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Rapid Screening and Isolation of a Fungus for Sitosterol to Androstenedione Biotransformation

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Abstract Androstenedione (AD) is a steroid intermediate used in the pharmaceutical industry for the production of several important anabolic drugs. An important route for producing AD is by the microbial transformation of sterols. Microbes capable of efficiently transforming sterols to AD are few and newer strains need to be isolated. Conventional procedures for screening and isolation are time consuming. A new procedure was used for screening and isolation of fungal microorganisms capable of biotransformation of sitosterol to androstenedione. In this procedure, Basic Alignment Search Tool (BLAST) and Position-Specific Iterative BLAST were employed to obtain a parent set of candidate microorganisms. The parent set was reduced using heuristics and constraints to obtain a manageable number of microorganisms that may be tested experimentally. For this work, screening of the entire NCBI database yielded a parent set containing 64 microorganisms. Among these, only two microorganisms, *Aspergillus oryzae* and *Aspergillus nidulans* FGSC A4, qualified to the experimental stage. Sitosterol biotransformation experiments were carried out using *A. oryzae* and the production of AD in culture medium was confirmed.

Keywords Androstenedione · Biotransformation · BLAST · β-Sitosterol · Isolation · Screening

Introduction

Androstenedione (AD) is a key intermediate of microbial steroid metabolism. It belongs to the 17-keto steroid family and used as starting material for the preparation of several pharmaceutically important steroids. AD is a compound that has structural and pharmacological resemblance to testosterone [1]. For a long time, AD has been the starting material for the preparation of androgens and anabolic drugs and more recently for the

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production of spironolactone. Biotransformation of sterols to AD(D) requires a cascade of enzymes working in series in a single microbial cell. Therefore, the literature data on selective side chain cleavage of sterols is confined mainly to *Mycobacterium* sp., *Moraxella* sp., and *Lactobacillus bulgaricus* [2]. Among these, *Mycobacterium* sp. is the most widely used biocatalyst for AD(D) production by selective side chain cleavage of sterols.

Sripalakit et al. [3] had attempted to screen some randomly selected bacterial and fungal species for their ability to convert natural sterols to AD. The bacterial species taken into consideration were *Arthrobacter citreus* TISTR 820, *Bacillus sphaericus* ATCC 13805, *Bacillus stearothermophilus* TISTR 329, *Corynebacterium aquaticum* TISTR 823, *Pseudomonas acidovorans* TISTR 356, *Streptomyces peucetius* TISTR 3355, *Mycobacterium* sp. NRRL B-3805, and *Mycobacterium* sp. NRRL B-3683, while the fungal strains *Aspergillus niger* TISTR 3254, *Aspergillus terreus* TISTR 3109, *Cunninghemella elegans* TISTR 3370, *Curvularia lunata* TISTR 3292, *Penicillium siamensis* TISTR 1253, and *Rhizopus arrhizus* TISTR 3188 were screened for sterol side chain cleavage activity using β-sitosterol as substrate. None of the species of bacteria or fungus mentioned above showed positive result except the already reported *Mycobacterium* sp. NRRL B-3683 and NRRL B-3805. The problem associated with screening microorganisms capable of biotransforming sitosterols to AD(D) by conventional methods is that there are no selection criteria for obtaining positive isolates. Each isolate would need to be tested experimentally for biotransformation capability. Consequently, the screening process is labor intensive and time consuming and yet possesses a lower success rate.

The present study was done with the aim of screening and isolating novel fungal microorganisms capable of transforming sitosterol to AD, by carrying out a minimum number of targeted wet-lab experiments. A reverse-genetics approach has been applied to identify microorganisms with requisite functional attributes. Extensive data mining was carried out using bioinformatic tools to predict a set of microorganisms capable of converting sitosterol to AD. Constraints were applied to select microorganisms possessing a higher probability of success. One of these microorganisms was selected to verify the hypothesis experimentally.

Experimental

Chemicals

β-Sitosterol was purchased from Acros organics, USA. Analytical grade 4-androstene-3,17-dione (AD) was purchased from Sigma. Nutrient broth (NB), potato dextrose agar, and anisaldehyde were purchased from Himedia, India. High-performance liquid chromatography grade chloroform, diethyl ether, toluene, acetic acid, and sulfuric acid were purchased from Merck, Germany while ethyl acetate and methanol were purchased from Qualigen, India.

Microorganisms and Maintenance

Aspergillus oryzae (NCIM 634) was obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. These were maintained on potato dextrose agar slant. Cultures were subcultured at a regular interval of 2 weeks. All the cultures were preserved at 4 °C. NB was used to prepare the seed culture of *A. oryzae* (NCIM 634). For biotransformation experiments, 2 g l^{-1} of sitosterol was added to the NB.

Computational Screening of Candidate Organisms Capable of Sitosterol to AD Biotransformation

Computational screening was carried out in two steps followed by an experimental verification of predicted sitosterol transformation ability. In the first step, information from metabolic pathway of *Mycobacterium* sp. was used to identify 11 enzymes occurring in the pathway converting sitosterol to AD (Fig. 1). The protein sequences of these enzymes were obtained in FASTA format from NCBI, BacMap, BRENDA, and *Mycobacterium* databases and were used as the query sequences in next step.

