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Novel Approach of Adaptive Laboratory Evolution: Triggers Defense Molecules in *Streptomyces* sp against Targeted Pathogen

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Abstract

Adaptation and evolution of microorganisms under selective pressure is the major cause of development of antibiotic resistance. However, our present study represents Adaptive Laboratory Evolution as an efficient tool to mine for and develop targeted bioactive molecules. Cryptococcal meningitis is an emerging neurological disease, with limited therapeutic choices. In the present study, a new marine anticryptococcal strain of *Streptomyces variabilis* AFP2 was co-cultured with *C. neoformans* with a periodic transfer for 30 days. AFP2 showed improved anticryptococcal activity post co-culture versus the parental type. The metabolomics of parental and evolved strain were analyzed by HPLC-UV and GC-MS. The phenotype and chemotype changes between parental and evolved strain were analyzed to determine the evolution of new traits. About 21 new metabolites alien to the parent strains were seen to be produced by evolved strain. Among which few of the induced compounds molecules like dl-Alanyl-l-leucine, dihydro-3,3-dimethyl-2 (3H)-Furanone; Enanthamide; 1,3,5-Cycloheptatriene; 1-Aziridineethanol were not reported for antifungal activity. In addition, these molecules were not reported in *S. variabilis*. The evolutionary fitness analysis reveals that 64 fold or 98% reduction in *C. neoformans* growth in evolved strain S3, with ~52 % of over expression of compounds. Hence the present study confirms that, integrated approach of adaptive laboratory technique and co-culturing techniques are useful to explore potential molecules against targeted pathogen.

Keywords: Adaptive Laboratory Evolution; marine *Streptomyces variabilis* AFP2; *C. neoformans*; Co-culture; antifungal bioactive molecule.

Introduction

Invasive fungal infections have been emerging as a great challenge in medicine. Their incidence and mortality rates have dramatically increased in the last few decades [1]. Although patients with weak immunity are highly susceptible and prone to these infections, reports on healthy individuals being affected are also in the rise. The most common cause for invasive fungal infection in healthy individuals are being surgery and long term antibiotic treatment [2–4]. *Cryptococcus neoformans* is an emerging opportunistic fungal pathogen that causes meningitis, a neurological infection in immunocompromised patients and is particularly predominant in patients with HIV [5,6]. *C. neoformans* has unique virulence properties such as capsule production and melanization during infection. Although the availability of antiretroviral therapy helped patients to become less prone to cryptococcus infection, it is still a major problem under resource limiting conditions, where HIV is still prevalent [7]. Recently, Center for Disease Control estimated approximately one million new cases of cryptococcal meningitis each year, resulting in the death of 625,000 people worldwide [8,9]. Therapeutic choices for the management of cryptococcal infections are limited. The current therapy consists of amphotericin B and flucytosine (5-FC) cocktail but 5-FC remains largely unavailable in Asia and Africa where cryptococcal meningitis is common [10]. However, fluconazole intake for a prolonged period of time can lead to other yeast infections such as fluconazole resistant candidiasis. Whereas, in the case of highly efficient lipid based amphotericin B, its cost and inability to pass through the blood brain barrier are major setbacks. Thus the need for research into novel, effective and safe drugs to treat cryptococcal meningitis has become a relevant question in the scientific community [11,12].

Majority of the antimicrobial therapies in clinical use are natural products obtained from microorganisms [13]. Ocean constitutes one such source for developing antimicrobials owing primarily to their rich biodiversity. Marine organisms, especially plants and invertebrates, have received a lot of attention for their ability to produce antimicrobials. Along with marine plants and animals, marine microbes, found distributed in every niche of the ocean ecosystem form poorly explored resource for antimicrobials [14]. Though new antimicrobials from actinomycetes are being identified, they nevertheless are mostly related compounds or derivatives. To explore more diversified antibiotics, we have co-cultivated the actinomycetes sp with the pathogen,

thereby helping trigger defense and related metabolite production. It has been shown that co-cultivation is an efficient method for identifying and harnessing new secondary metabolites that are not detected in axenic cultures of the producing strain [15]. For example, during co-cultivation fungal derived enniatin derivatives showed increased antagonistic alterations against MRSA [16]. Co-cultivation is identified as a powerful emerging tool for enhancing the chemical diversity in microbes. Though this technology is found to be promising in biomedical research, it is still in its infancy.

There are various elicitation methods that are being used by various research groups to induce secondary metabolites which were not actually expressed in monocultures. Such as, elicitation by microbial co-cultivation [17–19], microbial lysates [20], cellular components [21,22], chemical elicitation [23], and molecular elicitation [24]. Adaptive laboratory evolution (ALE) is also reported as an acceleration method to induce the production of secondary metabolites [25–27].

ALE is an important scientific approach in the analysis of evolutionary phenomena [28]. ALE is a well known method to generate new improved phenotypes of industrial importance. In addition, ALE is used as a laboratory tool to investigate ethanol tolerance, osmotic stress, antibiotic resistance, and to increase antibiotics production [26,28–31].

In the present study, we developed a novel approach to trigger and improve the activity of bioactive molecule by the combined approach of ALE and co-culture. A new strain of *Streptomyces variabilis* AFP2 displaying anticryptococcal activity was co-cultivated with *C. neoformans*. The technique was carried over for a period of 30 days which comprised of 3 serial passages of *S. variabilis* AFP2. The metabolomes of wild and evolved strains were compared by HPLC-UV and GC- MS methods.

Methodology

Isolation of marine actinomycetes spp

The marine sediment samples were collected from Rameshwaram sea, Tamil Nadu, India (latitude: 9.145105 to 9.06412677; longitude: 79.453812 to 79.31699753) at different depths ranging from surface till 250 cm. The sediment samples were mixed together and preserved at 4˚C until further processing. A simple pretreatment method was followed to enhance the

isolation of actinomycetes spp. The soil samples were incubated at 60°C for 40 min and re-suspended in 0.9% saline water. The re-suspended mixture was diluted with 100 ml of saline containing 1.5% (v/v) phenol and shaken for 30 min at 28°C [32]. The pretreated samples were plated on various media like zobell marine agar [33], actinomycetes isolation agar [34], starch agar [35], yeast extract malt extract agar [21] and starch casein nitrate agar [35]. The media were supplemented with gentamycin (1µg/ml) and fluconazole (50 µg/ml) after sterilization to inhibit the growth of bacteria and fungi respectively [36,37]. The plates were incubated for 21 days at 37^{O}C. The isolates obtained from each media were stored in 15% glycerol at -80^{O}C.

Antagonistic assay against *C. neoformans*

Test strain

The test strain *C. neoformans* 14116 was purchased from Microbial Culture Collection Centre, Chandigarh. The strain was maintained in PDA slants at 4˚C and 15% glycerol stocks at -80˚C.

