with a control organism *Actinoplanes utahensis* in the production media were studied under submerged fermentation conditions. These strains were inoculated into Streptomyces seed media 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 and incubated at 30°C for 3 days in an orbital shaker at 230 rpm. Samples (1 mL) were withdrawn at an interval of 24 h from the time of inoculation till end of incubation, centrifuged at 3000 rpm for 15 min and the supernatant was analysed for the formation of Echinocandin B nucleus by HPLC (as described in section 2.2.2) [15].

Effect of pH

The production medium was adjusted to pH ranges (5.5, 6.0, 6.5, 7.0 and 7.5) with 10% H_3PO_4/10% NaOH before sterilization. The sterile medium was supplemented with the substrate Echinocandin B (3 g/L) before they were inoculated with the cultures.

Effect of temperature

The inoculated production medium was incubated at different temperature (22°C, 26°C, 30°C and 34°C) for 72 h in an orbital shaker at 230 rpm. Samples were withdrawn at regular interval of time (every 24 h) and analysed by HPLC.

Effect of substrate concentration

The inoculated production medium was fed with Echinocandin B at different concentrations (2, 3, 4, 5 g/L) incubated at 26°C for 72 h in an orbital shaker at 230 rpm. Samples were withdrawn at regular interval of time (every 24 h) and analysed by HPLC.

Effect of feeding time of substrate

The inoculated production medium was incubated at 26°C in an orbital shaker at 230 rpm and fed with Echinocandin B (3 g/L) at regular intervals from the time of inoculation (0, 24, 48, 72, 96, 120 h). Samples were withdrawn at regular interval of time (every 24 h) and analysed by HPLC.

Effect of media volume on bioconversion

In general, the acylase activity varies depending on the volume of the medium used in the shake flasks. The pre-grown culture in the seed medium was inoculated into production media (500 mL in 2000 mL conical flask adjusted to pH 7.0) and incubated at 30°C for 72 h. Echinocandin B was fed at a concentration of 3.0 g/L and incubated at 26°C in an orbital shaker and samples were withdrawn at 24 h interval and processed for HPLC analysis.

Molecular level Identification of the selected Actinomycete

Two strains (BICC-8848 and BICC-8547) were identified to produce the enzyme. Genomic DNA from the selected Actinomycetes was extracted; 16S rRNA gene was amplified using 16 S universal primers. The amplified PCR product was sequenced, the obtained sequence was checked in NCBI BLAST analyser and identification is made based on the maximum homology to the available sequence in the NCBI database [16].

RESULTS

Qualitative plate assay for screening for Echinocandin B acylase producing strains

A total of 140 isolates belonging to Actinomycetes were screened by plate assay method and 53 isolates (37%) were found to be positive for converting the Echinocandin B, an antifungal compound, into a non-effective molecule, Echinocandin B Nucleus. Growth of *Candida albicans* around the Actinomycete colony in the soft agar was an indication of bioconversion. Satoshi et al. [14] screened 3300 Actinomycetes and 500 fungal strains and were able to select 22 Actinomycetes (0.6% only) and 2 fungal strains (0.06% only) positive for the bioconversion.

Quantitative assay for production of acylase under submerged fermentation (SMF)

Actinomycetes isolates (53 in number) selected from the plate assay method of screening were subjected to second round of screening by submerged fermentation, for the selection of strains with higher Acylase activity. Two Streptomyces species were selected, coded as BICC 8547 and BICC 8848. Among the two, BICC 8547 showed acylase activity equivalent to that of the control micro-organism **(Figure 2).** However, BICC 8848 showed higher activity (25 to 30% more) of Acylase compared to the control organism and therefore selected for further studies. The cell free supernatant fed with 1 g/L Echinocandin B showed very poor conversion.

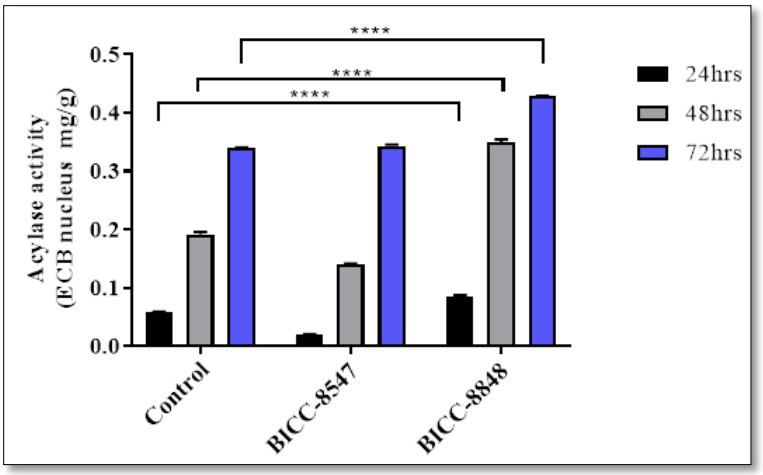

Figure 2. Acylase production in SMF.