The second step pertains to screening. Each query sequence was used separately to perform protein–protein Basic Alignment Search Tool (BLAST) and Position-Specific Iterative BLAST (PSI-BLAST) search in the nonredundant database restricted to fungi. The primary screening was performed by using minimum acceptable criteria of E value<0.005 (percent identity >25%), so that a set of all probable microorganisms was obtained. A set of 64 fungi obtained in this process formed the primary screen set (Table 1). Finally, the additional filtering criterion that all the 11 query sequences must be simultaneously satisfied was used. This resulted in a secondary set that contained only two fungi, which were targeted as the candidate microorganisms. The general procedure for rapid screening of microorganisms possessing desired traits has been presented in Fig. 2. In fact, the number of stages of primary screening may be repeated until a manageable size of microorganisms is obtained for testing. In case the experiments do not result in a positive isolate, the metabolic information should be reviewed to redefine query sequences and the process should be repeated.

Experimental Verification of Sitosterol to AD Biotransformation Ability of Computationally Screened Microorganism

One of the theoretical isolates, *A. oryzae* obtained from the computational screening procedure, was then experimentally screened for sitosterol biotransformation ability. A well-sporulated slant of *A. oryzae* was used for preparation of spore suspension, which was used as inoculum for biotransformation experiments. The number of spores was counted using a hemocytometer and the concentration of spores in the suspension was found to be 2×10^7 spores ml^{-1}. Biotransformation experiments were performed in 20 ml of NB containing 2 g l^{-1} of sitosterol taken in 250 ml Erlenmeyer flasks. Since sitosterol has low solubility, the sitosterol granules added was dispersed in NB medium by sonication. The biotransformation studies were carried out at 30 °C and constant agitation of 200 rpm for 24 h. Two flasks were prepared for each day of sampling and each flask was inoculated with 2 ml of the spore suspension. Flasks were incubated at 30 °C for 8 days with constant agitation speed of 200 rpm in an orbital shaking incubator (Orbitek, India). All the experiments were carried out in duplicate.

Extraction of Metabolites from Biotransformation Medium

For extraction of the metabolites produced as the result of biotransformation of sitosterol, the total media was taken from the individual flasks and mixed with an equal volume of chloroform. Samples were extracted twice by vigorous shaking for 2 h on each occasion. The organic phase was collected on each occasion and finally pooled together. The collected organic phase was then evaporated to dryness in a hot air oven at 60 °C. The solid residue obtained was dissolved in 2 ml of ethanol and filtered through a 0.45-μm nylon syringe filter into a sampling vial.