Antifungal assay

Anticryptococcal activity of 60 marine actinomycetes isolates was assayed to select a potential strain according to Ramakrishnan et al., (2009). Spore suspensions of individual isolates were spot inoculated (10 µl per spot) on Muller Hinton agar plates, and incubated at 30°C for 3 days. The cells were then killed with chloroform vapors and were subsequently over laid agar at last with 15 ml of medium containing 1% (w/v) agar, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract and swabbed with 100 µl of test isolate *C. neoformans* on the agar surface. The resulting clear zone of inhibition (ZOI) was measured after 2 days of incubation. The experiment was repeated thrice. Mean diameter of ZOI and standard deviations were calculated. Strain AFP2 which exhibited the maximum antagonistic activity against *C. neoformans* was selected for taxonomical investigation [38,39].

Taxonomical investigation of AFP2

Genomic DNA from AFP2 was isolated using the procedure described by Kimura (1980) [40]. The gene fragments were amplified by using PCR Kit (GENEI Pvt, Ltd, India) and 16S rRNA gene was amplified using Eppendorf Mastercycler pro thermal cycler with the following profile:

initial denaturation at 95°C for 4 min, 30 amplification cycles of (95°C for 1 min, annealing temperature at 50°C for 60 s, 72°C for 1 min) and a final extension step at 72°C for 4 min. The PCR product was electophoresed and purified from 1.5% agarose gel, using QIAquick PCR purification kit (QIAGEN) and sequenced using the primers 8F and U1492R [41,42]. Sequencing was done at Chromous Biotech, Bangalore, India using ABI 3100 sequencer (Applied Biosystems). The sequence was edited using FinchTV (Geospiza Inc.) and BioEdit (Ibis Biosciences, Abbott Labs). Sequence similarity search was made using 16S rRNA gene and taxid specific BLAST tool [39].

Adaptive Laboratory Evolution by competition based co-culture

Adaptive laboratory evolution by competition based co-culture was begun by growing AFP2 and *C. neoformans* separately each in 50 ml of starch broth at 37˚C for 48 hrs and 24 hrs respectively in orbital shaker at 120 rpm (Step 1). Following logarithmic growth period, competitive co-culture was initiated by transferring 10% of AFP2 (48 hrs old culture) and 5% inoculum of *C. neoformans* (24 hrs old culture) in 500 ml starch broth in a 1000 ml conical flask and growing them together at 37˚C over a 10-day period (Step 2). The culture was serially passed every 10 days for 30 days. The isolate whichever showing the enhanced anticryptococcal activity than the parental strain was selected for each serial passage. AFP2 was then cultured pure using quadrant streak technique on actinomycetes isolation agar medium at 37˚C for 72 hrs. Different colonies with distinct morphology and were checked for anticryptococcal activity. The isolate that produced largest ZOI (here after referred to as S1) was selected for a second round of adaptive competitive co-culture (Step 3). The serial passages of AFP2 (S1) were continued till 3rd cycle of adaptive co-culture experiment (Step 4 - 7). The best evolved strain from 3rd serial passage of adaptive phase was selected that showed higher antagonistic activity against *C. neoformans* than the parental and other evolved strains. However, we did not proceed to further serial passages as S3 shows suboptimal growth compared to parental strain, also it was presumed that, over the time, AFP2 will reach its maximum defense level against *C. neoformans*. During this selective pressure study, we have selected 3 potential evolved strains from each cycle of adaptive evolution whichever displaying improved activity than the parental one (**Figure 1)**. The evolved isolates S1, S2, S3 were selected for further analysis of secondary metabolite production (Step 8). The evolved strains were stored in 20% glycerol at -80ºC [26,28,43].

Production and Extraction of antifungal metabolites

A loopful of spores of parental type- *S. variabilis* AFP2, S1, S2, S3, and *C. neoformans* as control were cultivated in 25 ml of starch broth. The seed media was incubated at 37˚C for 48 hrs for parental strain, S1 and S2; 72 hrs for S3 and 24 hrs *C. neoformans* for logarithmic growth period. 10 % (v/v) of each seed inoculum was used to seed 2.5 L of production medium composed of Yeast extract (0.1%), Peptone (0.5%), Glucose (1%), KH_2PO_4 (0.1%), $MgSO_4$ (0.05%) at 30°C with a shaking speed of 180 rpm for an incubation period of 10 days. Each fermented broth was centrifuged at 8000 X g for 10 minutes. The culture filtrates were extracted twice with equal volume of ethyl acetate using cross current method. Residual ethyl acetate was concentrated *in vacuo* to dryness. Finally the extracts were dissolved in phosphate buffer (pH7). The yield of each crude extract was noticed and then evaluated for antifungal activity by well diffusion method, with a volume of 100 µl of compound in each well.

Differential secondary metabolite production by HPLC-UV-PDA

To monitor the differential secondary metabolite production in parental and evolved strains, ethyl acetate extracts of parental, S1, S2 and S3 were analyzed using Semi-Preparatory HPLC (1260 Infinity Agilent). Reverse-phase analytical C18 column (4.6 mm × 250 mm) was used with a linear gradient from 1% to 60% MeOH and 99% to 40% water over 30 minutes at a flow rate of 1mL/min at 25°C. First 4 minute MeOH concentration was linearly increased upto 20%. At twentieth minute, MeOH concentration was linearly increased to 50%. Finally, MeOH concentration was increased to 60% at 30 min. The chromatograms were obtained at different wavelength (200 nm, 225 nm, 250 nm, 300 nm), to explore the possible metabolite profiles at various wavelengths [18].

Antifungal activity of Parental and S3 HPLC fractions

The HPLC fractions of parental and S3 strain were collected using peak based method. The fractions were dried in oven at 50°C, dissolved in 50 µl PBS and checked for anticryptococcal activity (10^5 cells in each well) in 96 well plate, triplicates were maintained for each fraction. The 96 well plate was incubated at 37°C for 48 hrs in humid condition to reduce media evaporation. The optical density of each well was recorded using an ELISA reader device (Sunrise, Tacan) at 620 nm [15].

Evaluation of evolution in *Streptomyces variabilis* AFP2

In general, morphological variants are evident in *Streptomyces* mutants [43]. Hence, phenotypic changes in parental and evolved strain were evaluated by observing the changes of colony and spore morphology. The spore morphology of 14 days old culture sample of parental strain and S3 strain were examined using light and scanning electron microscope. The spore morphology was studied by examining gold-coated dehydrated specimens using the Field Emission Scan Electron microscope, JSM-6701F, JEOL, Japan Make.

Also, increment in antagonistic activity of S3 were analyzed by culturing in following conditions, (i) monoculture of parental strain (ii) monoculture of S3 strain (iii) monoculture of *C. neoformans* (iv) co-culture of parental and *C. neoformans* and (v) co-culture of S3 and *C. neoformans*. The experiments were carried out for 14 days at 37^{O}C. Each day, 100 µl of co-culture broth were withdrawn at 24 hrs time intervals for 14 consecutive days and cell counts of *C. neoformans* were enumerated using hemocytometer. In addition, changes in capsule size, the polysaccharide matrix which is surrounding *C. neoformans* were assessed.