The acylase activity is directly proportional to the conversion of ECB to ECB nucleus. Therefore, the activity of the acylase was determined by the titre value of ECB nucleus which was quantified by HPLC analysis

Effect of pH and temperature

The optimal pH and temperature for acylase activity (for bioconversion) of BICC-8848 was found to be optimal at pH of 7.0 and at temperature of 26°C **(Figures 3 and 4).**

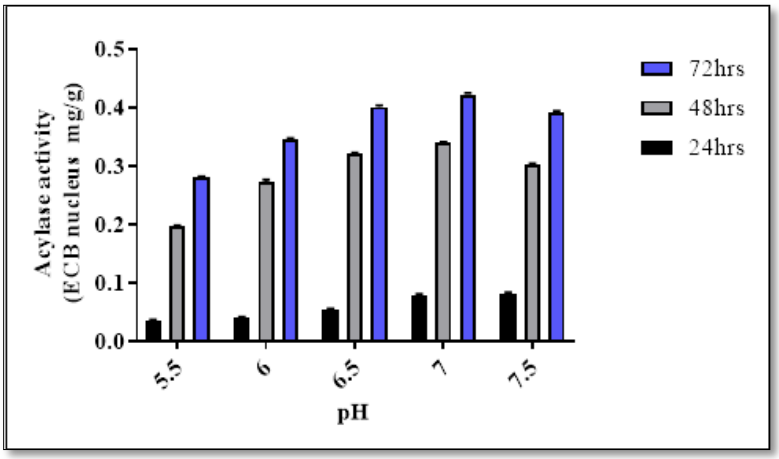

Figure 3. Effect of pH on acylase activity.

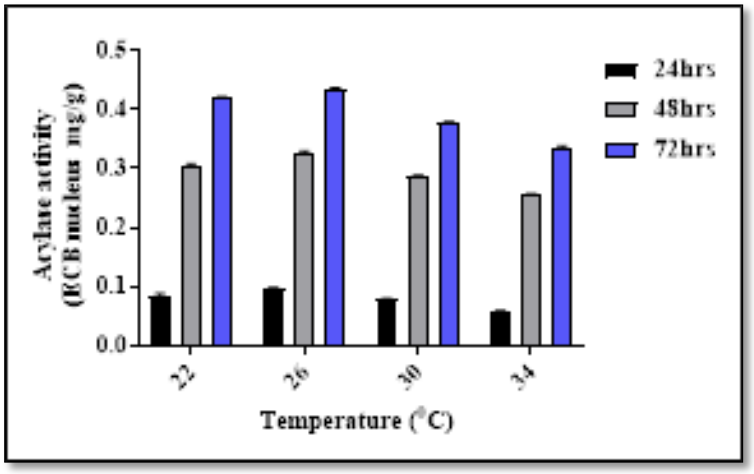

Figure 4a. Effect of temperature on acylase activity.

