Identification of Hydrophobins in Fungal Strains Using Bioinformatics Tools

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Abstract: **Hydrophobins are proteins produced by various fungi. They can self-assemble at hydrophilic hydrophobic interfaces into an amphipathic film. Hydrophobins are used to stabilize foam in food products; and immobilize enzyme, antibodies, cells, and an organic molecule on surfaces. Hydrophobins are the surface active proteins with good foaming capacities. Bioinformatics study was done to identify the presence of hydrophobins in *Aspergillus* and *Penicillium* strains. This was done by using BLAST and CDS tools in NCBI. Using these tools, hydrophobin domain families of *Aspergillus* species and *Penicillium islandicum* was identified and described. Also, surface tension reducing capacity of fermentated broths obtained from these strains was tested.**

Keywords: *Aspergillus*, BLAST, Hydrophobin, Malted barley waste, *Penicillium*.

I. Introduction

Hydrophobins are a large family of small proteins (about 100 aminoacids), produced by filamentous fungi. They can self-assemble at hydrophobic/hydrophilic interfaces into amphipathic biofilm. Hydrophobins are 70-350 amino acids in length. They contain eight conserved cysteine residues that form four disulfide bridges, which connect C1-C6, C2-C5, C3-C4, and C7-C8 (Hakanpää *et al.* 2004, 2006a, b; Kershaw *et al.* 2005; Kwan *et al.* 2006, 2008; Morris *et al.* 2012; Ren *et al.* 2013a, b; Pille *et al.* 2014).

The hydrophobins are classified in two types, namely, Class I hydrophobins and Class II hydrphobins. Class I hydrophobins are very stable, and contains amyloid-like membrane that can only be dissociated using trifluoroacetic acid (TFA) and formic acid (Wessels *et al.* 1991; de Vries *et al.* 1993). Class II hydrophobins can be dissociated in 60% ethanol, in 2% SDS, or by applying pressure (Wösten and de Vocht 2000). Hydrophobins, is a fungal protein are found to be useful in number of pharmaceutical fields. Class I hydrophobin SC3 has been used to increase oral drug bioavailability (Haas Jimoh Akanbi *et al.* 2010), and Class II hydrophobin HFB II-coated porous silicon nanoparticles have been used to improve the biocompatibility and change the biodistribution after intravenous injection (Sarparanta *et al.* 2012).