Measurement of capsule thickness by India ink staining

A drop of India ink was mixed with an aliquot of *C. neoformans* on a glass slide. The samples were examined using Nikon (Eclipse C*i*-L) and images were taken with a SLR, Canon D5100, Camera. To calculate relative size of capsule, diameter of whole cell, including capsule (Dwc) and cell body weight limited by cell wall (Dcb), were measured using ImageJ 1.48v software (National Institutes of Health, Washington, D.C.). The size of the capsule relative to that of the whole cell was defined, as percentage {[(Dwc - Dcb)/Dwc] X 100}. Eight cells were measured for each determination and average was calculated [44–46].

GC-MS analysis

Crude extract of S3 strain, was subjected to comparative analysis against extract from parental strain using Gas Chromatography and Mass Spectrometry (GC-MS) with the help of National Institute of Standard and Technology spectral library [47]. The analyses were performed using a PerkinElmer Clarus 500 GC-MS system. The program was set at a temperature of 50°C for a duration of 1 min and raised at 10°C/min to 150°C (1 min hold), at 8°C/min to 250°C (1 min hold), at 15°C/min to 300°C (3 min hold). Helium (1 mL/min) was used as carrier gas. The injector temperature was maintained at 280°C and the mass range was 40–450 Amu. One µl of sample dissolved in ethanol was injected into the system. The compounds were identified by comparing mass spectra with data base spectra.

Result

Isolation of *S. variabilis* AFP2

During this study, 60 morphologically different actinomycetes spp from marine sediments were isolated using various agar medium recommended for actinomycetes isolation. However, only two isolates were shown to have anticryptococcal activity, among which strain AFP2 isolated from starch agar displayed maximum ZOI of 12 mm. Thus the strain AFP2 was selected for 16S rRNA sequencing. The sequence similarity search using BLAST tool reveals that AFP2 (KJ716228) belongs to a distinct phyletic line in *S. variabilis*. The isolate was closely related to the type strain of *S. variabilis* strain NRRL B-3984, *S. labedae* strain CSSP735, *S. lateritius* strain CSSP722 and *S. griseoincarnatus* strain CSSP407 sharing a homology of 99%.

Laboratory Adaptive evolution by competition based co-culture

ALE by competition based co-culture was carried out to trigger the expression of anticryptococcal metabolites in AFP2. After 30 days of serial passage (~ 180 generations) of AFP2 under competition based co-culture with *C. neoformans*, three isolates S1, S2 and S3 were derived from a single evolved population in three adaptive cycle. During adaptive evolution, certain changes in characteristics of *S. variabilis* are expected to occur, hence to confirm the putative effect, the evolved isolate from each adaptive phase were checked for anticryptococcal activity. Because, the best phenotype is not necessarily the one with the highest ability to survive in competitive condition, but the one that showed increased activity [28]. The ZOI of evolved strains were comparatively higher than parental strain suggesting that antagonistic activity are

higher in evolved strains than parental strain. A similar kind of adaptive evolution was used by Charusanti et al. (2012) to enhance the activity of *S. clavuligerus* against *S. aureus*, however the experiment design was different from the present study [26,48,49].

Enhancement of antifungal activity in evolved strains

Parental-type, S1, S2, S3 strains were cultivated in production medium for 10 days at 37°C. The culture supernatant were then centrifuged and extracted with ethyl acetate. The yield variation between the strains were noticed (parental type- 11.8 mg; S1 and S2-12.6 mg; S3- 8.6 mg, and *C. neoformans* - 3 mg, for 100 ml of production medium). The significant increment in antifungal activity of the crude extracts of adapted strains are depicted in **Figure 2**, parental strain - 12 mm; S1 and S2 - 15 mm; S3 - 26 mm ZOI. Interestingly, the compound yield and antagonistic activity were not directly proportional, probably suggesting that adaptation could most likely enhance the synthesis of more specific inhibitory molecules than wild-type.

Secondary metabolite profiles by HPLC-UV-PDA

The secondary metabolite profiles of parental-type, S1, S2, S3 were monitored by HPLC-UV-PDA. The maximum absorbance was observed at 225 nm for all the strains. The metabolome of evolved strains showed a different chemo-type than parental-type (Figure 3). The comparison of all chromatograms implies that some new peaks were noticed in S1, S2 and S3 between the retention times of 4-8 minutes. In addition, the elevation of peak area in S3 chromatogram confirms that the triggered molecules and existing molecules are over-expressed. The comparison of HPLC chromatograms of parental and evolved strains suggests that progressive adaptations of the strain over several passages are useful to trigger and enhance the expression of metabolites. Many reports have demonstrated the chemotypic differences in axenic and mixed culture using HPLC-UV analysis [15,18,50], however it is a presumptive test to confirm the metabolome variation. Though the samples were analyzed at different wavelengths to screen out the maximum number of peaks, it is mandatory to identify the chemotype of parental and evolved strains.

Antifungal activity of parental and S3 HPLC fractions

The HPLC fractionation of crude extract of wild type and S3 yielded 22 and 32 fractions respectively. **Figure 4** shows that nearly 12 molecules were newly expressed in evolved strain. Among which, 3 fractions were shown to have antagonistic activity of 35-45% in terms of reduction in *C. neoformans* growth compared to positive control. However, 22nd fraction which was found to be expressed in both parental type and S3 exhibited 65% reduction in growth *C. neoformans*. Hence the induction and over-expression of antifungal molecules are evident in evolved S3 strain.

Evaluation of evolution in AFP2

A notable phenotypic change in terms of colony morphology was observed in S3 strain (**Figure 5 I**). Scanning Electron Micrograph analysis of 14 day old spores of parental strain shows the presence of wart-like projections and spines on spore surface. However, S3 strain was characterized by tightly coiled spiral spore chains with spores having large numbers of long projections (**Figure 5 II**), unlike the somewhat straight and loosely coiled spore chains with fewer projections over the spore surface of the parent type.

The changes in growth profiles were also observed for evolved and parental strain. The evolved strain shows suboptimal growth when compared with parental strain (**Figure 6 I)**. The suboptimal growth of S3 strain reveals that it has undergone a competition for nutrients during co-cultivation. However, maximum growth rate of microbe is not a common criteria to determine evolutionary fitness [28,51]. Nevertheless, biomass yield and its tolerability to survive in competitive environment are always co-related with secondary metabolite production [52]. Interestingly, increased activity was noticed even in suboptimal growth of S3. **Figure 6 II** represents the reduction of *C. neoformans* growth during mixed culture with parental strain. However, much remarkable reduction in fungal growth was observed in co-cultivation of S3 and *C. neoformans*. Meanwhile, a notable change in reduction of capsule thickness by evolved strain was clearly evident in **Figure 7** with a reduction of 25% capsule thickness.