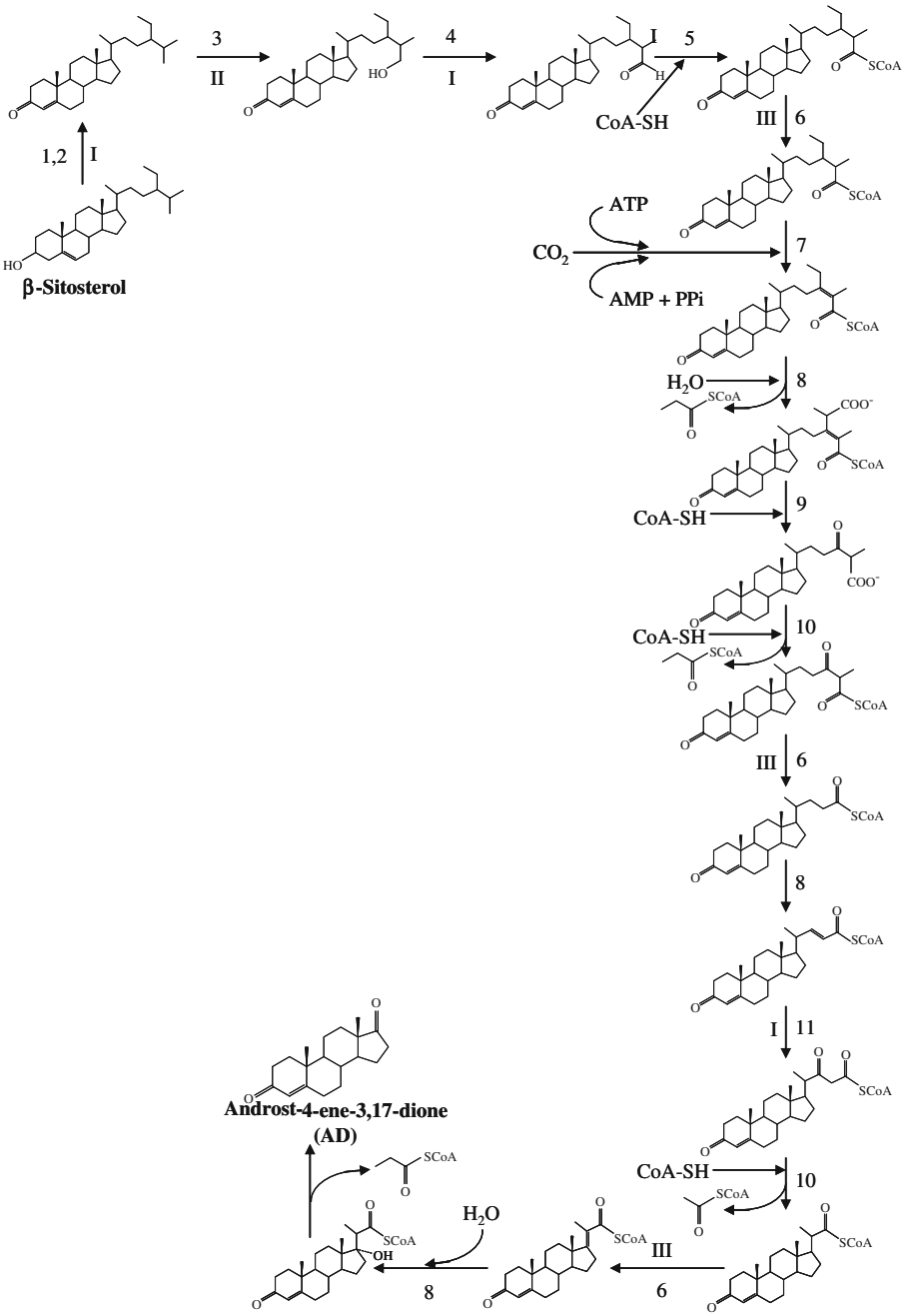

Fig. 1 Metabolic pathway for biotransformation of β-sitosterol to androstenedione is shown in this figure. This is a cofactor-dependent pathway and a cascade of 11 different enzymes is required for selective side chain degradation of sitosterol to androstenedione. Acetyl CoA and propionyl CoA enzymes are produced as by products [12]. *1* 3-β-Hydroxysteroid dehydrogenase, *2* 3-ketosteroid isomerase, *3* ω-oxygenase, *4* alcohol dehydrogenase, *5* aldehyde dehydrogenase, *6* acyl SCoA dehydrogenase, *7* methylcrotonyl carboxylase, *8* acyl SCoA enoyl hydratase, *9* acyl SCoA thiophorase, *10* β-keto-thiolase, *11* β-hydroxyacyl SCoA [*I*] $NAD^+ \rightarrow NADH + H^+$ [*II*] $NADH + H^+ \rightarrow NAD^+$ [*III*] $FAD \rightarrow FADH_2$

Table 1 List of fungi constituting the primary set obtained by computational screening of entire NCBI fungi database.

Serial no.	Fungi	Enzymes[a]										
		1	2	3	4	5	6	7	8	9	10	11
1	*Aspergillus nidulans FGSC A4*	++	++	++	++	++	++	++	++	++	++	++
2	*Aspergillus oryzae*	++	++	++	++	++	++	++	++	++	++	++
3	*Aspergillus fumigatus Af293*	++	–	++	++	++	++	++	++	++	++	++
4.	*Aspergillus niger*	–	–	–	++	–	++	–	–	–	–	–
5.	*Aspergillus aculeatus*	–	–	–	++	–	++	–	–	–	–	–
6.	*Ustilago maydis 521*	++	++	–	++	++	++	++	++	++	++	++
7.	*Chaetomium globosum CBS 148.51*	++	–	++	++	++	++	++	++	++	++	++
8.	*Yarrowia lipolytica*	++	–	–	++	++	++	++	++	++	++	++
9.	*Debaryomyces hansenii*	++	–	–	++	++	–	++	++	++	++	++
10.	*Cryptococcus neoformans var JEC2*	++	–	–	++	++	++	++	++	++	++	++
11.	*Cryptococcus gatti*	–	–	–	++	–	–	–	–	–	–	–
12.	*Candida albicans SC5314*	++	–	–	++	++	–	++	++	++	++	++
13.	*Candida glabrata*	++	–	++	++	++	–	++	++	–	++	++
14.	*Candida tropicalis*	–	–	–	–	–	–	–	–	–	++	–
15.	*Candida boidinii*	–	–	–	++	–	–	–	–	–	–	–
16.	*Candida parapsilosis*	–	–	–	++	–	–	–	–	–	–	–
17.	*Candida metapsilosis*	–	–	–	++	–	–	–	–	–	–	–
18.	*Candida orthopsilosis*	–	–	–	++	–	–	–	–	–	–	–
19.	*Sachhoromyces cerevisiae*	++	–	–	++	++	++	++	++	++	++	++
20.	*Saccharomyces bayanus*	–	–	–	++	–	–	–	–	–	–	–
21.	*Saccharomyces pastorianus*	–	–	–	++	–	–	–	–	–	–	–
22.	*Saccharomyces kluyveri*	–	++	–	++	–	–	–	–	–	–	–
23.	*Schizosaccharomyces pombe*	++	–	–	++	++	++	++	++	–	++	–
24.	*Gibberella zeae PH-1*	++	++	–	++	++	++	–	++	++	++	++
25.	*Encephalitozoon cuniculi GB-M1*	–	–	–	–	–	–	–	–	–	++	–
26.	*Cladosporium fulvum*	–	–	–	–	++	–	–	–	–	–	–
27.	*Magnaporthe grisea 70–15*	++	++	++	++	++	++	++	++	–	++	++
28.	*Neurospora crassa*	++	–	–	++	++	++	++	++	++	++	++
29.	*Mycosphaerella graminicola*	–	–	–	–	–	–	–	–	–	++	–
30.	*Kluyveromyces lactis NRRL-1140*	++	–	–	++	++	–	++	++	++	++	–
31.	*Kluyveromyces wickerhamii*	–	–	–	++	–	–	–	–	–	–	–
32.	*Kluyveromyces marxianus*	–	–	–	++	–	–	–	–	–	–	–
33	*Ashbya gossypii ATCC 10895*	++	–	++	++	++	–	++	++	++	++	++
34.	*Lauaria bicolor*	–	–	–	–	–	–	–	–	–	++	–
35.	*Emericella nidulans*	–	–	–	++	++	–	++	++	++	–	++
36.	*Alternaria alternate*	–	–	–	–	++	++	–	++	–	–	–
37.	*Agaricus bisporus*	–	–	–	–	++	–	–	++	–	–	++
38.	*Mucor circinelloides*	–	–	–	++	–	–	–	–	–	–	–
39.	*Metarhizim anisopliae*	–	–	–	++	–	–	–	–	–	–	–
40.	*Davidiella tassiana*	–	–	–	–	++	–	–	–	–	–	–
41.	*Pichia stipitis*	–	–	–	++	–	–	–	–	–	–	++
42.	*Pichia methanolica*	–	–	–	++	–	–	–	–	–	–	–
43.	*Pichia anamola*	–	–	–	++	–	–	–	–	–	–	–
44.	*Pichia angusta*	–	–	–	++	–	–	–	–	–	–	–
45.	*Pichia jadinii*	–	–	–	++	–	–	–	–	–	–	–
46.	*Paracoccidioides brasiliensis*	–	–	–	–	–	++	–	–	–	–	–
47.	*Cochliobolus lunatus*	–	–	–	++	–	–	–	–	–	–	–
48.	*Monascus anka*	–	–	–	++	–	–	–	–	–	–	–