Genus *Aspergillus* classified under ascomycetes includes few hundred mold species. Species from this genus found several applications in Biotechnology. Many strains of *Aspergillus* are industrially important for production of different enzymes, acids etc. Fifty hydrophobins were identified in *Aspergillus* by studying nine full genome sequences. Many of the identified hydrophobins could not directly be allocated to either class I or class II. On species level twenty of the identified hydrophobins have not previously been mentioned in other studies. All hydrophobin genes identified for *Aspergillus niger* strain are new and not reported in other studies. Out of fifty hydrophobin genes twenty three could be classified as class I hydrophobins based on hydropathy pattern and cysteine spacing pattern. Majority of the hydrophobins could not be classified under any one class but they are intermediate in nature. *Aspergillus terreus* is the only strain which has potential to express both class I and II hydrophobins. These hydrophobins not have been physically isolated or characterized, these can obviously only tentatively be classified as a class I and class II hydrophobin respectively (Jensen *et al.* 2010). With reference to Jensen *et al.* work *Aspergillus fumigatus* has the potential of expressing up to five different hydrophobins. Only two of them Rod A and Rod B present on the surface of mature conidia, have been characterized so far. In this work of characterization these two proteins were found on conidial outer cell wall. However only Rod A was found to be directly involved in rodlet layer formation and Rod B was found to be responsible for overall conidial development of fungi. Before this work numerous genes encoding putative hydrophobins expressed during condition have been cloned but the hydrophobin proteins of the conidial rodlet layer have been isolated in only three fungal species: *Neurospora crassa, B.bassiana*, and *Magnaporthe grisea*. (Beever *et al.* 1979; Talbot *et al.* 1993; Templeton *et al.* 1995) Molecular weights of RodA and RodB was found to be 16 and 14 kDa. Additionally there is information available about 16 and 14 kDa proteins present in *A.niger* and *A.nidulans* (Paris *et al.* 2003). In the same work scientists have compared the similarity between signal sequences of DewA of *A.nidulans* and RodB of *A.fumigatus*. Results indicated that both proteins share 26% similarity of their amino acid sequences and their hydropathy profiles are different. Formation of the rodlet layer is the one of the cause for human pathogenecity of *A.fumigatus*, so to develop mechanism for disruption of this rodlet structure the self-assembly of RodA protein in to a three-dimensional multilayer system was studied recently. It could help to design disruption mechanism of *A.fumigatus* and to decrease pathogenecity of it against human beings (Zykwinska *et al.* 2014). As mentioned earlier two hydrophobins are present in *A.nidulans* RodA and DewA. Hydrophobin isolation and characterization from *Aspergillus niger* is not done directly but the entire genome sequencing of CBS 513.88 strain was completed. This particular strain is the ancestor of currently used enzyme production strains. In this work to study cell wall composition of *A.niger* fed batch fermentation was followed and biomass has been collected for mRNA analysis after 72 and 120 h. Two out of seven of the predicted hydrophobin genes were expressed during submerged fermentation (Pel *et al.* 2007). Fungi of Ascomycetes family have many industrial applications one of the industrially important fungi is Aspergillus oryzae which has been explored for production of soy sauce for several years. Study was extended for the application of the species in large scale biological monomers recycling systems for biodegradable plastics such as polybutylene succinate - coadipate (PBSA). During the fermentative process of degradation the expression of hydrophobin orthologous to Rod A-like biosurfactant protein was observed. This expression of a gene is nothing but Rol A hydrophobin of A.oryzae. RolA in A.oryzae localized at cell surface in presence of PBSA. RolA possesses a typical signature of eight cysteine residues observed in class I Hydrophobin and exhibits a hydropathy profile similar to the profiles of class I Hydrophobins (Pel *et al.* 2007). In another review paper the molecular mechanisms underlying growth and development of *Aspergillus* was studied. In this study the role of Hydrophobins explained well for the asexual development. The main role of Hydrophobins in *Aspergillus* is to help escape of hyphae into the air and make aerial structures hydrophobic. Hydrophobins affects the cell wall architecture (Schuren *et al.* 1990) and mediate attachment to hydrophobic substrates (Wosten *et al.* 2001). The *A.nidulans, A.fumigatus, A.oryzae,* and *A.niger* genomes contain 6, 4-5, 2 and 8 hydrophobin genes, respectively (Jensen *et al.* 2010). Altogether the study of hydrophobins in *Aspergillus* is helping more to explore different species for their industrial application and also the remedies on pathogenic strains of the group.

In this work, two species of isolated *Aspergillus* were studied for presence of hydrophobins using bioinformatics tools. Using BLAST and CDS, isolated *Aspergillus* species were used and compared with the hydrophobin sequences of already present *Aspergillus* species. This showed that our *Aspergillus* strains contain hydrophobin peptide sequences which have similar sequences like many other *Aspergillus* species. Unlike the *Aspergillus* family fungal species *Penicillium* fungal species have been rarely explored for the study of presence of hydrophobins. Species like *P.chrysogenum, P.oxalicum* have been investigated for presence of hydrophobin like protein by studying hydrophobic characteristics of the strains (Pascul *et al.* 2000). *P. camemberti* hydrophobin gene was studied using bioinformatics approach and its expression was studied in solid and liquid medium (Boualem *et al.* 2008). In our work we have isolated *P. islandicum* strain from malted barley waste. We have studied the presence of hydrophobin in isolated *Penicillium islandicum* using BLAST and CDS which was not studied previously. This showed that hydrophobin was present in *Penicillium islandicum.*

II. Materials and Methods

A. Materials

The fungal strains were isolated from eight waste samples taken from consecutive stages of malt barley wastes.

B. Methods

1. Strain Isolation

Potato Dextrose Broth (PDB HiMedia) supplemented with 15 g/l agar (Agar Agar Type I, HiMedia) was used medium for 'isolation of different fungal strains'. The fungal strains were isolated by classical techniques like spread plate, zig-zag streak method and direct sample inoculation. Sterilized glass petri dishes (75 mm x 100 mm) containing approximately 30 ml of sterile Potato Dextrose Agar were prepared. One gram of sample was suspended in 10 ml of sterile saline. Ten tubes with 50% dilutions were prepared last four dilutions were selected for spreading. A suspension of 0.2 ml was spread on PDA plates. The plates were incubated at 30°C for 48 hrs.

