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Chapter I

Gut Fungi: Classification, Evolution, Life Style and Application

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Abstract

Gut fungi represent a special anaerobic group of microscopic eukaryotes inhabiting the digestive tract of ruminant as well as many non-ruminant herbivores. Every trait of these fungi is singular and exceptional. Morphologically, gut fungi are the only representatives of kingdom Fungi demanding anaerobiosis, and therefore, possessing hydrogenosomes instead of mitochondria. Genetically, anaerobic fungi represent organisms with the lowest G+C content ever found in Nature and with duplication of a variety of important hydrolytic genes, which is a trait not common among eukaryotes. Enzymologically, anaerobic fungi possess cellulosomes, the enzyme factories known only in some bacteria, carrying the most active (hemi)cellulases. These special features have always complicated the determination and phylogenetic position of anaerobic fungi. Taxonomical problems have been resolved recently by establishment of the separate phylum as a result of reconstruction of the large group of flagellated fungi called chytridiales. The new phylum comprises a single class (Neocallimastigomycetes), one order (Neocallimastigales) and a single family (Neocallimastigaceae), which covers six genera of anaerobic fungi including *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpinomyces*, *Anaeromyces* and *Cyllamyces*. All species of anaerobic gut fungi secrete a variety of hydrolytic enzymes, including cellulases, xylanases, mannases, esterases, glucosidases, and glucanases, which effectively hydrolyze plant biomass consisting mainly from cellulose and hemicellulose. These enzymes exhibit higher activities than commercial enzyme products derived from *Trichoderma reeseii* or *Aspergillus niger*. The

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capacity of extracellular plant cell-wall enzymes from anaerobic fungi to degrade cellulose, hemicellulose, and various lignocellulosic compounds indicates high potential feasibility of gut fungi hydrolases in commercial processes. Their experimental applications in livestock production, paper industry, biofuel and renewable energy production are discussed in this chapter.

1. Introduction

J. W. Foster declared in 1949 the opinion, that molds are highly oxidative and unable to metabolize carbohydrates anaerobically [1]. This statement known as mycological dogma has been probably the main reason of relatively late discovery of anaerobic fungi. Flagellated microorganisms observed in rumen fluid already at the beginning of last century were wrongly considered as protozoans [2, 3] and it took more than sixty years to determine them as fungi. The discovery, identification and early exploration of these microorganisms is closely related to the personality of the British scientist Colin Orpin, who recognized these cells as anaerobic fungi, described their major stages of life cycle [4], discovered chitin as the main structural polysaccharide of their cell walls [5], and classified the first species of anaerobic fungi [6-8].

Table 1. Survey of known strains of anaerobic fungi

Genus	Species	References
Neocallimastix	*frontalis*	[16]
Neocallimastix	*hurleyensis*	[17]
Neocallimastix	*patriciarum*	[8]
Neocallimastix	*variabilis*	[18]
Anaeromyces	*elegans*	[19]
Anaeromyces	*mucronatus*	[20]
Caecomyces	*communis**	[6]
Caecomyces	*equi*	[21]
Caecomyces	*sympodialis*	[22]
Cyllamyces	*aberensis*	[23]
Orpinomyces	*bovis*	[24]
Orpinomyces	*intercalaris*	[25]
Orpinomyces	*joyonii***	[26]
Piromyces	*citronii*	[27]
Piromyces	*communis*	[21, 24]
Piromyces	*dumbonica*	[28]
Piromyces	*mae*	[28]
Piromyces	*minutus*	[29]
Piromyces	*polycephalus*	[30]
Piromyces	*rhizinflata*	[31]
Piromyces	*spiralis*	[32]

* Originally described as *Sphaeromonas communis.*

** Originally described as *Neocallimastix joyonii.*

The taxonomical shift of anaerobic fungi from Phycomycetes [4] to Chytridiomycetes [9-14] has been driven both by their extraordinary morphology and rDNA composition. The final determination as Neocallimastigomycetes has been achieved by the modern methods of molecular biology based on multi-gene phylogeny of DNA sequences, mainly originating from the nuclear ribosomal RNA operon [15].

