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Aspects in lipase screening

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Abstract

Lipases have become increasingly important for the industry over the last 10 years, and heterologous gene expression has played a key role. Screening of the biological diversity that Nature provides in order to identify lipases suitable for industrial use is a key issue. This paper will discuss some of the aspects to consider when carrying out a lipase screening program.

Keywords: Lipases; Screening; Industrial use

1. Introduction

Fungi provide us with a huge potential of enzymes. In particular, enzymes for use as additives in detergents have gained increased importance over the last decade. Traditionally, proteases of bacterial origin have been used for the last 30 years in household detergents. But cellulases, lipases and amylases have also found their way into households world wide. Many of these enzymes are of fungal origin. Examples of fungal enzymes are triacylglycerol lipase from *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*) used in stain removal and β-1,4-glucanase (cellulase) from *Humicola insolens* used for color clarification.

An industry supplying these enzymes, and enzymes for industrial use in general, must carefully contemplate how to bring enzymes with the best possible characteristics to the market in order to meet competition. This paper deals with some of the things to consider when carrying out a screening that has the objective of finding a lipase particularly well suited for industrial use of various kinds. The paper will deal only with fungi, although a handful of bacterial lipases have already been identified, cloned, characterized and used industrially.

As we know, microorganisms occur everywhere on Earth. As shown in Table 1, the latest estimate for the number of fungal species alone is 1.5 million which means that fungi are only exceeded by insects which count about 6 million species [1]. It is furthermore anticipated that only about 1–2% of the existing fungal species have been found and characterized.

Many, or even most, of these fungi produce lipase extracellularly, either readily or subjected to an inducing environment. It is therefore actually a major requirement to find the very best lipase for a specific application. It will be much too time-consuming to grow all lipase-producing fungi, or even clone and express more than

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Table 1
Comparisons of the numbers of known and estimated total species in the world of selected groups of organisms

Group	Known species	Total species	Percentage known
Vascular plants	220000	270000	81
Bryophytes	17000	25000	68
Algae	40000	60000	67
Fungi	69000	1500000	5
Bacteria	3000	30000	10
Viruses	5000	130000	4

just a few of them. Obviously, one needs a screening strategy.

2. Taxonomic screening

First of all, you try to maximize the diversity of the microorganisms to participate in the screening program. A screening program for a specific type of enzyme will usually include representatives for most taxonomic groups as far as this is possible. You also maximize diversity by using different sample types from a wide range of habitats and climates for the isolation of microorganisms. However, usually not all taxonomic groups will be represented, because many microorganisms are difficult to handle in the laboratory. The possibilities for molecular screening being available as the DNA and protein databases grow may widen the range of screening programs and will be further discussed later in this paper.

Because of the unwillingness of some fungi to grow readily on a given medium, however, it is extremely important to apply different isolation methods. The number of isolates, their diversity and the ability to classify microorganisms isolated from a single soil sample depends heavily on the isolation method. For the fungal taxonomist, a prerequisite for identifying a given fungus is usually that it is spore forming, because the shape and appearance of the spores is used to assign the fungus to a certain genus. Obviously, it is necessary to identify the fungi to ensure a wide diversity in a screening. And therefore, the value of a large and well characterized strain collection is evident.

Fungi have a tremendous way of adapting to a given environment by differentiation, i.e. changes in gene dosage upon an external stimulation. Differentiation will most often lead to a change in morphology and thus difficulties in determining whether two morphologies represent two differential states or two different fungi. Thus, knowledge about the behavior of the fungi

Table 2
Plant genera giving rise to lipase producing endophytes

Plant genus	Family	Order	Oil/resin	Number of cultures
Pithecolobium	Mimosaceae	Leguminales	+	5
Jasminum	Oleaceae	Oleales	+	5
Buxus	Buxaceae	Tricoccae		3
Pittosporum	Pitosporaceae	Saxifragales	+	3
Eucalyptus	Myrtaceae	Myrtales	+	3
Callistemon	Myrtaceae	Myrtales	+	2
Eribotrya	Pomaceae	Rosales		2
Cinnamomum	Lauraceae	Polycarpicae	+	2
Rhododendron	Rhodoraceae	Bicornes		2
Pinus	Abietaceae	Pinales	+	2
Juniperus	Cuppressaceae	Pinales	+	1
Fraxinus	Oleaceae	Oleales	+	1
Viburnum	Caprifoliaceae	Polycarpicae	+	1
Mahonia	Berberidaceae	Polycarpicea	+	1
Nandina				1
Pleipblastus				1

on different media is invaluable, and the use of many different media from the point of the sampling time is mandatory in order to obtain a diversity widely represented taxonomically.