2. Surface Tension Measurement

Considerable lowering of surface tension is a very important property of hydrophobin. It has been reported in literature that surface tension of hydrophobins ranged from 45 to 27 mN/m. Surface tension of fermented broth of isolated fungal species was measured using surface tensiometer (Data Physics) (Tchuenbou-Magaia, *et al.* 2009).

3. Bioinformatics Approach

A bioinformatics approach was used to study the presence of hydrophobin domain family in few of the isolated strains such as *A.versicolor* and *A.glaucus*. For these strains, presence of hydrophobin family has not been reported in bioinformatics approach. NCBI Blast was run to check the sequence similarity between the strain and various other strains from *Aspergillus* family reported to have presence of hydrophobins in them. The conserved domain was also studied (Littlejohn *et al.* 2012).

4. BLAST and CDS

BLAST (Basic Local Alignment Search Tool) is an algorithm used for comparing primary amino acid sequences of proteins or the nucleotides of DNA sequences. BLAST is one of the most widely used bioinformatics programs for sequence searching (Casey *et al.* 2005). It addresses a fundamental problem in bioinformatics research (Littlejohn *et al.* 2012). This BLAST was used to search respective protein sequences in the isolated *Aspergillus* species and *Penicillium islandicum.* FASTA format was also used for representing the amino acid sequence of the above isolated species.

It is necessary to identify hydrophobin sequences in the genome of *Penicillium islandicum* to ascertain the possibility of hydrophobin production by the species. Also, this provides an assessment of the class of hydrophobins that the species encodes and possibly produces. This knowledge would aid in designing type-specific isolation and purification protocols. Annotated and widely-used hydrophobin protein sequences of different classes and species were run using the *Penicillium islandicum* species as filter on UNIPROT as well as NCBI-BLAST. The hits obtained were then run on PFAM, Interpro, SMART and NCBI Conserved Domain search to identify protein family and domains.

The protein sequences corresponding to the translations of coding sequences (CDS) in GenBank are collected for each GenBank release. CDS were used to check the presence of hydrophobin domain family in *A.versicolor, A.glaucus,* and *P.islandicum*.

III. Results and Discussion

A. Strain Isolation

The samples were isolated from malted barley waste sources are shown in (Table I). Previously many microorganisms have been isolated from malted barley including 100 of fungal strains. Effect of these microorganisms on quality of beer has been studied. Potential of the strains isolated from malted barley have not been explored for presence of hydrophobins using Bioinformatics tools.

Table I: Sample Source Nomenclature

Sr. No.	Sample	Nomenclature
1.	Barley Grader Waste	S1
2.	Barley Grader Waste (1)	S2
3.	Malt Extract Plant Dust Collector Waste	S3
4.	Pre-Cleaner Waste Chiff Grain	S4
5.	Pre-Cleaner Dust Collector Waste	S5
6.	Barley Grader Dust Collector Waste	S6
7.	Sieve Drum Worter	S7
8.	Pre-Cleaner Dust	S8

The strains were identified on the basis of their morphological characters. The strain identification was done at Agharkar Research Institute, Pune and given in Table II. Most of the isolated strains were from *Aspergillus* family. One species of *Penicillium islandicum* was isolated from malted barley sample which has shown good reduction in surface tension of fermentation broth.

Table II: Details of the Fungal Strains Isolated from Culture

Sr. No.	Culture	Identification Remarks	Family
1.	S2-1	*Aspergillus versicolor* gr.	*Aspergillaceae*
2.	S2-2	*Aspergillus glaucus* gr.	*Aspergillaceae*
3.	S2-4	*Aspergillus flavus var. oryzae*	*Aspergillaceae*
4.	S2-8	*Aspergillus candidus* gr	*Aspergillaceae*
5.	S5-7	*Aspergillus flavus* Link	*Aspergillaceae*
6.	S5-9	*Aspergillus terreus* gr	*Aspergillaceae*
7.	S7-5	*Penicilliumis landicum*	*Trichocomaceae*
8.	S1-7	*Aspergillus fumigatus* gr.	*Trichocomaceae*
9.	S1-8	*Aspergillus flavus* gr.	*Trichocomaceae*
10.	S8-4	*Aspergillus flavus* Link	*Trichocomaceae*
11.	S8-6	*Aspergillus flavus* Link	*Trichocomaceae*

B. Surface Tension Measurement

All fungal strains isolated from malted barley found to decrease surface tension efficiently.