GC-MS analysis

About 21 new metabolites alien to the parent strains were seen to be produced by S3 (**Figure 8).** The list of compounds that are commonly expressed in parental and evolved strain S3 are given in **Table 1.** The triggered molecules in S3 and those that are absent in wild strain

are provided in **Table 2**. In addition to interpretation of chemical diversities, the % peak area data represents the compound yield. The increment in compound yield is a noticeable result to prove that integrated approach of ALE and co-cultivation is a suitable method to enhance metabolite production. For instance, the yield of 2-methyl propanamide was 56% fold increased, Butanamide (54% fold increase); 3-methyl- Butanamide (53% fold increase) and hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione (41% fold increase) were recorded when compared with parental type *S. variabilis*. The overproduction was clearly evident as shown in **Figure 8 and Table 1**. Few of the induced compounds and their derivatives were previously reported for antifungal activity (Table 2). . Nevertheless, the molecules like dl-Alanyl-l-leucine (yield, % peak area - 3.1%); dihydro-3,3-dimethyl-2 (3H)-Furanone (2.7%); Enanthamide (2.7%); 1,3,5-Cycloheptatriene (1.6%); 1-Aziridineethanol (1.6%) were not reported for antifungal activity. In addition, these molecules were not reported in *S. variabilis*. Hence it confirms that our approach enables the triggering of silent gene cluster(s) resulting in the synthesis of novel molecules.

The evolutionary fitness analysis reveals that 64 fold or 98% reduction in *C. neoformans* growth were noticed in evolved strain S3. In addition, remarkable differences in induction and over production of antifungal molecules were also evident in S3 strain, with an increase of ~52 % of compounds and 21 triggered compounds. Hence the present study confirms that an integrated approach of adaptive laboratory technique and co-culturing techniques are useful to explore potential molecules against targeted pathogen.

Table 1: Common compound present in both Wild strain and S3 strain analyzed by GC-MS

S. No.	Name	Formula	MW	Wild			S3			Overproduced of compound in S3 (% fold increase)
				Retention time	Peak area	%Peak area	Retention time	Peak area	%Peak area	
1.	2-methyl-Propanamide,	C_4H_9NO	87	4.74	39873944	3.7718	4.82	1.39E+08	8.6307	56.29787
2.	Butanamide	C_4H_9NO	87	5.42	13945809	1.3192	5.48	46590024	2.8877	54.31658
3.	N-Hydroxymethylacetamide	$C_3H_7NO_2$	89	7.87	4336799	0.4102	6.29	4697297	0.2911	-
4.	3-methyl-Butanamide	$C_5H_{11}NO$	101	6.48	25926508	2.4525	6.61	85550336	5.3025	53.74823
5.	Pentanamide	$C_5H_{11}NO$	101	9.12	43524428	4.1171	9.26	91229608	5.6545	27.18896
6.	Octanamide	$C_8H_{17}NO$	143	13.64	8076171	0.7639	11.66	17206876	1.0665	28.37318
7.	Octanamide	$C_8H_{17}NO$	143	-	-	-	13.70	6632707	0.4111	
8.	[(methoxymethoxy)methyl]-Benzene	$C_9H_{12}O_2$	152	14.24	3329908	0.315	14.25	4154936	0.2575	-
9.	Tridecane	$C_{13}H_{28}$	184	14.92	1007684	0.0953	14.92	696186	0.0432	-
10.	Benzeneacetamide	C_8H_9NO	135	15.76	2.85E+08	26.9762	16.10	4.72E+08	29.244	7.754753
11.	2,4-bis(1,1-dimethylethyl)-Phenol	$C_{14}H_{22}O$	206	18.38	24139044	2.2834	18.40	2209378	0.1369	-
12.	Tridecane	$C_{13}H_{28}$	184	14.92	1007684	0.0953	14.92	696186	0.0432	-
13.	Tridecane	$C_{13}H_{28}$	184	-	-	-	20.19	608988	0.0377	-
14.	hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	27.05	35255676	3.3349	27.16	67217472	4.1662	19.95343
15.	4-methyl-, 1-methylethyl ester Benzoic acid	$C_{11}H_{14}O_2$	178	28.23	24750376	2.3412	28.33	51245500	3.1762	26.28928
16.	hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	29.41	21280488	2.013	29.52	55535760	3.4422	41.51996
17.	hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	30.05	79494712	7.5196	30.17	1.29E+08	7.9727	5.683144
18.	hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210	30.36	28916384	2.7353	30.48	67372280	4.1758	34.49638
19.	Dodecanamide	$C_{12}H_{25}NO$	199	31.83	94955744	8.9821	34.00	2033383	0.126	-
20.	hexahydro-3-(phenylmethyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{14}H_{16}N_2O_2$	244	40.47	72263544	6.8356	41.06	80100008	4.9647	-

Table 2: Compound present in S3 strain analyzed by GC-MS

S. No.	Name	Formula	MW	Retention time	Peak area	%Peak area
1.	1,3-dimethyl-Benzene	C_8H_{10}	106	3.86	4746478	0.2942
2.	1-Aziridineethanol	C_4H_9NO	87	7.08	26188024	1.6232
3.	1,1'-[ethylidenebis(oxy)]bis[2-methyl-Butane	$C_{12}H_{26}O_2$	202	7.39	1686723	0.1045
4.	3-Butenoic acid, 2-methyl-, methyl ester	$C_6H_{10}O_2$	114	7.6	3654044	0.2265
5.	Butanedioic acid	$C_4H_6O_4$	118	13.12	1403604	0.087
6.	4-methylene-5-Hexenal	$C_7H_{10}O$	110	7.79	2322845	0.144
7.	dihydro-3,3-dimethyl-2(3H)-Furanone	$C_6H_{10}O_2$	114	8.32	44006676	2.7276
8.	4-Acetylbutyric acid	$C_6H_{10}O_3$	130	10.49	2265020	0.1404
9.	6-methyl-6-Azabicyclo[3.2.1]octane	$C_8H_{15}N$	125	10.69	196201	0.0122
10.	Enanthamide	$C_7H_{15}NO$	129	11.45	44932220	2.7849
11.	1,3,5-Cycloheptatriene	C_7H_8	92	12.76	26539802	1.645
12.	dihydro-4,4-dimethyl-2(3H)-Furanone	$C_6H_{10}O_2$	114	13.91	1851129	0.1147
13.	Benzamide	C_7H_7NO	121	14.38	2886997	0.1789
14.	2-methyl-4H-3,1-Benzoxazin-4-one	$C_9H_7NO_2$	161	15.43	698960	0.0433
15.	Phenylpropanamide	$C_9H_{11}NO$	149	18.91	1882071	0.1167
16.	4-methyl-Pentanamide	$C_6H_{13}NO$	115	21.04	3508549	0.2175
17.	4-hydroxy-Benzeneacetic acid	$C_8H_8O_3$	152	22.21	6275494	0.389
18.	3-(1-aziridinyl)-3-(dimethylamino)-2-Propenal	$C_7H_{12}N_2O$	140	24.37	14641184	0.9075
19.	dl-Alanyl-l-leucine	$C_9H_{18}N_2O_3$	202	25.78	51295148	3.1793
20.	4-Hydroxyphenylacetamide	$C_8H_9NO_2$	151	26.4	8670816	0.5374
21.	4-ethyl-5-methyl-Heptanamide	$C_{10}H_{21}NO$	171	26.68	14915393	0.9245
22.	2,6,10,15-tetramethyl-Heptadecane	$C_{21}H_{44}$	296	33.79	582825	0.0361