3. Ecological screening

The second approach, termed ecological screening as opposed to taxonomic screening is the targeted screening of relevant niches and is an obvious possibility in screening for certain enzymes. If one is looking for heat stable enzymes you would focus on a screening from organisms from hot springs. If you are looking for lipases, one would consider samples that are rich in oil and other fatty substances. As an example, in our lipase screening at Novo Nordisk A/S, we made a comparison between endophytes isolated from the interior of plant tissues which contain oil, with endophytes isolated from non-oil-containing plant tissue. Endophytes grow out readily from the surface of sterilized infected leaf material when placed on a growth medium and are in most cases easily handled. Nevertheless, this group of fungi has not yet been studied thoroughly.

Many endophytes were isolated representing oil containing and non-oil-containing hosts equally, and scored for lipase activity. Table 2 shows that only 9 out of 35 lipase producing cultures originated from plants without oil or resin. This indicates some selection for lipase producing endophytes in oil containing plants and stresses the need for considering the utilization of ecology in screening programs.

Another example of targeted lipase screening related to ecological screenings is to focus on plant pathogenic species, many of which are known to produce cutinase for the penetration of the plant cuticle. This approach has been undertaken by many researchers in the past. In particular plant pathogenic species of *Fusarium* have been given much attention, and the cutinase from *Fusarium solani var. pisi* has been cloned [2].

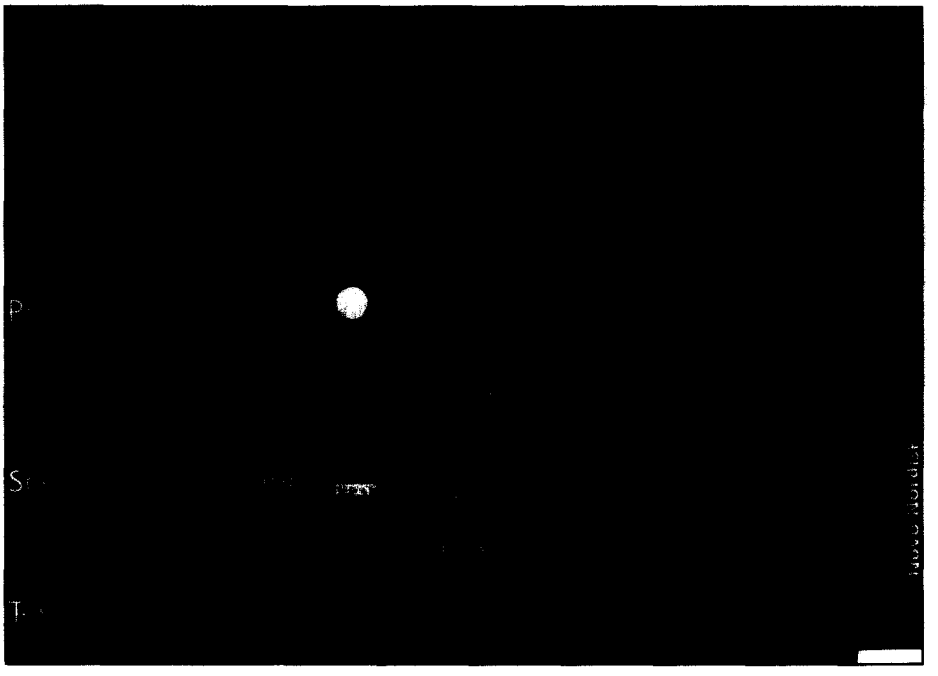

Fig. 1. The workflow of a screening process.

4. The screening workflow

Now having decided on a screening strategy and having isolated a large number of potentially interesting cultures, the next step is to find out which of these cultures produce interesting lipases.

Fig. 1 shows a typical work flow of a screening program. In the primary screening, typically about 10000 cultures are handled. For this step a high-throughput assay is needed and usually only lipase activity is scored at a defined set of conditions. For a detergent lipase this could for example be at pH 7 and 10 in order to select alkaline lipases. The primary screening has to be so selective that only a few organisms pass on. In the secondary screening we grow the cultures in shakeflasks and assay them further which is much more time demanding and definitely not applicable to 10000 cultures.