(Lumsdon *et al.* 2005) showed that hydrophobins coat surfaces and so lower the surface tension. The surface tension will be decreased more in air/water interfaces than in liquid/liquid interfaces. Thus, surface tension reduction is a very strong indicator of potential production of hydrophobin by the strain. In general lowering of surface tension by hydrophobins is a characteristic dependent on structure of hydrophobin. As hydrophobin amino acid sequence and structure slightly changes from species to species surface tension varies from 28 to 47 mN/m.

C. Bioinformatics Approach

All the isolated *Aspergillus* species were previously studied for presence of Hydrophobin protein sequence using bioinformatics tools. Almost all the isolated *Aspergillus* species has shown the presence of hydrophobin as per the previous study. Further two random *Aspergillus* species from isolated species were selected and presence of hydrophobin protein sequence was BLAST with many other *Aspergillus* species. Result of this experiment are compiled and presented in following tables (Table III and IV). BLAST and CDS results have given the accession numbers and length of the hydrophobins present in the isolated strains. *Aspergillus glaucus* was used as query sequence for other *Aspergillus* species (Table III) using bioinformatics tool. It was found that peptide sequence of *Aspergillus glaucus* was similar to the following strain which contains the hydrophobins. Here *Aspergillus glaucus* contains hydrophobin partial peptide sequence which was comparable to hydrophobin sequence of *A.fischeri, A.lentulus, A.similis, A.omanensis, A.astellatus A.undulatus* and *A.discophorus.* Also it contains hydrophobin precursor which is comparable to the sequence of *A.fumigatus.* It was studied that exact hydrophobin family was also present in our query strain which was similar to sequence of *A.udagawae* and *A.flavus.* Also the length of the hydrophobin sequence was wider compared with *A.flavus* in our strain. The unnamed protein product was also present in the isolated *Aspergillus glaucus* and it was similar to the peptide sequence of *A. nidulans.* This protein product may contain other type of peptide sequence which may present in our species.

In Table IV, isolated *Aspergillus versicolor* hydrophobin sequence was used as a query sequence for other *Aspergillus* species. BLAST and CDS results have identified the accession numbers and length of the hydrophobins present in the isolated strains. It was studied that *Aspergillus versicolor* contains fungal hydrophobins sequence which was comparable to *A.lentulus, A.udagawae, A.fumigatus* and *A.steallatus.* It was also studied that exact hydrophobin family protein was also present in *Aspergillus versicolor* strain which was similar to protein sequence of *A.flavus* and *A.niger.* Also the length of the hydrophobin sequence was compared with *A.flavus.* The unnamed protein product had shown similarity with given query sequence of the isolated *Aspergillus versicolor* and it was also similar to the peptide sequence of *A. nidulans.*

Hydrophobin sequences of *A.glaucus* and *A.versicolour* have shown similarity with common *Aspergillus* species hydrophobin protein sequences like of *A.flavus* and *A.nidulans*. The bioinformatics approach study has been done for various *Aspergillus* species which are already present (Littlejohn *et al.* 2012).

To study presence of hydrophobin protein in strain *Penicilium islandicum* hydrophobin sequences of *Trichoderma reesei*, *Aspergillus* and other *Penicillium* species was compared with *P. islandicum*. These species did not return any significant hits in Protein-BLAST. Both classes of annotated hydrophobin protein sequences available from these species were used. The hits obtained matched very small regions and did not possess domains characteristic of hydrophobin proteins. A hydrophobin 1 sequence from *Agaricus bisporus* hypA (UNIPROT entry name: HYP1_AGABI, Primary (citable) accession number: P49072) returned significant hits with BLAST. One sequence showed considerable similarity with the hydrophobin domain of hypA called 'hypothetical protein PISL3812_00028'. This is an uncharacterised predicted protein sequence (UNIPROT entry name: A0A0U1LI41_TALIS; Primary (citable) accession number: A0A0U1LI41). It has not been reported in literature to being isolated or characterised earlier. Running its sequence on PFAM and Conserved domain search identified hydrophobin superfamily (pfam01185) from 28-103 bp. Interpro indicated Hydrophobin family- IPR001338, and SMART returned one hit- HYDRO-SM00075.