Thus thirty-two years after the discovery, the anaerobic fungi are classified into a separate phylum, the Neocallimastigomycota, which encompasses one class (Neocallimastigomycetes), one order (Neocallimastigales) and a single family, the Neocallimastigaceae [15]. Inside this family six genera of anaerobic fungi including *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpinomyces*, *Anaeromyces* and *Cyllamyces* have been determined up to now (Table 1).

However, recent research dealing with diversity of animal gut fungi indicates that the number of 6 genera and 21 species of anaerobic fungi is neither terminal nor most likely confined to ruminant and large herbivores only [33, 34].

2. Role of Gut Fungi

Gut fungi are symbiotic microorganisms occupying a unique ecological niche, the anaerobic environment of the rumen and gastrointestinal tract of large herbivores. In the digestive tract, fungi contribute, in association with bacteria and protozoa, to hydrolyzation of diet fiber resulting in the production of end fermentation products (volatile fatty acids) that can be utilized by host animal as source of nutrition. The complete set of enzymes necesary for plant cell-wall degradation provides anaerobic fungi to penetrate plant cell walls, access fermentable substrates, degrade and weaken plant tissue and reduce the size of diet particles. Released zoospores attach to the feed fragments, encyst them and germinate to produce a fungal thallus, which is composed of rhizoids and the sporangium. The rhizoid system can be highly branched and is able to penetrate deeply into stems and thus possibly aids to decompose substrate also mechanically [35]. Due to the capability to colonize the recalcitrant plant components like sclerenchyma and vascular system, gut fungi are known to decompose lignin-containing plant walls, even if they do not utilize phenolics or lignin [36]. Thus, the role of gut fungi seems to be lucid. However, the factual state, number, representation, distribution and the extent of their participation in the fermentation processes has not been yet fully clarified.

A study of Lee et al. [37] suggested that the contribution of the rumen fungi to cell wall degradation may greatly exceed the fermentation part of bacteria. It is believed that gut fungi are the very first colonizer of diet fiber, that enzymatically as well as physically open up the plant tissue for surface-acting bacteria. Rumen bacteria attach the phytomass more quickly; however, they preferentially colonize sites of damaged plant material. The anaerobic fungi and bacteria are probably in a complicated relation with each other, which may even be contraproductive. The inhibition of rumen fungi by some cellulolytic strains of *Ruminococcus albus* [38], *R. flavefaciens* [39-41], and *Butyrivibrio fibrisolvens* [42] was demonstrated by several *in vitro* studies. On the other hand, these results cannot be generalized because the type of interaction with fibrolytic bacteria depends on the content and diversity of the anaerobic fungal genera. Moreover, the interaction of anaerobic fungi with nonfibrolytic

bacteria was found to be beneficial and a close positive association of gut fungi with methanogens is well known [43, 44]. The syntrophic co-cultures with *Archaea* increase amount of fungal biomass and markedly improve rate and extent of fungal cellulolysis and xylanolysis [45].