Discussion

Natural products from actinomycetes spp are considered as an important source for novel antimicrobial compounds due to its diversified secondary metabolites [53,54]. Recently the rate of discovery of new compounds from terrestrial actinomycetes spp has been decreasing, while the rate of rediscovery of known compounds has increased [55,56]. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes spp have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds. They form a stable, persistent population in various marine ecosystems [57]. There are some recent discoveries of new antifungal molecules namely, bahamaolides A and B, polyene-polyol-macrolides [58], ikarugamycin derivatives from marine *S.*

variabilis [59–61] and new tetramic acid glycoside, aurantoside K, obtained from marine sponge [62,63]. In addition, more information was provided by Xu et al., (2015) in a recent review which reports 116 new antifungal and antibacterial compounds from marine fungi during 2010-15[64]. Many studies propose the existence of unexplored antifungal compounds from marine actinomycetes spp. The present study exploited marine sediments for actinomycetes spp exhibiting anticryptococcal activity. During the course of this study, we found a new strain of *S. variabilis* AFP2 having inhibitory effect against *C. neoformans,* indicating the production of possible new antifungal metabolites. However, the chances of rediscovering the tens of thousands of known molecules are high. The main challenges behind this are the inability to cultivate potential microbes and finding a suitable trigger that enables the expression of silent biosynthetic gene clusters under normal laboratory conditions. Novel approaches are being implemented by various researchers to explore antimicrobials such as, genome mining and soil metagenome[43,48,65]. Till now various co-culture strategies were employed to mimic natural habitations to facilitate the exploration of novel secondary metabolites [66,67]. Mixed cultivation of microbes has drawn the attention of most investigators as the interspecies communication and defense mechanisms are found to trigger the expression of silent biosynthetic gene clusters [68,69]. However, mixed fermentations are still in its infancy probably due to lack of reproducibility [70]. More recently, two new butyrolactone derivatives was isolated during the co-cultivation of *A. terreus* with the bacteria *Bacillus subtilis* and *Bacillus cereus* [71]. Similarly, a new molecule Rhodostreptomycin was expressed during the co-cultivation of *Rhodococcus fascians* and *S. padanus* [72], which was not detected in axenic culture. However, co-culture may not always be suitable for exploring novel molecules. For example, Levorin (existing molecule) was isolated during co-cultivation of *Candida tropicalis* and *Actinomyceslevoris* [73,74]. Similarly, 6-Methylsalicylic acid, cyclo-(phe-phe) dipeptides were isolated during the co-cultivation of two unknown marine endophyte fungi [75]. Also, in a recent study, co-cultivation of *Aspergillus fumigatus* and *S. bullii* was reported to produce seven metabolites belonging to diketopiperazine alkaloids with antimicrobial and antiprotozoan activity [21]. Likewise, significant numbers of known compounds were reported and thus mixed fermentation are not likely to yield novel molecules always [76]. Hence, till now various co-culture strategies were employed to mimic natural habitations to facilitate the exploration of novel secondary metabolites [67]. In the present study, we followed a different approach by combining the adaptation laboratory evolution and

mixed fermentation. Our idea was to evolve improved phenotypes showcasing efficient defense mechanism against the targeted pathogen by over expression and induction of novel molecules. In general, ALE involves cultivation of microbes in controlled conditions for prolonged periods in the range of weeks to years, which allows the selection of improved phenotypes. Evolutionary engineering is proven to be an efficient method for the improvement of industrial strains. The technique is successfully established to generate strains with improved activity such as tolerance to thermal stress, nutrient stress, osmotic stress, ethanol stress, acid stress and also used for enhanced substrate utilization[77–79]. The primary motive of the present study was to apply the evolutionary principle to generate improved antagonistic property with the possible methods to activate silent gene cluster by co-cultivation. During our study, with adaptive evolution and competitive co-culture of *S. variabilis* AFP2 and *C. neoformans* for periods of 30 days, we have selected 3 improved phenotypes with higher anticryptococcal activity and the metabolomes were analysed.

In general, the common analytical technique for metabolome analysis includes LC-MS, GC-MS and NMR. GC-MS is recognized as one of the most versatile analytical method in metabolomics study of biological samples, because of its reliable and reproducible results [80,81]. However, the reports on microbial metabolomics with GC-MS are limited [82]. Cevallos-Cevallos et al., (2011) demonstrated the suitability of GC-MS for microbial metabolomics of *E. coli, Bacillus* sp and *Pseudomonas* sp. More frequently researchers used HPLC/ LC-MS followed by NMR to elucidate the structural details of triggered molecules [21,71,83]. However, as our intention was to compare metabolic profiles of the parental and evolved strain thus GC-MS platform was used. The GC-MS analysis showed clear metabolome comparisons of parental and S3 strain with respect to peak identification, peak relative area, retention time and mass spectra data (**Figure 8)**. Structural elucidation using NMR are future directions of our lab. Though GC-MS analysis determines the over-expression and induction of compounds we believe that biological assays are also mandatory. Thus, we analyzed the extracts of parental, S1, S2 and S3 against *C. neoformans* and its capsule growth. The capsule is the most prominent and well-studied antiphagocytic factor in *C. neoformans* [84,85]. The efficiency of the phagocytosis is regulated by capsule size. The increase in capsule size is found to decrease the complement mediated phagocytosis [86,87]. Hence, evaluation of *in vitro* activity of the extracts of parental and evolved strains on influence of capsule size of *C. neoformans* was considered to be another significant result of the present

study, which is clearly depicted in **Figure 7**. The evolved strain showed 64 fold or 98% reduction in *C. neoformans* growth when compared with parental strain. The enhanced antifungal activity was possibly due to the induction of new molecules and over expression of compounds in S3 strain. GC-MS analysis depicts elevation of ~52 % metabolite production. Hence the present study concludes with evidence, that adaptive laboratory evolution by competition based co-culture is a novel and suitable method of strain engineering to induce and overproduce specific molecules against the targeted pathogen.

Conclusion

The main challenge behind the discovery of novel antiinfectives from microbial source is to find a suitable trigger that enables the expression of silent biosynthetic gene clusters under normal laboratory conditions. Novel approaches are being implemented by various researchers to explore novel antimicrobials such as, genome mining and soil metagenome and co-culture techniques employed to mimic natural habitations to facilitate the exploration of novel secondary metabolites. However, co-culture may not always be suitable for exploring novel molecules. Our results suggests that the proposed integrated approach of adaptive laboratory evolution and competition based co-culture is a novel and suitable method of strain engineering to induce and overproduce specific molecules against the targeted pathogen.

Conflict of Interest Statement

The authors declare no conflict of interests.

Author and Contributors

All authors have equal contribution.

Funding

Department of Science & Technology (DST), New Delhi, under the Fast Tack Young Scientist Scheme (No: SB/FT/LS-249/2012) and EMR scheme SR/S0/HS-0073/2012.

Acknowledgements

This study was financially supported by the Science and Engineering Research Board (SERB), Department of Science & Technology, New Delhi, under the Fast Track Young Scientist Scheme (No: SB/FT/LS-249/2012) and HPLC facility EMR scheme SR/S0/HS-0073/2012 to JP. We thank SASTRA University for providing us the infrastructure to carry out our research work.