Presence of hydrophobin sequence in the genome of *P.islandicum* is indicative of the potential production of this hydrophobin by the species. As it showed greater similarity to *A.bisporus* type I hydrophobin, it indicates the need to employ measures akin to those used for *A.bisporus* or hydrophobin I proteins in order to effectively produce, isolate and purify the protein. This is the first report of study of presence of hydrophobin protein sequence in species *P.islandicum* using bioinformatics tools.

Table III: BLAST and CDS Results I: Query Sequence Used: *Aspergillus glaucus*

Sr. No.	Strain Used	Accession Number	Length	Type of Protein
1.	*A.fischeri*	AAC13531.1	115	Hydrophobin partial
2.	*A.lentulus*	BAF02782.1	115	Hydrophobin partial
3.	*A.similis*	BA194607.1	117	Hydrophobin partial
4.	*A.fumigatus*	AAL27804.1	140	Hydrophobin precursor
5.	*A.niger*	CAK46216.1	68	Unnamed protein product
6.	*A.udagawae*	GA083590.1	147	Hydrophobin

Sr. No.	Strain Used	Accession Number	Length	Type of Protein
7.	*A. nidulans*	CAL63967.1	157	Unnamed protein product Partial
8.	*A.omanensis*	BA194604.1	126	Hydrophobin partial
9.	*A.astellatus*	BA194593.1	136	Hydrophobin partial
10.	*A.flavus*	KOC12585.1	242	Hydrophobin family Protein
11.	*A.undulatus*	BA194609.1	135	Hydrophobin partial
12.	*A.discophorus*	BA194597.1	134	Hydrophobin partial
13.	*A.luchenesis*	GAT20765.1	131	Hypothetical proteinRIB2604_00802380

Table IV: BLAST and CDS Results II: Query Sequence Used: *Aspergillus versicolor*

Sr. No.	Strain Used	Accession Number	Length	Type of Protein
1.	*A.lentulus*	GAQ08117.1	142	Fungal Hydrophobin
2.	*A.udagawae*	GA081860.1	150	Fungal Hydrophobin
3.	*A.fumigatus*	XP_747504	155	Fungal Hydrophobin
4.	*A.oryzae*	BAR73404.1	207	Capsid mutational Protease
5.	*A.steallatus*	BAI94611.1	137	Fungal Hydrophobins
6.	*A.niger*	CAK46216.1	68	Hydrophobin
7.	*A.flavus*	KOC12585.1	242	Hydrophobin family Protein
8.	*A. nidulans*	CAL63967.1	157	Unnamed protein product Partial

D. P.Islandicum Blast Against Hypa of Agaricus Bisporus

hypothetical protein PISL3812_00028 [*Penicillium islandicum*]

GenBankCRG82684.1

Identical Proteins FASTA Graphics

Go to:

LOCUSCRG82684 107 aa linear PLN 29-APR-2015

DEFINITION hypothetical protein PISL3812_00028 [*Penicillium islandicum*].

ACCESSION CRG82684

VERSION CRG82684.1

DBLINKBioProject: PRJEB8788

BioSample: SAMEA3344808

DBSOURCEembl accession CVMT01000001.1

KEYWORDS.

SOURCETalaromycesislandicus

ORGANISM Talaromycesislandicus

Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae;

Talaromyces.

REFERENCE 1

AUTHORSWibberg, Daniel.

TITLEDirect Submission

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IV. Conclusions

Different *Aspergillus* and *Penicillium* species have been isolated and studied for hydrophobic characteristics. Screened strains showing lowered surface tension were further evaluated for presence of hydrophobin using bioinformatics tools like BLAST and CDS. Hydrophobin protein sequence was identified in three fungal strains *Aspergillus glaucus, Aspergillus versicolour* and *Penicillium Islandicum*. Both the *Aspergillus* strains have shown similarity with hydrophobin sequences of many other *Aspergillus* species. Hydrophobin sequence of *Penicillium Islandicum* has shown similarity with Hydrophobin sequence of *Agaricus bisporous*.

Acknowledgement

We are thankful to Dr. Kalpana Joshi, Head, Department of Biotechnology, Sinhgad College of Engineering and Dr. Sanjay Nene, Innovation Biologicals Pvt. Ltd. for their continuous guidance and support for the work.

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