3. Special Features of Anaerobic Fungi

Anaerobic fungi differ considerably from all known members of the kingdom Fungi in many characteristics. This deviation from aerobic fungi is very probably caused by the specificity of their biotope, which is digestive tract of herbivores. The most apparent exceptional feature is the ability to cope with anaerobiosis and the inability to cope with aerobiosis resulting in the fact that mitochondria were replaced by hydrogenosomes during secondary rearrangements at the cellular level. Rumen or gut fungi are the only fungi that do not only need no oxygen for their life cycle, but oxygen is toxic for them. Anaerobic fungi cannot „breathe“, and therefore do not require mitochondria with their whole machinery of the respiratory chain. The energetic center of anaerobic fungi is represented by hydrogenosomes. These organelles, under anoxic conditions, decarboxylate malate into acetate, CO_2, and H_2 with concomitant production of energy in the form of ATP [46, 47]. Hydrogenosomes of gut fungi are unique and followed probably an alternative way to adapt to anaerobic environments. The available functional and phylogenetic evidence allows the conclusion that the hydrogenosomes of anaerobic fungi, trichomonads and ciliates are substantially different, and that they evolved independently from each other. Yarlett and Hackstein [48] suggested the hypothesis that the hydrogenosomes of anaerobic fungi evolved from fungal mitochondria, the hydrogenosomes of trichomonads from a hydrogen-producing protomitochondrion-like ancestral organelle, and the hydrogenosomes of *Nyctotherus ovalis* (hydrogenosome containing their own DNA) from the mitochondria of an aerobic ciliate retaining substantial feature of a classical mitochondrion. On the other hand, Benchimol et al. [49] demonstrated a sharp similarity between hydrogenosomes of anaerobic fungi and trichomonad protozoa based on observations of this organelle division. In consequence, Benchimol [50] suggests that hydrogenosomes are homologous organelles in unrelated species, weakens the hypothesis of their polyphyletic origin, and reinforces the hypothesis that fungal and trichomonad hydrogenosomes are derived from an ancestral endosymbiont preceded by a singular event of endosymbiosis during the course of evolution.

A phylogenetic tree which reconstructs the evolution of anaerobic gut fungi in relation to other related aerobic fungal lineages is presented in figure 1. Anaerobic gut fungi (marked in the rectangle) appear as monophyletic group (BP = 100 %).

Within this group the *Orpinomyces* clade contains the monocentric anaerobic species *Neocallimastix frontalis* and *Piromyces communis* (BP = 68 %), whereas *Anaeromyces* forms a well supported monophylum (BP = 78 %). The aerobic chytrids (indicated in bold font) forming a single opisthokont flagellum at the end of their zoospores appear to be the closest phylogenetic ancestors of the anaerobic gut fungi whose zoospores form more than a single flagellum. Thus, the presence of flagellum appears to be an important synapomorphic character for the group of zoosporic fungi as a whole taxon. The type of flagellation will be elucidated in the following section.