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Figure Captions

Figure 1: Adaptive Laboratory Evolution by competition based co-culture: **Step 1**: Mono-culturing of AFP2 and *C. neoformans* for 48 h and 24 h respectivly; **Step 2**: coculturing of 10% AFP2 and 5% *C. neoformans* for 10 days; **Step 3**: Isolation of discrete colonies of AFP2 from coculture broth and selection of phenotype with improved antifungal activity (evolved strain designated as S1); **Step 4**: co-culturing of S1 and *C. neoformans* for second round of ALE; **Step 5**: Isolation of discrete colonies of S1 from co-culture broth and selection of phenotype with improved antifungal activity (the strain designated as S2); **Step 6 and 7**: the above precedure is repeated for 3rd round of ALE; **Step 8**: selection of potential evolved strain for monitoring the induction and overproduced antifungal molecules, compared with parental strain.

Figure 2: Antagonistic assay of EtoAc extracts of AFP2 parental and evolved strains (A- D): ZOI of AFP2 parental, S1, S2, S3 strains.

Figure 3: Comparative study of HPLC profiling of EtoAc extracts of AFP2 parental and evolved strains. HPLC chromatograms of AFP2 Parental, S1, S2, S3 and *C. neoformans* at 225 nm.

S3 chromatogram (*) represents newly expressed peaks (^) overproduced peaks.

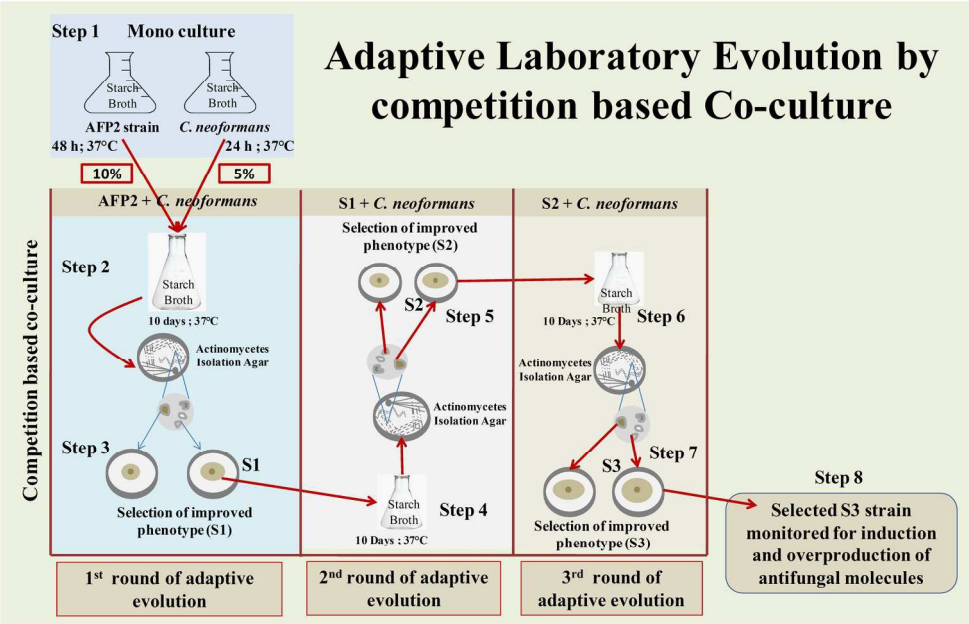
Figure 4: Antifungal activity of AFP2 parental and evolved S3 HPLC fractions. (-)ve represents negative control (media without culture); (+)ve represents positive control (media with culture).

Figure 5: I. Morphology variants of parental strain and evolved strain: (A) and (B): colony morphology on AIA agar; II. SEM micrograph of AFP2 and S3 strain.

Figure 6: I. Growth profile of monocultures (Control): (A): *C. neoformans*; (B): AFP2 parental strain; (C): S3 evolved strain; II. Antagonistic effect of parental and evolved strain during co-culturing: (D): Parental Vs *C. neoformans*; (E): S3 evolved Vs *C. neoformans*

Figure 7: Comparison of *C. neoformans* capsule growth during cocultivation with parental and S3 evolved strain (A); The percentage of the capsule thickness for ≥8 cells per group is shown. Bars represent standard errors. (B-D). Light microscopic view (100 X) of *C. neoformans* capsule.

Figure 8: GC-MS profiling of AFP2 parental and S3 evolved strain: S3 chromatogram (*) represents induced molecules (^) represents overproduced molecules. 1: dihydro-3,3-dimethyl-2 (3H)-Furanone; 2: Enanthamide; 3: 1,3,5-Cycloheptatriene; 4: dl-Alanyl-l-leucine.



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SASTRA Manuscript Revised 2209
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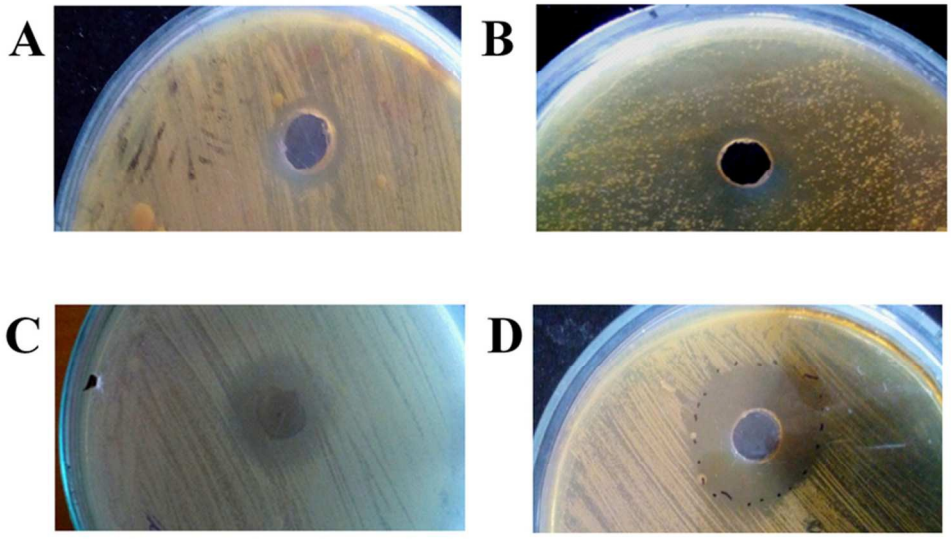