Table 1 (continued)

Serial no.	Fungi	Enzymes[a]										
		1	2	3	4	5	6	7	8	9	10	11
49.	*Cerospora zeae-maydis*	−	−	−	++	−	−	−	−	−	−	−
50.	*Filobasidiella neoformans*	−	−	−	++	−	−	−	−	−	−	−
51.	*Glomus intraradices*	−	−	−	++	−	−	−	−	−	−	−
52.	*Coccidioides immitisRS*	++	−	−	−	−	−	++	−	−	−	++
53.	*Phanerochaete chryosporium*	−	−	−	−	−	−	−	−	−	−	++
54.	*Fusarium sporotrichioides*	−	−	−	−	−	−	−	−	−	−	++
55.	*Debaromyces hanseniiCBS 767*	++	−	−	−	−	−	−	−	−	−	−
56.	*Cryptococcus neoformans var. grubii H99*	++	−	−	−	−	−	−	−	−	−	−
57.	*Zygosaccharomyces rouxii*	++	−	−	−	−	−	−	−	−	−	−
58.	*Coprinellus disseminatus*	++	−	−	−	−	−	−	−	−	−	−
59.	*Hypocrea jecorina*	++	−	−	−	−	−	−	−		−	−
60.	*Cochliobolus heterostrophus*	++	−	−	−	−	−	−	−	−	−	−
61.	*Sporidiobolus salmonicolor*	++	−	−	−	−	−	−	−	−	−	−
62.	*Gibberella moniliformis*	−	++	−	−	−	−	−	−	−	−	−
63.	*Torulaspora delbrueckii*	−	++	−	−	−	−	−	−	−	−	−
64.	*Tolypocladium inflatum*	−	++	−	−	−	−	−	−	−	−	−

The search was performed against the query sequence of each of the 11 enzymes of the pathway for biotransformation of β-sitosterol to androstenedione

++ the presence of the enzyme, − the absence of the enzyme

[a] The numbers 1 to 11 denote the enzyme with the corresponding number in Fig. 1

HPTLC Analysis of Metabolites

Analysis of Product Formed

Sample extracts were spotted in 2-μl aliquots onto Kieselgel 60 F_{254} fluorescent thin-layer chromatography (TLC) plates (Merck, Germany). The presence of AD in the extract obtained from biotransformation experiment was confirmed by comparing the TLC profile of standard AD with those of extracted samples. For the resolution of AD along with the different products produced during sitosterol transformation, 1:1 (*v*/*v*) composition of chloroform and diethyl ether was used as mobile phase. The TLC plates were dried using hair dryer and scanned at 251 nm with a Camag TLC Scanner III in absorbance mode operated by WinCats software (Version 1.2.0). Steroid products were observed as black spots on a yellow green fluorescent background (Fig. 3). Analytical grade AD was used to prepare standard curves for each high-performance thin-layer chromatography (HPTLC) analysis. The concentration of AD was determined from the standard curve for the corresponding band area of the sample.

Analysis of Sitosterol (Substrate)

For analysis of sitosterol, 2-μl aliquots of experimental extracts were loaded onto the Kieselgel 60 F_{254} fluorescent TLC plates (Merck, Germany). For resolution of the sitosterol, 8:2 (*v*/*v*) composition of toluene and ethyl acetate was used as mobile phase. After the sample run was complete, plates were dipped in anisaldehyde-sulfuric acid reagent (sulfuric acid/methanol/acetic acid 1:17:2) for 1 s. The TLC plates were then heated