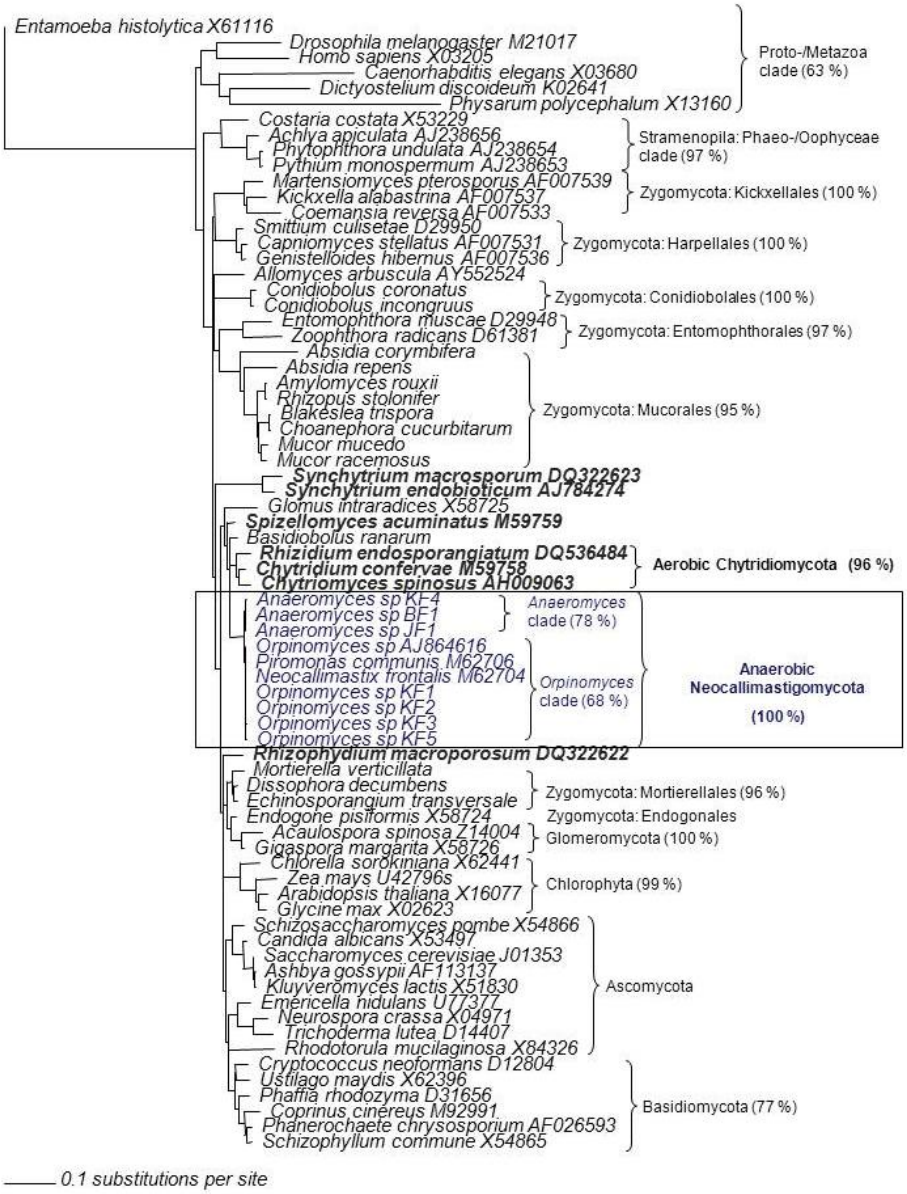

Figure 1. Neighbor-Joining phylogenetic tree of 589 aligned nucleotide characters of the 5´part of the 18S rDNA of 73 taxa. Numbers behind taxon names represent accession numbers as obtained from GenBank at www.ncbi.nlm.nih.gov. Numbers behind clade designations indicate bootstrap proportions (BP) of 1,000 neighbor-joining replicates of Jukes-Canor distances for measurement of the clade stability of each node in the phylogenetic tree.

Polyflagellate zoospores are another exceptional morphological feature of anaerobic fungi. Flagellate zoospores are responsible for asexual reproduction of Chytridiomycota, Blastocladiomycota and Neocallimastigomycota, however the first two phyla produce exclusively uniflagellate zoospores and only two genera of gut fungi produce polyflagellate motile cells. This singular feature is found in polycentric *Orpinomyces* and monocentric *Neocallimastix* species. Their zoospores have 7 – 20 flagella inserted in two rows. Zoospores

of other anaerobic fungal genera are uniflagellate, however sometimes bi- and tri-flagellate cells can be observed. Flagellate fungi are assumed to form early-diverging clades within the kingdom Fungi, because the simple aquatic forms with flagellate spores are believed to be the fungal ancestor [51]. The loss of flagellum was certainly an important evolutionary event leading to the diversification of terrestrial fungi. From this point of view, Neocallimastigomycota are extremely interesting because multiflagellate zoospores indicate the outstanding and extraordinary evolutionary history of anaerobic fungi, which is, to date, not well-understood yet.

Not only morphological, but also genetic singularities are harboured by anaerobic fungi in secrecy. The percentual representation of guanine and cytosine bases ranging from 5 to 20 % in the genomic DNA of gut fungi exhibits the lowest GC content ever reported in any organism. This extreme nucleotide bias is reflected in both the coding and non-coding regions of the genome, with codon usage tending towards more AT-rich codons [52]. The non-coding regions are even more AT-rich with many sections expected to be near or above 95 mol% AT content [53-56]. The codon bias resulting from the low G+C content leads to a depletion in amino acids coded by GC rich codons (e.g. arginine, proline, glycine) and an overabundance of amino acids coded by AT rich codons (e.g. lysine, phenylalanine, tyrosine) influencing thus the amino acid composition of encoded proteins [57].