The cultures are grown under conditions that allow induction of lipase activity, usually by adding oil as the sole or supplementary carbon source. The broth which results from this cultivation is applied for further studies of the enzyme properties. These studies typically consist in determining the pH profile, pH stability profile as well as temperature stability etc. Other properties depend much on the application we are aiming at. For use in detergents, the enzyme needs to be detergent stable and stable towards activated bleach systems. Household detergents contain builders responsible for chelating calcium. Thus, the enzyme needs to be working in absence of calcium. When used for paper manufacturing, the lipase should have a lower pH activity optimum, and the requirements for thermostability are much more stringent. The screening assay must, generally speaking, resemble the working conditions for the enzyme in the industrial process. But since the yields of lipases are quite low, this usually hampers the assay functionality because of other components produced by the fungus, and the conditions must therefore most often be less strict.

Because the evaluations of the enzyme properties at this stage are based on crude broths containing low amounts of enzyme, assay development is rather crucial. The results are not always reliable, particularly stability tests of various kinds, and the results obtained are usually only rough indicators for performance in application trials, but a prerequisite in a screening program.

Returning to the workflow, there is still a relatively large number of cultures to look at, and a limited capacity for fermentation and fine-purification from still low-yielding cultures makes it crucial to distinguish/deselect known enzymes from unknown and thus prevents that an enzyme is processed more than once in these steps. Duplicates need to be avoided and the largest possible diversity in the enzymes selected must be ensured. Partially because purification is time consuming due to low yields, but also because it is often chosen to clone the enzymes directly.

5. Molecular taxonomy in screening

There are a number of ways to easily distinguish genetically between related strains. One way is DNA sequencing of the ribosomal DNA complex. As seen in Fig. 2, the ribosomal DNA

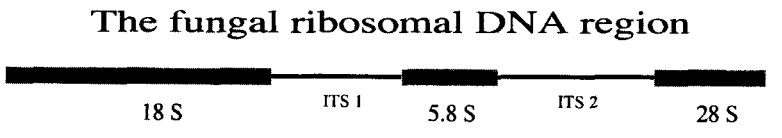

Fig. 2. The fungal ribosomal DNA complex, which is composed of genes encoding 18S, 5.8S and 28S ribosomal RNA. Between the genes, ITS1 and ITS2 sequences are located (internal transcribed sequence). The different regions vary to a different degree through evolution, and are therefore suitable as an analysis tool for phylogenetic relationships.