Figure 2: Antagonistic assay of EtoAc extracts of AFP2 parental and evolved strains (A- D): ZOI of AFP2 parental, S1, S2, S3 strains.
SASTRA Manuscript Revised 2209
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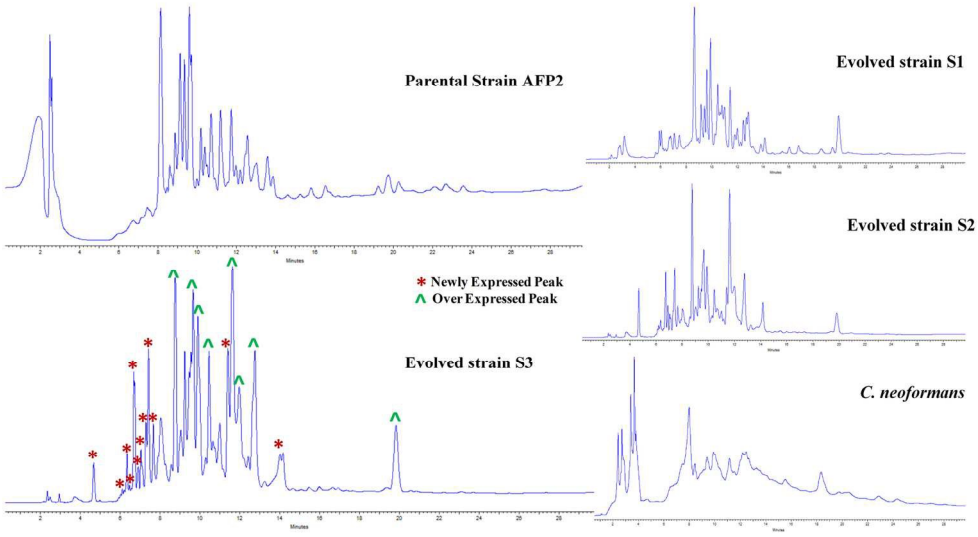

Figure 3: Comparative study of HPLC profiling of EtoAc extracts of AFP2 parental and evolved strains. HPLC chromatograms of AFP2 Parental, S1, S2, S3 and C. neoformans at 225 nm. S3 chromatogram (*) represents newly expressed peaks (^) overproduced peaks.
SASTRA Manuscript Revised 2209
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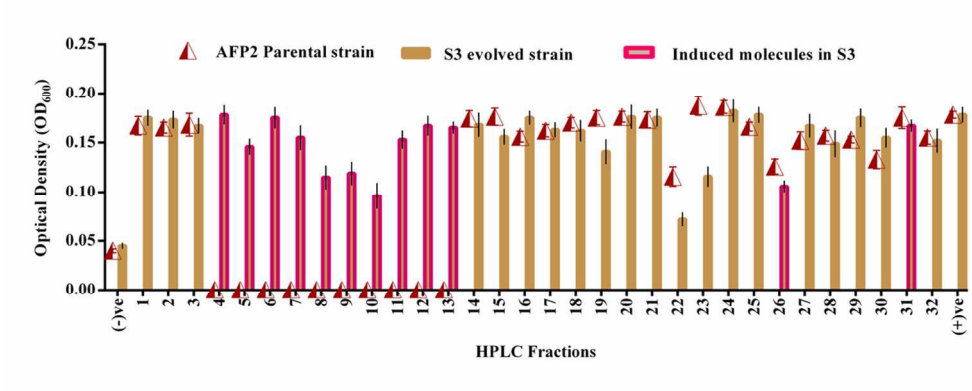

Figure 4: Antifungal activity of AFP2 parental and evolved S3 HPLC fractions.
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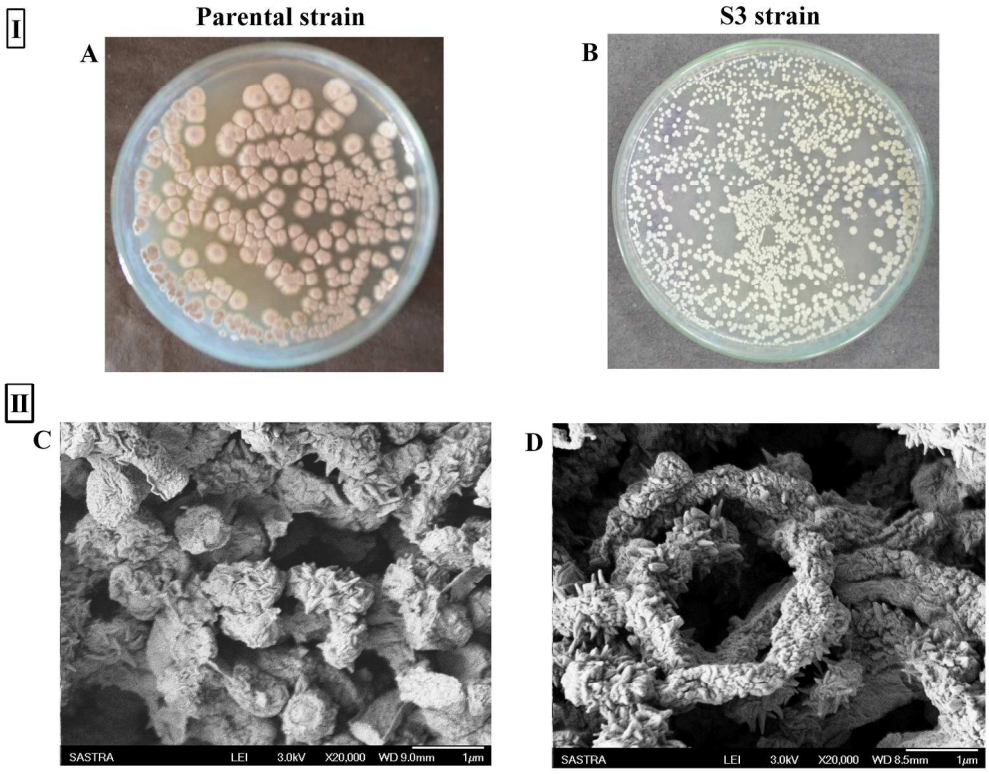

Figure 5: I. Morphology variants of parental strain and evolved strain: (A) and (B): colony morphology on AIA agar; II. SEM micrograph of AFP2 and S3 strain.
SASTRA Manuscript Revised 2209
214x170mm (300 x 300 DPI)