The main attractive feature of anaerobic fungi is, however, their excellent enzymatic system, which is connected with unique ability to organize hydrolases in large multienzyme complexes called cellulosomes. The Neocallimastigomycota is the only known member of the kingdom Fungi possessing cellulosomes and this can explain their cellulolytic superiority over aerobic cellulolytic fungi. These exocellular enzyme complexes are extremely active and can degrade both amorphous and crystalline cellulose [58]. Research indicates that cellulosomes of anaerobic fungi are similar to the most investigated cellulosome of bacteria *Clostridium thermocellum*, but on the other hand some important differences have been already proven. The composition of fungal cellulosome is similar to bacterial one including the nonenzymatic scaffolding protein(s) with enzyme binding site called cohesins and variety of cellulosomal enzymes with dockerins, which interact with the cohesins in the scaffolding protein [59, 60]. Fungal noncatalytic dockerin domain has function similar to bacterial dockerins [61], but no sequence homology to clostridial dockerins has been found [62-66]. Moreover, so far no scaffolding polypeptide has been isolated from a cellulosome of any anaerobic fungus, and there is no detailed knowledge regarding cohesins in scaffolding peptides from gut fungi. Fungal cellulosomes have been described up to now for *Neocallimastix*, *Piromyces*, and *Orpinomyces* species and molecular biological studies indicate that enzymes associated with the fungal cellulosomes from these three genera are modular like those of anaerobic bacteria.

4. Amazing Enzymes of Anaerobic Fungi

Anaerobic fungi synthetize a variety of hydrolytic enzymes, including cellulases, xylanases, mannases, esterases, glucosidases, and glucanases, which effectively hydrolyze plant biomass consisting mainly from cellulose and hemicellulose, the most abundant polysaccharides in the biosphere. Gut fungi decompose complicated structure of cellulose

chain by several types of enzymes including endoglucanase (cellulase), which splits β-1,4-glycosidic bonds randomly within the cellulose chain, exoglucanases (cellobiosidase or cellobiohydrolases), which cleave cellobiose from the ends of the cellulose chain, and β-glucosidases (cellobiases), which convert cellobiose and other low molecular mass cellodextrins into glucose. Cellulolytic hydrolases of anaerobic fungi pertain according to amino acid sequences, hydrophobic clusters, and stereochemistry to the several different glycoside hydrolase (GH) families. β-glucosidases represent usually family GH1 or sometimes GH3, exoglucanases are registered in GH6. Endoglucanases are generally of family GH5, but some are classified in family 6. For *Piromyces* genus unusual cellulases of family 9 [54, 55], 45 [67-69] and 48 [70] have been discovered. Cellulases of family 5 from anaerobic fungi are intronless and their catalytic domains show homology with those of GH from several anaerobic bacteria. It has been suggested that the family 5 cellulase gene of anaerobic fungi was originally transferred from a ruminal bacterium and subsequently underwent gene duplication [52]. Cellulases of family 6 from anaerobic fungi are often intron-containing and this finding is considered to be a good evidence for fungal (eukaryotic) origin of this gene.

Also enzymatic conversion of xylan, the principal type of hemicellulose, to its monomeric components requires the participation of several enzymes, which are effectively secreted by anaerobic fungi. The main hydrolase is xylanase (endoxylanase) cleaving β-1, 4-xylosidic linkage randomly within the main xylan chain and xylosidase, which removes successive D-xylose residues from the non-reducing termini. The ester bonds on xylan and the side chains are hydrolyzed by feruloyl acid esterases, acetylxylan esterases, arabinases, or α-glucuronidases depending on the type of branching group. Xylanases of anaerobic fungi belong usually into GH family 11, rarely to family 10. Other types of hemicellulases like licheninase (1, 3-1, 4-β-D-glucan 4-glucanohydrolase) and mannanase are registered in GH families 16 and 26, respectively. Esterases of gut fungi are also very various pertaining to carbohydrate esterase (CE) families 1, 2, 3 and 6.