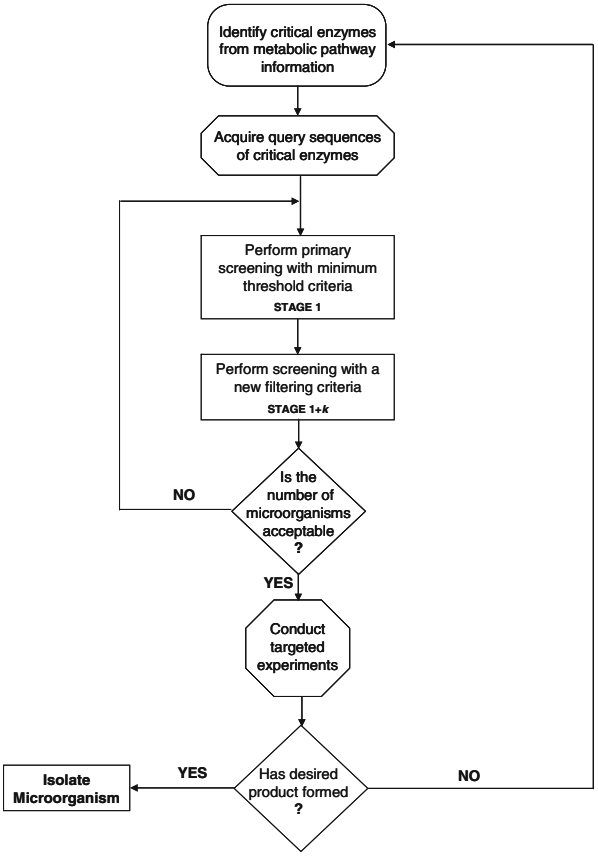

Fig. 2 Flow diagram for computational screening and isolation of organisms possessing the required enzymes for carrying out a desired series of reactions. The total number of screening depends on the number of filters (*k*) used to obtain an acceptable set of candidate microorganisms

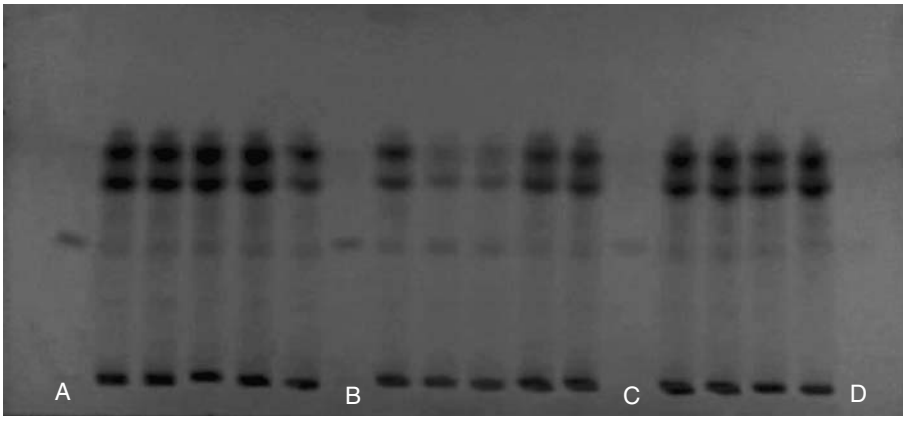

Fig. 3 HPTLC plate showing the metabolites produced in fermentation broth by biotransformation of sitosterol using *A. oryzae* (NCIM 634). *Lanes A*, *B*, *C*, and *D* represent the bands for 300, 200, 100, and 50 ng standard androstenedione, respectively. *Other lanes* represent samples extracted from the culture medium of the biotransformation experiments. Presence of additional bands indicates the formation of some additional metabolites that may be the intermediates of the androgen and estrogen metabolic pathway

at 100 °C for 5 min. Sitosterol was observed as black spot on a white background. The TLC plates containing derivatized sitosterol were then scanned at 366 nm with a Camag TLC Scanner III in absorbance mode operated by WinCats software (Version 1.2.0). Analytical grade sitosterol was used as reference standard for each HPTLC analysis. The concentration of sitosterol in the samples was determined from the internal sitosterol standard calibration curve [4].

Results and Discussion

Computational Screening of Microorganisms Capable of Sitosterol to AD Biotransformation

After carrying out the primary screening from NCBI database, we obtained a set of 64 fungi each having one or more of the 11 enzymes of the β-sitosterol to AD pathway (Table 1). This includes five *Aspergillus*, seven *Candida*, four *Saccharomyces*, and five *Pichia* species. Among these, three of *Aspergillus* sp. have ten or more enzymes, one each of the *Candida* and *Saccharomyces* species have more than nine enzymes while all the *Pichia* sp. have less than two enzymes of the pathway. Also, only 18 fungi in the primary set have more than six enzymes of the pathway. Thus, it appears that the enzymes of the β-sitosterol to AD pathway are not prevalent among fungi. Only two of the fungi out of these 64, *A. oryzae* and *Aspergillus nidulans* FGSC A4, were found to possess all 11 enzymes of the pathway. BLASTP and PSI-BLAST results for all the 11 enzymes of sitosterol biotransformation pathway in *A. oryzae* and *A. nidulans* FGSC A4 have been shown in Table 2. These two *Aspergillus* sp. were theoretically screened as positive isolates.

The method of computational screening proposed here is based on the premise that microorganisms possessing the enzymes of a pathway converting a given precursor to a desired product would exhibit this capability if conditions are conducive. The method requires a priori information about the metabolic pathway and databases of the reference microorganism. This has become possible with the enrichment of public databases in the post genomic era. Query sequences are defined using the a priori information and the databases are searched for candidates showing acceptable homology. Constraints are then imposed to filter out candidates with a higher probability of success.