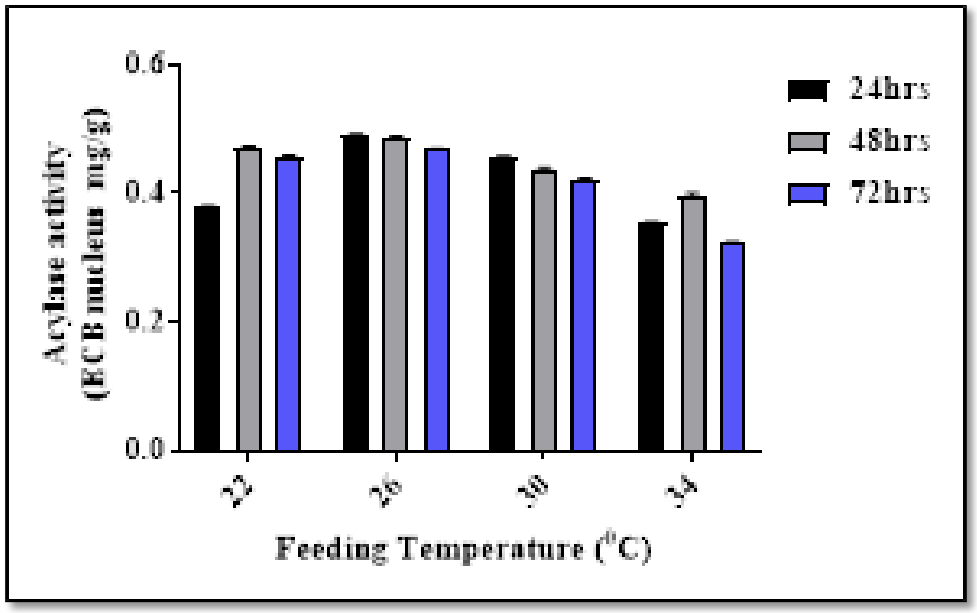

Figure 4b. Effect of feeding temperature on acylase activity.

Effect of substrate concentration

Any substrate beyond a particular concentration can be toxic to cells or can reduce the activity of the enzyme. Echinocandin B was fed at different concentrations to production broth and we could observe higher conversion rate at 4 g/L, but, however, the conversion rate was decreased when the concentration of the substrate was increased **(Figure 5)**.

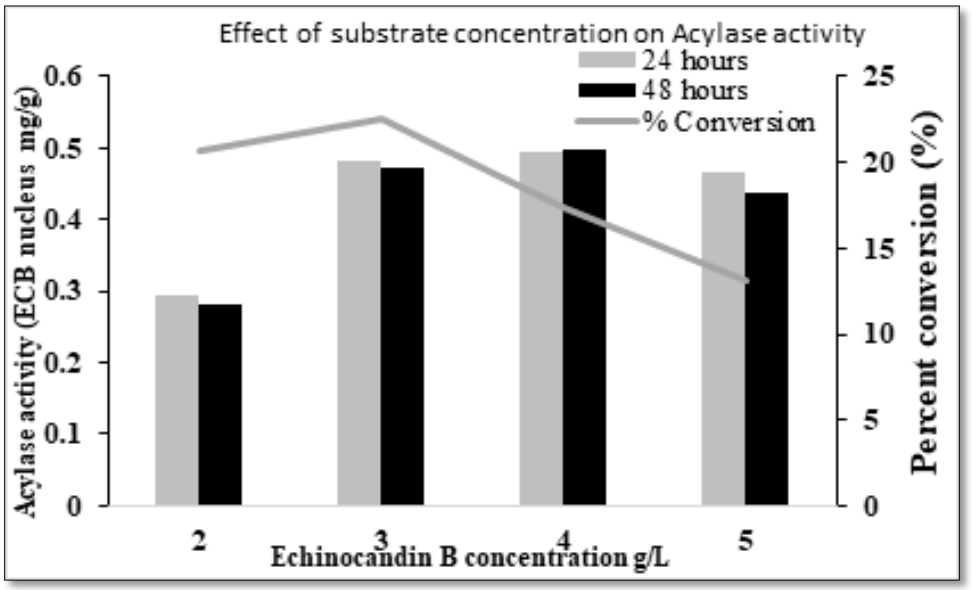

Figure 5. Effect of substrate concentration on acylase activity.

Effect of feeding time of substrate on conversion rate

In the process of determining the optimal pH and temperature for bioconversion, it was observed that the impurity level was higher than the control. Therefore, an attempt was made to decrease the level of impurity by adding the substrate at different duration of growth of the culture in production media. Interestingly, it was observed that BICC-8848 was able to convert the substrate in 24 h by lowering the impurity. However, it was also observed that the concentration of the product decreased from 48 h onwards with increased impurity **(Figure 6)**.