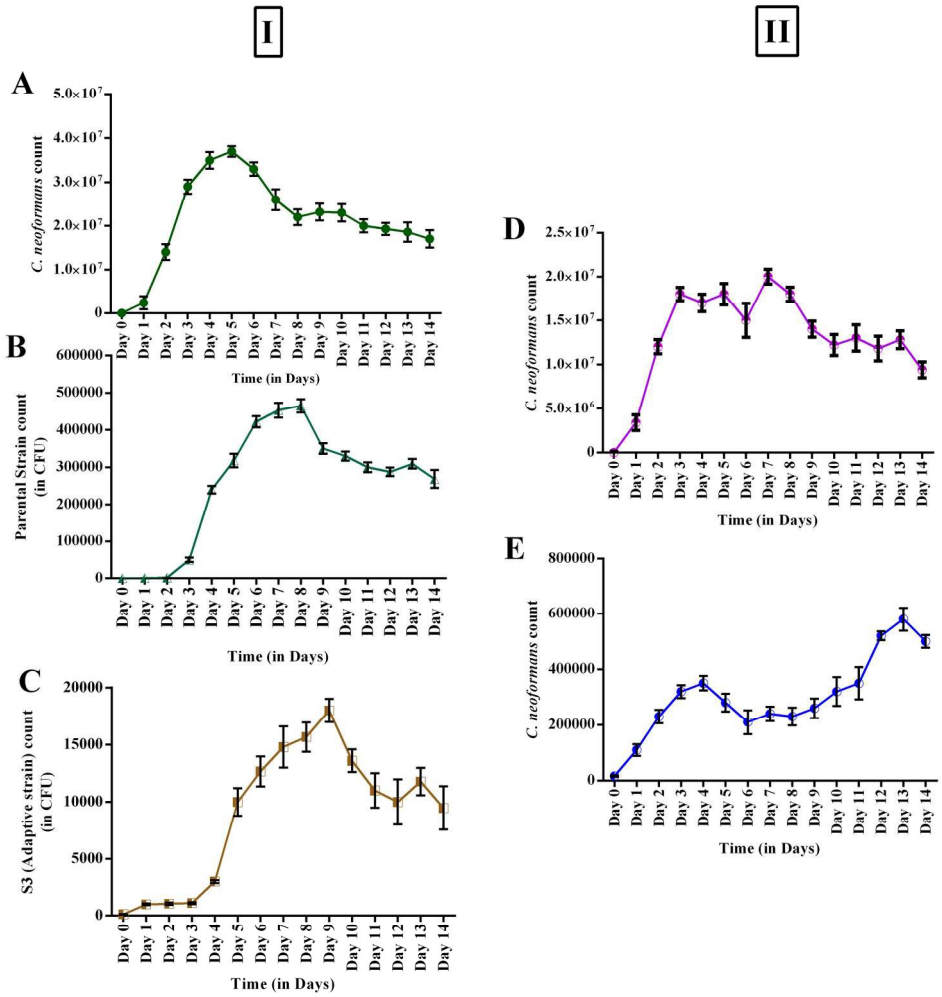

Figure 6: I. Growth profile of monocultures (Control): (A): C. neoformans; (B): AFP2 parental strain; (C): S3 evolved strain; II. Antagonistic effect of parental and evolved strain during co-culturing: (D): Parental Vs C. neoformans; (E): S3 evolved Vs C. neoformans
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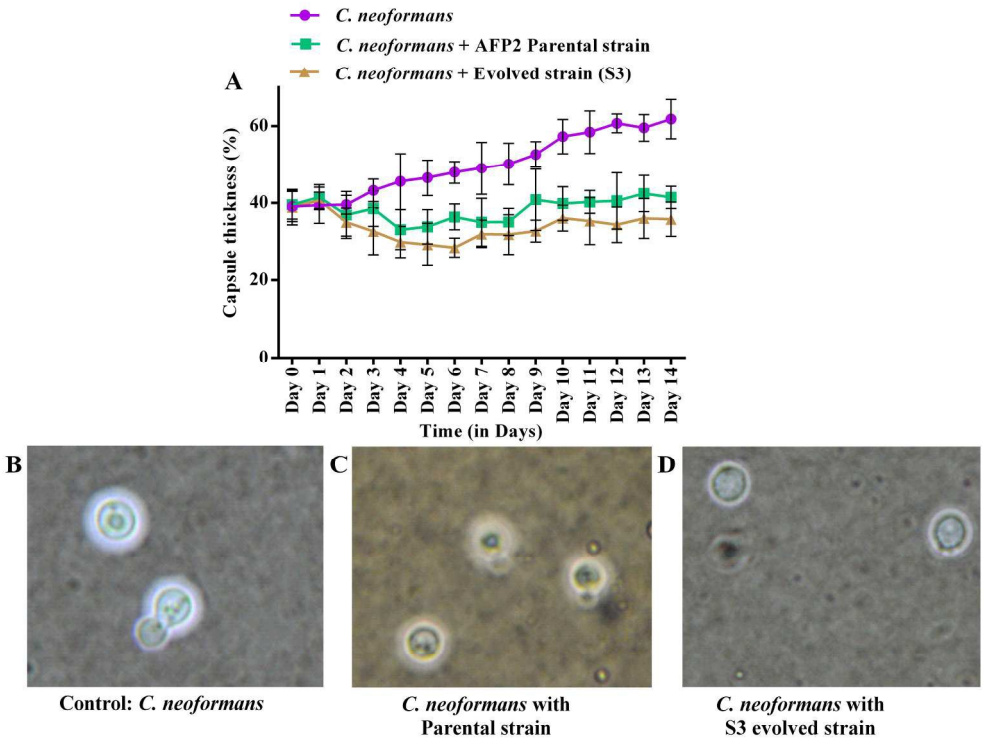

Figure 7: Comparison of C. neoformans capsule growth during cocultivation with parental and S3 evolved strain (A); The percentage of the capsule thickness for ≥8 cells per group is shown. Bars represent standard errors. (B-D). Light microscopic view (100 X) of C. neoformans capsule.
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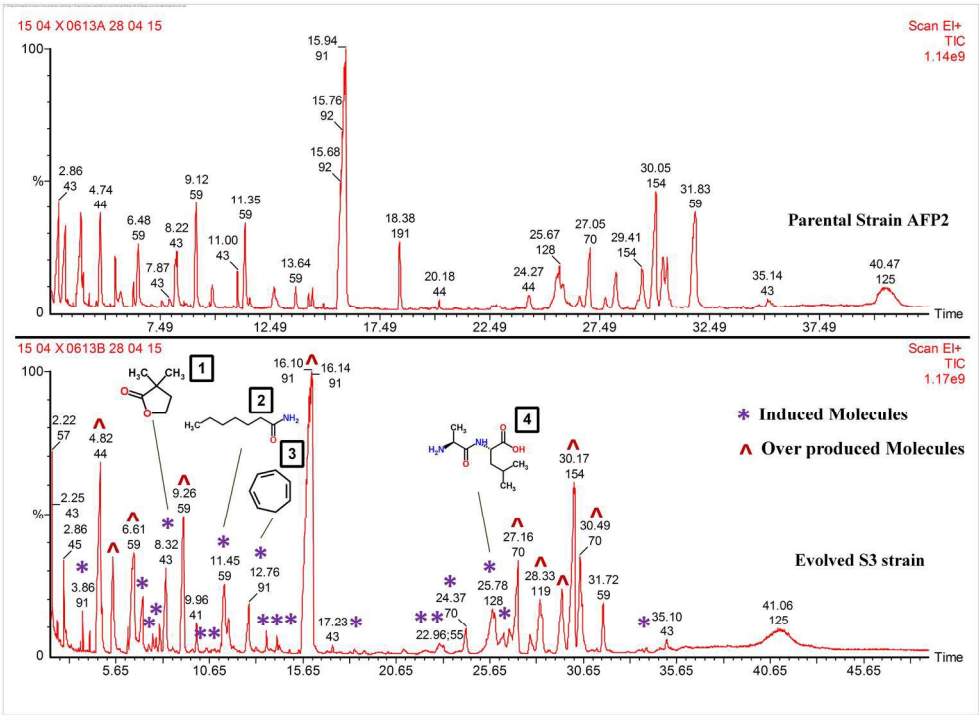

Figure 8: GC-MS profiling of AFP2 parental and S3 evolved strain: S3 chromatogram (*) represents induced molecules (^) represents overproduced molecules. 1: dihydro-3,3-dimethyl-2 (3H)-Furanone; 2: Enanthamide; 3: 1,3,5-Cycloheptatriene; 4: dl-Alanyl-l-leucine.
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205x151mm (300 x 300 DPI)