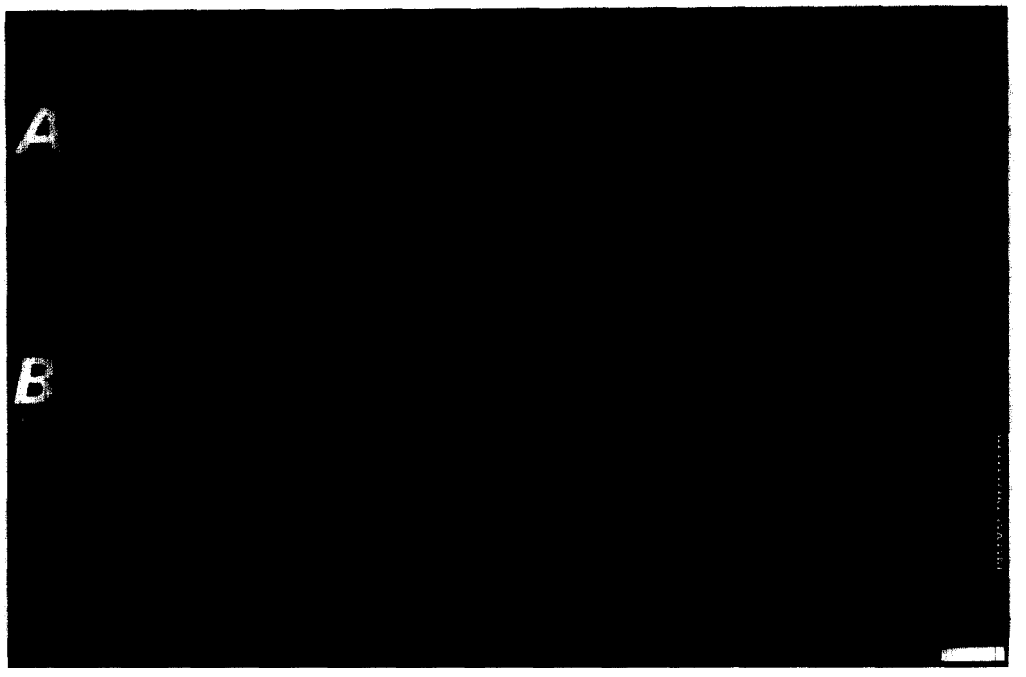

Fig. 3. From [3]: D. Stahl compared phylogenetic relations based on two different sets of data: ribosomal and enzyme-encoding sequences and found the same phylogenetic trees.

complex consists of genes encoding 18S, 5.8S and 28S RNA's. Some of these regions, in particular the 18S gene, has been used systematically to distinguish between families and genera of both fungi and bacteria. And with the development of DNA isolation methods, PCR techniques, DNA sequencing techniques and the rapidly growing number of 18S sequences in DNA databases, species determination (and phylogeny) by ribosomal sequencing has become much less time consuming.

6. Evolution versus diversity

Variation in the 18S sequence is coupled to evolution as a time clock, and variations in the 18S sequence will reflect evolutionary distance. The crucial question to a screening microbiologist is of course whether or not and to what extent evolutionary changes in some DNA sequences reflect changes in other sequences. When using ribosomal sequences in phylogeny, it is anticipated that changes in the sequences are neutral, i.e. not reflecting external selection pressure. Enzymes and variations in their encoding DNA sequences may very likely be influenced by external pressure throughout evolution, or horizontal gene transfer in the evolution process may have occurred. These events will of course destroy the possibility of predicting the degree of enzyme diversity on the basis of ribosomal DNA sequences. However, Stahl [3] has compared ribosomal sequences and cellulase sequences, run phylogenetic analyses and shown that DNA changes in the enzyme encoding genes reflect the same taxonomic relation as ribosomal sequencing does. The relations are shown in Fig. 3. Thus, in some cases, ribosomal sequencing can be used to predict the diversity in enzyme sequences, which are not readily sequenced.

7. Deselecting using RAPD

Having screened a large number of fungi ending up with a number of strains producing indistinguishable lipases, one approach to distinguish between these very related isolates is the RAPD (random amplified polymorphic DNA) technique. Fig. 4 shows the principle behind RAPD. A 10-mer oligonucleotide hybridizes randomly, but specifically for a given chromosome, to sites on the genome. By amplification using PCR and electrophoresis on an agarose gel, a band pattern (fingerprint) reflecting the base sequence of the genomic DNA, appears. Fig. 5 shows an example where we used this technique to distinguish between 8 different fungal strains suspected to be strongly related and each producing an interesting lipase. In fact, RAPD could sort the fungi into 4–5 distinct groups of genetically related fungi. Since strains within each group were considered genetically closely related, only one strain from each group was further considered interesting for cloning of the lipase encoding gene.

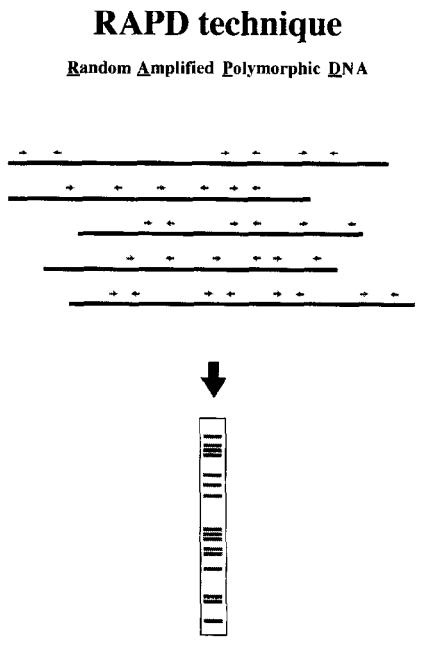

Fig. 4. The principle of RAPD (random amplified polymorphic DNA): Small 10-mer oligonucleotides hybridize in a random manner, but specifically for a given chromosome. Upon PCR (polymerase chain reaction), DNA fragments of different lengths are formed, and a pattern specific for a given strain shows on a gel when the bands are separated.