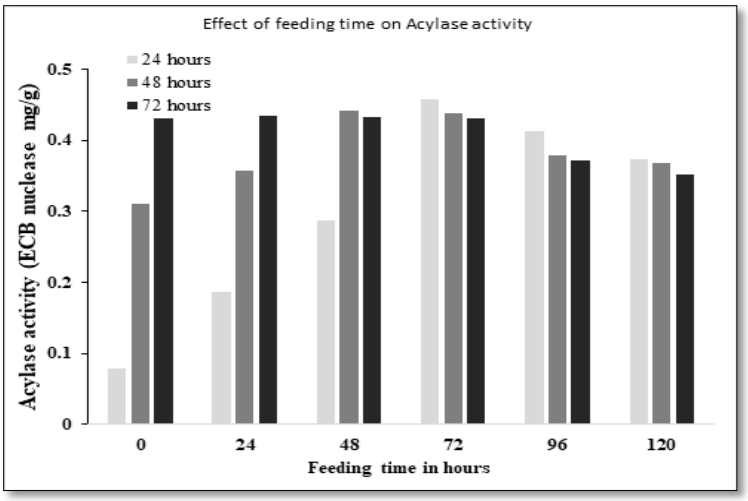

Figure 6. Effect of feeding time on acylase activity.

Effect of media volume on bioconversion

An attempt was made to evaluate the efficiency of the conversion with respect to volume of the media taken. The percentage conversion was observed to be generally lower in lower volume of broth, therefore trial was performed in higher volume, i.e., 500 mL of media (in 2 L conical flask) with the above set parameters and was observed that the conversion rate was as high as 55.0% when compared to 22.5% in 100 mL medium after 24 h of incubation. The conversion in BICC-8848 was completed in 24 h while the conversion in control strain proceeded till 72 h and the maximum conversion was 40.2% at the end of 72 h **(Figure 7)**. It is evident that maximum conversion is achieved with higher volume with respect to mass balance ratio.

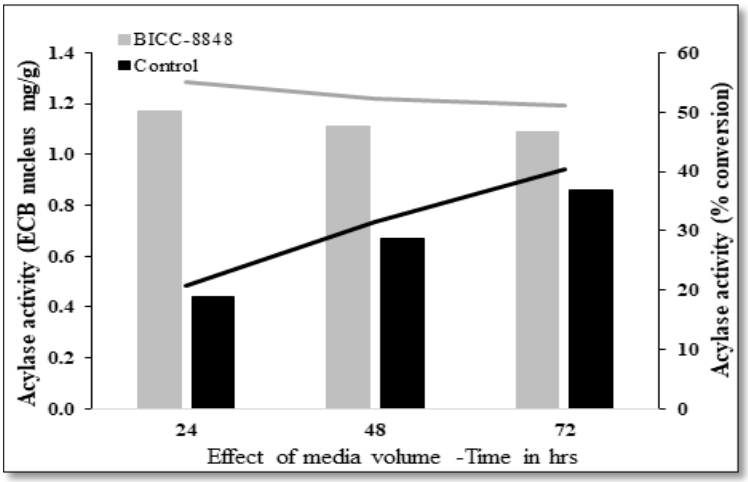

Figure 7. Effect of media volume on acylase activity.

Molecular level identification of culture

The actinomycetes strain BICC-8848 when subjected to ribotyping match only 99% with other Streptomyces species, showing only 98% homology to S*treptomyces* spp. B180, *Streptomyces parvus* and *Streptomyces sindenensis*. These *Streptomyces* spp. have not been reported so far to possess the acylase activity and the strain *Streptomyces* sp. BICC-8848 is a strain with better Acylase activity (better than *Actinoplanes utahensis* NRRL 12052) which can be studied further with detailed taxonomic studies such as genome sequencing and comparative studies to identify it till species level.

GenBank accession number SUB4743646 SEQ1 MK123323

DISCUSSION AND CONCLUSION

Deacylation is a critical step in the semi-synthetic production of various novel lipopeptides. The enzymatic deacylation of Echinocandin B to Echinocandin B nucleus is an important step followed by chemical reacylation with an optimized acyl group to get a pharmacologically important antifungal drug, Anidulafungin [17]. These steps are

conveniently and efficiently carried out by microbes when incubated with suitable substrate. The present investigation was taken up in search of an Actinomycete with better acylase activity. The preliminary data obtained in our present study revealed that the selected Actinomycete (*Streptomyces* spp. BICC-8848) possess acylase with good conversion rate (up to 55% conversion rate compared to 22% in control organism) at standard growth conditions than few Streptomyces and fungal organisms reported earlier. The molecular characteristics of our isolate of Streptomyces show only 99% similarity to any of the species within the genus described to date [18-20]. This species of Streptomyces could be a novel one, an additional Actinomycete member, under the family of acylase producers. Structural elucidation of acylase protein of this novel Streptomyces species would be studied further, to understand its structure-function relationship [21].

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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