In connection with xylan degradation, the fate of pentose's yielding from the hydrolysis of hemicellulose should be mentioned. Also in this metabolic conversion the anaerobic fungi have chosen the unusual way, which has been elucidated only recently in *Piromyces* sp. [71]. Enzymatic and molecular analysis of this gut fungus provided evidence that xylose is converted to xylulose by xylose isomerase and xylulose is phosphorylated by D-xylulokinase to xylulose-5-phosphate, which is a key intermediate in pentose metabolism. This metabolic route resembles situation in bacteria, because in the majority of yeasts and fungi xylose is converted via xylose reductase and xylitol dehydrogenase to xylulose, which is subsequently phosphorylated [72, 73]. Xylose isomerase from several strains of anaerobic fungi has been intensively studied with respect to its biotechnological exploitation in ethanol production as mentioned below in chapter regarding application.

All genera of anaerobic fungi degrade cellulose and hemicellulose, but the range of their hydrolysation depends on the degrees of crystallinity. Moreover gut fungi exhibit genus-dependent differences in their activity towards (hemi)celluloses and recalcitrant substrates. *Neocallimastix* and *Piromyces* sp. are supposed to be the most active genera [74-76], while *Caecomyces* sp. the least effective genus [77, 78]. Except for this set of (hemi-)cellulolytic enzymes, anaerobic fungi secrete numbers of other less important enzymes including disaccharidases, amylases and amyloglycosidases, pectin lyase, chitinase and proteases. The

range of enzymes was found to be similar for anaerobic fungi isolated from the foregut and hindgut of nonruminant herbivores and for strains from forestomach of ruminants [79]. Some of these enzymes act individually and are free in solution, whereas others are constituents of large (hemi)cellulase multienzyme complexes mentioned above as cellulosomes. Enzymes organized in cellulosomal complexes containing endo- and exo-cellulases, hemicellulases and esterases have been described for *Piromyces*, *Orpinomyces* and *Neocallimastix* species. Another remarkable feature of anaerobic fungi insist in containing more than one copy of genes encoding (hemi)-cellulases in their genome. This gene duplications result in clusters of almost identical genes arranged head-to-tail on the genome. In contrast to other eukaryotes, this phenomenon appears frequently in anaerobic fungi [55]. Genome of anaerobic fungi thus can serve as an excellent model for study of horizontal gene transfer. Sequence comparison of broad range of hydrolases produced by anaerobic fungi including catalytic as well as non-catalytic domains indicates both bacterial and eukaryotic (fungal or even animal) origin of these enzymes.

5. Potential Application

The broad range of potent polysaccharide-degrading enzymes of anaerobic fungi has always attracted the attention of scientific and biotechnological community. The ambitions of exploitation of the effective hydrolytic facilities of gut fungi entered several fields, especially food, animal feed, brewery, textile, paper and biofuel industries. The applications naturally face many problems and the difficulties to set up continuous-flow cultures to produce anaerobic fungal enzymes represent one of the biggest limitation. Processes using immobilized growing cells seem to be more promising than traditional fermentations with free cells. The effort to immobilize anaerobic fungal mycelium and/or zoospores in alginate beads has been recently reported by several authors for monocentric *Piromyces* sp. [80, 81] and *Caecomyces* sp. [82] and polycentric *Orpinomyces* [80, 81] and *Anaeromyces* sp. [83]. These studies suggest that immobilization may be more suited for polycentric anaerobic fungi, because mycelial growth on the surface observed for monocentric *Piromyces* is undesirable effect leading to the growth in culture liquor and represents major problem that has to be overcome before immobilized fungi can find wide use in the fermentation industry. Also immobilization of enzymes has not been without problems. Immobilized proteins had difficulties to accommodate small substrate and had a lower rate of hydrolysis as has been shown by Mesta et al. [84] trying to immobilize chimeric endoxylanase from *N. frontalis* on metal-chelate matrix. Interesting results have been achieved during the expression of recombinant xylanase gene from *Neocallimastix patriciarum* in Canola seeds [85]. Oil-bodies extract from transgenic seed exhibited xylanase activity and immobilized enzyme could be recycled by floatation several times without loss of activity. Another experimental streams directed to enhanced xylose and cellulose degradation are represented by efforts to induce changes in catalytic domain of enzymes of anaerobic fungi and/or to produce hydrolytic enzymes of anaerobic fungi in microorganisms already used in commercial processes.