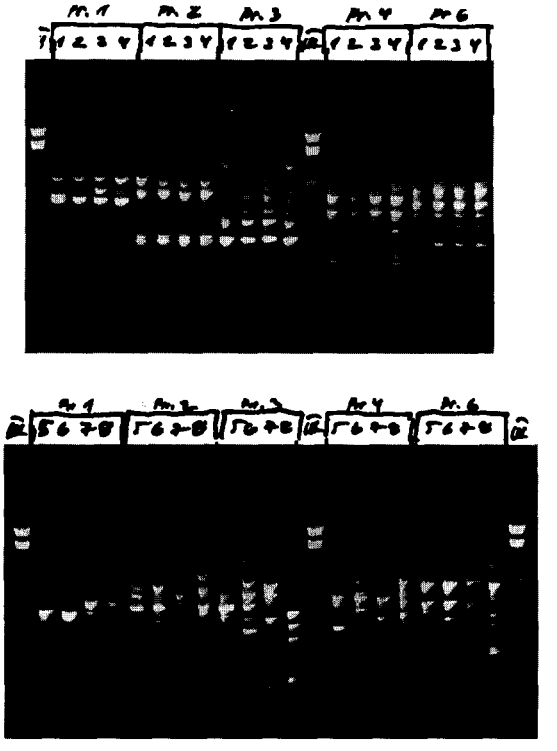

Fig. 5. RAPD analysis was carried out on 8 different fungal strains (1–8) of unknown interrelatedness. 5 10-mer oligonucleotides were used as primers in individual PCR reactions. The reaction products were separated on a gel, run again and this time ordered according to their band pattern. Strains 1–4 are related, 5 and 6 somewhat related and 7 and 8 are unrelated.

8. Molecular screening

The classical screening approach has combined knowledge about the taxonomic relationship with studies of immunological similarities between strains using antibodies raised against known enzymes. Even though this approach has been useful, there is no doubt that it will become substituted by DNA based methods.

The first fungal genome sequencing project aiming at sequencing the whole genome of *Sac-*