In this case study, the screening was carried out to identify fungal organisms, capable of biotransforming β-sitosterol to AD. The screening of fungi was performed because of their higher growth rates and capacity to produce secondary metabolites. Fungi having the homologs of all the 11 enzymes involved in the side chain cleavage of β-sitosterol may be considered as an alternative. Sitosterol biotransformation pathway of *Mycobacterium vaccae* was used as reference pathway (Fig. 1) and the protein sequences for different enzymes of the pathway were retrieved from various *Mycobacterium* sp. based on phylogeny.

NCBI BLAST program was used to search nonredundant fungi databases for homology against query sequences of the enzymes of the pathway. BLAST output provides some idea of whether the alignment is “good” and whether it portrays a possible biological relationship, or whether the similarity observed is attributable to chance alone [5, 6]. Sequences matching query sequence with cutoff *E* value in the range of 0.01 to 0.05 are usually considered as true homologs [7]. For a protein sequence to be homologous to the query sequence, 25% homology over the whole sequence is generally considered reliable [8]. To minimize the chances of obtaining nonhomologous sequences, we restricted our search to hits having *E* values$\leq$0.005 or identity $\geq$25%.

Table 2 BLASTP and PSI-BLAST results of computationally screened positive isolates *A. oryzae* and *A. nidulans* FGSC A4.

Enzyme name	BLASTP search				PSI-BLAST search			
	No. of hits			BLASTP RID number	No. of hits			PSI-BLAST RID number
	Total	Identity ≥25%	*E* value ≤0.005		Total	Identity ≥25%	*E* value ≤0.005	
Omega-oxygenase	1, 1	1, 1	–, –	RID: 1143088109-12146-91260858805.BLASTQ1	2, –	2, –	–, –	RID: 1155460488-26752-123418446836.BLASTQ1
Alcohol DH	23, 12	21, 11	23, 12	RID: 1143101497-2895-53468145192.BLASTQ4	21, 17	21, 17	21, 17	RID: 1145694041-22055-201314890272.BLASTQ4
Aldehyde DH	25, 18	16, 16	24, 18	RID: 1143102155-18667-141148230680.BLASTQ4	18, 22	18, 22	18, 22	RID: 1155563050-12434-35026436005.BLASTQ1
Acyl CoA DH	9, 12	2, 3	7, 6	RID: 1143105404-6110-90610740171.BLASTQ1	8, 10	7, 7	7, 7	RID: 1155563376-26280-130692095562.BLASTQ4
Methyl-crotonyl CoA carboxylase	2, 4	1, 4	2, 3	RID: 1146656067-27201-43428887886.BLASTQ1	2, 3	1, 3	2, 3	RID: 1155563852-18923-25430028384.BLASTQ4
Acyl SCoA enoyl hydratase	21, 16	16, 13	17, 14	RID: 1143124049-4529-22405175248.BLASTQ1	17, 14	15, 14	15, 14	RID: 1155564211-2553-121718337865.BLASTQ1
Acyl SCoA thiophorase	4, 4	4, 4	3, 3	RID: 1143106721-10322-24010818504.BLASTQ1	5, 3	5, 3	4, 2	RID: 1155564530-22649-156106909623.BLASTQ1
β-Keto-thiolase	6, 11	4, 9	4, 8	RID: 1143010919-17414-68139396850.BLASTQ4	9, 13	8, 11	5, 8	RID: 1156324687-1555-66978104600.BLASTQ1
3-Hydroxyacyl CoA DH	34, 28	34, 28	34, 28	RID: 1143707116-32120-115320069102.BLASTQ4	6, 7	6, 7	5, 6	RID: 1155565199-20067-35961680210.BLASTQ4
3-β-Hydroxysteroid dehydrogenase	35, 20	11, 9	19, 6	RID: 1145686497-27641-21223664646.BLASTQ4	36, 15	21, 12	9, 6	RID: 1155565740-26235-111497691189.BLASTQ4
3-Ketosteroid isomerase	1, 1	1, 1	–, –	RID: 1145694041-22055-201314890272.BLASTQ4	1, 1	1, 1	–, –	RID: 1155565921-23618-107674860428.BLASTQ4

The first values are for *A. oryzae* and the second values are for *A. nidulans*-FGSC A4

– absence of hits within range of cutoff value

In a search of the entire NCBI fungi database, only *A. oryzae* and *A. nidulans* FGSC A4 were found to possess all the 11 enzymes of phytosterol side chain cleavage pathway within the restricted search criteria. It is evident from the BLAST results that omega-oxygenase and 3-ketosteroid isomerase have the lowest probability of occurrence in fungi. These two enzymes gave only ten and 13 hits, respectively, that too in the range beyond the cutoff criteria for assigning them as true homologs, and so, these were considered as critical enzymes for the proposed pathway with respect to their availability in the fungal species. BLASTP results have shown that the *E* values of omega-oxygenase for *A. oryzae* and *A. nidulans* FGSC A4 were 2.6 and 4.4, respectively, and that for 3-ketosteroid isomerase was 7.4 and 1.9, respectively. Although these values were above the theoretical values for accepting homology, their percent identities were within range. However, it has been established that use of a statistical score, such as the BLAST *E* value, is superior to percentage sequence identity in detecting remote homology or structural similarities by sequence comparison [9–11]. Therefore, it was necessary to perform biotransformation experiments to verify that these candidate fungi would be capable of converting β-sitosterol to AD.