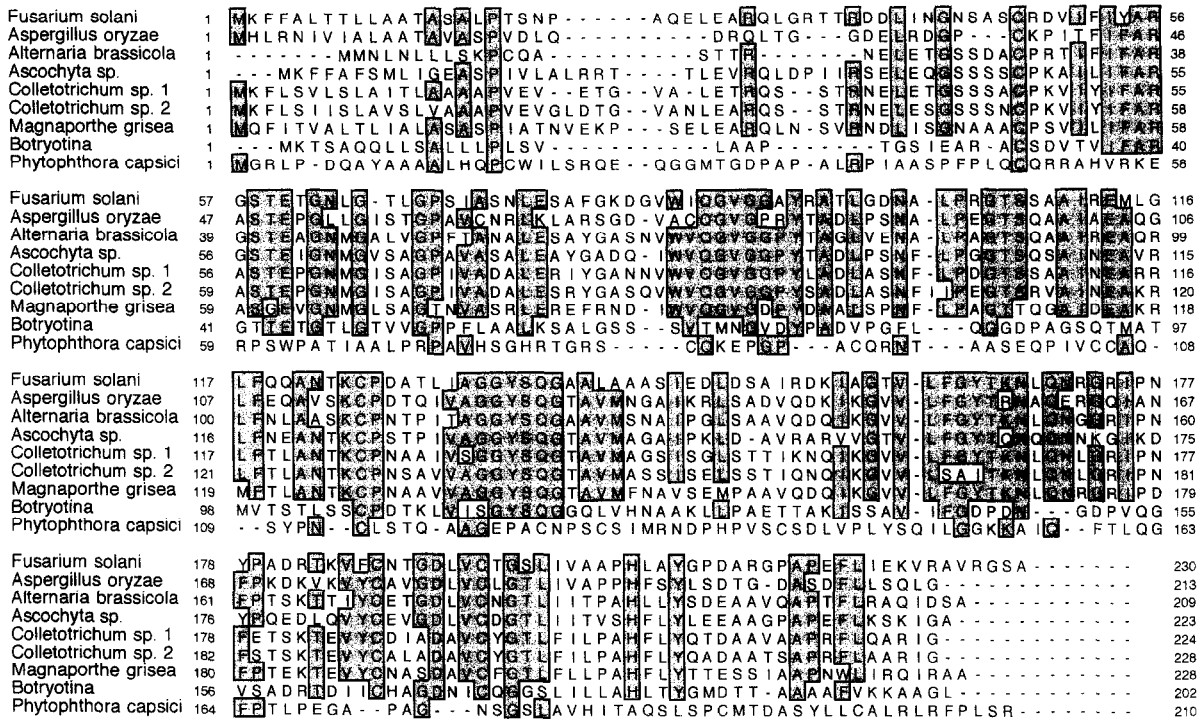

Fig. 6. Alignment of lipases (cutinases) from *Fusarium solani*, *Aspergillus oryzae*, *Alternaria brassicola*, *Colletotrichum sp.* 1, *Colletotrichum* sp.2, *Magnaporthe grisea*, *Botryotina sp.* and *Phytophthora capsici*. Sequences were gathered from GenBank, translated and aligned using the Pileup program from the UWGCG package (University of Wisconsin).

charomyces cerevisiae has been finished, and data will be published before the end of 1996. 3 microbial genomes (*from Haemophilus influenzae*, *Mycoplasma genitalis* and *Methanococcus jannaschii*) have been finished and 12 more are underway. Through these efforts and general data submission to the genetic databases, these grow with and average rate of about 2 million basepairs per day. It is anticipated that only about 2000–3000 protein structures make up the entire biological set of basic building blocks. At present, we are able to make multiple alignments of a large number of proteins. One example is the alignment of published cutinase sequences [4–9] represented in Fig. 6. The alignment reveals several areas exerting a high degree of homology on the protein level. In particular, of course, around the conserved region

Table 3
Genetically characterized producers of lipolytic enzymes

Genus	Family	Order	Class	Phylum	Kingdom
Fusarium	Hypocreaceae	Hyprocreales	Pyrenomycetes	Euascomycetes	Ascomycota
Alternaria	m. Loculoascomycetes	Dothideales	Loculoascomycetes	Euascomycetes	Ascomycota
Ascochyta	m. Loculoascomycets	Dothideales	Loculoascomycetes	Euascomycetes	Ascomycota
Aspergillus	Trichocomaceae	Eurotiales	Pyrenomycetes	Euascomycetes	Ascomycota
Colletrotrichum	Phyllachoraceae	Phyllachorales	Pyrenomycetes	Euascomycetes	Ascomycota
Magnaporthe	Phyllachoraceae	Phyllachorales	Pyrenomycetes	Euascomycetes	Ascomycota
Botryotinia	Sclerotiniaceae	Leotiales	Discomycetes	Euascomycetes	Ascomycota
Phytophthora	Pythiaceae	Perenosporales	Oomycetes		Chytridiomycota

containing the active serine G–X–S–X–G, but also in other regions. Cutinases belong structurally to the group of enzymes belonging to the group of α/β hydrolase fold enzymes [10,11]. Therefore, there are several structural motifs that will be reflected in the DNA sequence and thus potentially may be identified using oligonucleotide PCR primers in molecular screening, similar to what has been reported [12] for cloning new cellulase based upon the hydrophobic cluster analysis-based cellulase family classification [13]. In other words, the techniques being employed and used increasingly for cloning, will be expanded to be implemented in screening. At a certain stage it is almost certain that multiple representatives of each structure will be present in the databases, and the possibility of molecular screening will be dramatically increased.

9. Lipase screening at present

Triacylglycerol lipases have been identified and cloned from various fungi. Table 3 shows the taxonomic placement of the cutinases listed in Fig. 6. Furthermore, lipases have been cloned from *Thermomyces lanuginosus*, *Geotrichum candidum*, *Candida cylindraceae*, *Mucor miehei*, *Candida rugosa* and others, belonging to different lipase families. Common to all of them, *except Mucor miehei*, which belongs to the *Zygomycetes* and *Phytophthora capsici*, which belongs to the *Chytridiomycota* is that they all belong to the kingdom of *Ascomycetes*. The kingdom *Basidiomycota* is not represented by a triacylglycerol lipase. Thus, we are far from getting an overview of triacylglycerol lipase diversity. Have all families of lipases been identified? Do kingdoms other than *Ascomycota* and *Chytridiomycota* have cutinases? And if they have, how different are they? Has horizontal transfer of lipase genes occurred across kingdoms? These and many more questions are of extreme importance to elucidate the potential of enzymes of industrial use in the world of microbiology. Therefore, much more phylogenetic work and screening is needed.

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