It is possible that the organisms identified as positive using this method are not competent in performing the desired reaction that result in the synthesis of a product of interest. This may happen due to biological constraints such as changes in secondary structure of homologous proteins. Therefore, the proposed method may be extended to encompass other definitions of search criteria based on signal transduction or RNA transcripts, provided a priori information is available. Merely finding the hits within the cutoff value of acceptance of true homology will not ensure positive results because enzyme activity depends on many factors such as three-dimensional structure and active site. This method is only a "first estimate" procedure that takes advantage of the wealth of biological information in the form of sequenced genomes of different microorganisms, to compute quickly if a database microorganism would satisfy our requirements. However, this method ensures that the search for a candidate microorganism is limited only to a set of "probable" competent microorganisms.

Biotransformation of β-Sitosterol to AD by *A. oryzae*

PSI-BLAST results indicated the absence of any hits corresponding to omega-oxygenase in *A. nidulans* FGSC A4 (Table 2), and hence, *A. oryzae* was selected for carrying out the biotransformation experiments. The biotransformation experiment was setup and carried out as described in "Experimental Verification of Sitosterol to AD Biotransformation Ability of Computationally Screened Microorganism". Samples extracted from the biotransformation experiments were analyzed by HPTLC. HPTLC profile of extracts from *A. oryzae* culture medium was compared with that obtained for AD standard at R_f value of 0.47 (Figs. 3 and 4). Sample peaks with R_f values of 0.47 were identified as AD and the time profile of AD biotransformation was plotted in Fig. 5. These results validate the prediction of the computational screening method that the candidate fungi *A. oryzae* is capable of biotransformation of sitosterol to AD. A maximum of 3.43 mg l^{-1} of AD was produced under the given experimental conditions. The yield coefficient for biotransformation of sitosterol to AD was computed to be 0.01 g g^{-1}. The biotransformation of sitosterol to AD may be represented by the equation

$$y(t) = y_F\left(1 - \exp\left(-k_p(t-\theta)\right)\right) \quad (1)$$

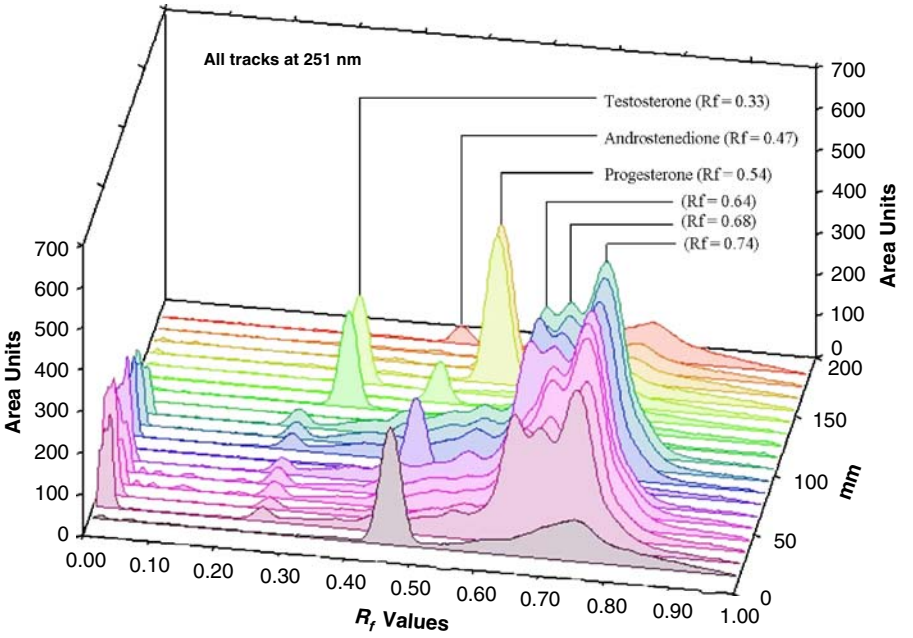

Fig. 4 Three-dimensional HPTLC profile of the metabolites present in extract from the sitosterol biotransformation medium using *A. oryzae* (NCIM 634). Peaks corresponding to standard androstenedione (R_f=0.47), testosterone (R_f=0.33), and progesterone (R_f=0.54) were observed in experimental extract. Some additional unidentified peaks (R_f=0.64, 0.68, and 0.74) were also observed in the HPTLC chromatogram

where $y(t)$ is the AD concentration at time t, y_F is the final AD concentration, θ is the lag time, and k_p is the rate of AD synthesis. From this equation and the data shown in Fig. 5, the value of k_p was calculated to be 0.012 h^{-1}. Since sitosterol has very low solubility in aqueous medium and excess sitosterol is dispersed in the medium by sonication, sitosterol is always available to the microorganism at the constant (maximum solubility) concentration. Equation 1 shows the time profile of AD concentration by biotransformation following first order kinetics when sitosterol is present in the medium at a constant concentration, that is, at the

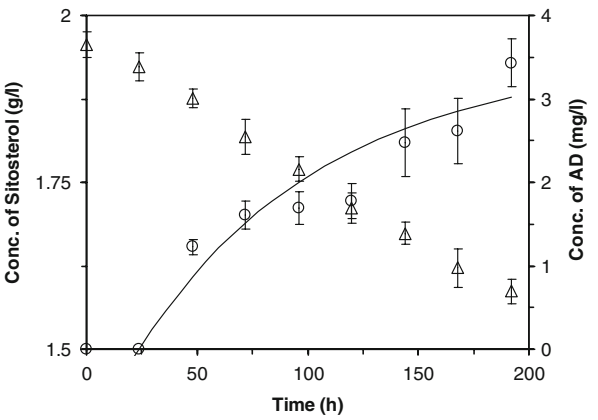

Fig. 5 Time profile for androstenedione production during biotransformation of sitosterol by *A. oryzae* (NCIM 634). Biotransformation experiment was carried out for 8 days and samples were extracted every 24 h. A maximum of 3.43 mg l^{-1} androstenedione was produced after eighth day of biotransformation. The response curve indicates androstenedione synthesis by first order kinetics. *Triangle* represents sitosterol and *circle* represents AD

concentration of its maximum solubility. The lag time θ is the time needed for the spores to become active.

The TLC chromatograms of the metabolites produced in culture medium during sitosterol biotransformation using *A. oryzae* (Figs. 3 and 4) show that in addition to the peaks corresponding to standard AD, there are some additional peaks. Two additional peaks with R_f values 0.33 and 0.54 corresponded to the R_f values of standard progesterone and testosterone analyzed under similar analytical conditions. Therefore, the presence of testosterone and progesterone in the experimental extract was confirmed. However, the identity of compounds corresponding to the R_f values 0.64, 0.68, and 0.74 (Fig. 4) could not be established. Since these additional compounds could be byproducts of AD metabolism, the androgen and estrogen metabolism pathway for *A. oryzae* (www.genome.jp/keg/pathway/map/map00150.html) was examined. It was observed that the reference pathway for *A. oryzae* did not show the presence of enzymes required for the biotransformation of AD to testosterone. However, our experimental results clearly demonstrate the presence of both AD and testosterone in culture medium. Therefore, further investigation using BLASTP and PSI-BLAST of all the listed AD-metabolizing enzymes in Kyoto Encyclopedia of Genes and Genomes for androgen and estrogen metabolism was performed. The BLAST results showed that several AD-metabolizing enzymes were present in *A. oryzae* within acceptable ranges of homology. These enzymes include 3-keto-1(2)-dehydrogenase, unspecific monooxygenase, 3-oxo-5α-steroid 4-dehydrogenase, 3α-hydroxysteroid dehydrogenase, steroid 11β-monooxygenase, 3(or 17)β-hydroxysteroid dehydrogenase, and estradiol 17β-dehydrogenase. An examination of these results showed that AD could be metabolized by any of the following four routes: (a) AD to testosterone, 19-hydroxytestosterone, 19-oxotestosterone, 17-β-estradiol, and estrone; (b) AD to 19-hydroxy-AD, 19-oxo-AD, estrone, and 17 β-estradiol; (c) AD to 5α-androstane-3,17-dione and androsterone; and (d) AD to 11 β-hydroxyandrost-4-ene-3,17-dione. Since any of these pathway metabolites could be present, the identification of the additional metabolites produced in culture medium was not possible at this stage.

Conclusions

Our results establish that the computational screening method is rapid and efficient and can be used universally for rapid screening of the microorganisms with requisite functional attributes. Therefore, by performing only few focused experiments, it is possible to isolate a strain of interest. Although the search is restricted to existing databases, this method is attractive as a first step of screening before initiating a more detailed program of screening and isolation of microorganisms. Consequently, its impact on minimizing time and effort devoted to wet-lab experiments is significant. It is a method that may be considered before embarking on a full-fledged experimental plan to screen microorganism by other methods.

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References

1. Jasuja, R., Ramaraj, P., Mac, R. P., Singh, A. B., Storer, T. W., & Artaza, J. (2005). *The Journal of Clinical Endocrinology and Metabolism*, *90*(2), 855–863. doi:10.1210/jc.2004-1577.

2. Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M., & Cabral, J. M. S. (2003). *Enzyme and Microbial Technology*, *32*, 688–705. doi:10.1016/S0141-0229(03)00029-2.
3. Sripalakit, P., Wichai, U., & Saraphanchotiwitthaya, A. (2006). *Journal of Molecular Catalysis. B, Enzymatic*, *41*, 49–54. doi:10.1016/j.molcatb.2006.04.007.
4. Malaviya, A., & Gomes, J. (2008). *Journal of Industrial Microbiology & Biotechnology*, *35*, 1435–1440. doi:10.1007/s10295-008-0444-4.
5. Altschul, S. F., Boguski, M. S., Gish, W., & Lipman, D. J. (1994). *Nature Genetics*, *6*, 119–129. doi:10.1038/ng0294-119.
6. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, H., Miller, W., & Lipman, D. J. (1997). *Nucleic Acids Research*, *25*, 3389–3394. doi:10.1093/nar/25.17.3389.
7. Mount, D. W. (2004). *Bioinformatics sequence and genome analysis* (2nd ed.). New York: Cold Spring Harbour Laboratory.
8. Bommarius, A. S., & Riebel, B. R. (2004). In *Biocatalysis*, 1st ed (pp. 413–432). Weinheim: Wiley.
9. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). *Journal of Molecular Biology*, *215*, 403–410.
10. Altschul, S. F., & Gish, W. (1996). *Methods in Enzymology*, *266*, 460–480. doi:10.1016/S0076-6879(96)66029-7.
11. Ye, J., McGinnis, S., & Madden, T. L. (2006). *Nucleic Acids Research*, *34*, W6–W9. doi:10.1093/nar/gkl164.
12. Szentirmai, A. (1990). *Journal of Industrial Microbiology*, *6*, 101–106. doi:10.1007/BF01576429.