The livestock production is the industrial field with high interest in application of fibrolytic enzymes of anaerobic fungi to improve the efficiency of feed utilization. The possible use of rumen fungi as probiotics has been already studied on sheep, buffalos, and

cows [86-89]. Direct supplementation of *Orpinomyces* sp., *Piromyces* sp. or *Anaeromyces* sp. cultures improved animal growth rate and feed efficiency. Administration of fungal cultures increased digestibility of cell-wall contents and crude protein, rumen fermentation parameters were improved, and the average daily weight gain was significantly higher. On the other hand the application of anaerobic fungal enzyme has not induced the response obtained with administration of live fungal culture. Another research is directed to use the ruminal fungi features for non-ruminant animals, especially economically important swines and poultry. Cereals, the main part of monogastric feed, contain in the cell walls hardly digestible nonstarch polysaccharides like β-glucan in barley and wheat, and arabinoxylans in rye and oats. The antinutritive effect of these compounds for monogastric animals is well known and unwanted [90]. Anaerobic fungi are able to split these hemicellulosic complexes and it has been already proved that application of their glycoside hydrolases to barley or wheat diets increases the growth of animals. Addition of fungal β-glucanase to the basic barley diet resulted even in 25% increase in growth of broiler chicks [91]. The high-priced production of enzymes from anaerobic fungi has induced the genetic engineering attempts to introduce xylanase and cellulase genes of gut fungi into the genome of bacterium *Lactobacillus reuteri*, which is natural component of microflora of broiler digestive tract [92, 93].

The great interest in enzymes of anaerobic fungi has been manifested by paper industry. Fungal cellulases and mainly xylanases are desired as environmental friendly substances to reduce the amount of alkalies and chlorines used for treatment of paper pulp. Xylanases of anaerobic fungal strain *Orpinomyces* have decreased the lignin content in paper stuff, increased the brightness of paper and positively affected the further bleaching with ozone [94]. Cellulases of the same genus have been used for deinking of recycled mixed office paper and significantly reduced both dirt count and residual ink area and increased brightness of recycled paper [95]. The pulp bleaching entails high temperatures and high pH and therefore genetic engineering aspires to construct thermophilic xylanase stable in alkaline environments. For this reason the catalytic domain of a xylanase from *Neocallimastix patriciarum* has been changed to be more alkalophilic [96] through directed evolution using error-prone PCR. The resulting composite mutant xylanase was alkalic tolerant maintaining 25% of its activity at pH 9. Moreover the mutant xylanase was more thermostable at $60^{O}C$ [97].

Enzymes of anaerobic fungi are involved also in tests dealing with fermentative production of ethanol from lignocellulosic residues. The wild - type of *Saccharomyces cerevisiae*, which is the most used industrial microbial strain for ethanol production, cannot metabolize xylose and arabinose, two important pentoses resulting from hemicellulose hydrolysis. The genetic engineering is therefore concentrated to modify this yeast to be able to metabolize xylan or at least monosaccharide xylose. Efforts with incorporation of rumen fungal xylanases [98, 99] or cellobiohydrolases [100] into different kinds of aerobic fungi have not been surprisingly as successful as introduction of xylose isomerase, the pentose cycle enzyme of gut fungi. Strain of *Sacharomyces cerevisiae* genetically modified to change xylose into xylulose using the isomerase of anaerobic fungi *Piromyces* and/or *Orpinomyces* [101-103] represents at this time the most promising species for industrial production of ethanol [104].

The unworn involvement of rumen fungi into the biogas production has been inspired by growing demands for renewable energy. The possibility of *Anaeromyces* and *Piromyces*

strains to integrate into biogas-producing anaerobic sludge bacterial ecosystem, to improve degradation of substrate polysaccharides and consequently to influence methane production has been already tested in laboratory conditions [105]. Even if small batch cultures have indicated positive influence of anaerobic fungi on biogas production, the application of this approach in commercial full-scale biogas does not seems feasible at this moment. However, the detection of gut fungi in landfill samples published by Lockhart et al. [106], the only report of the presence of anaerobic fungi outside the gastrointestinal animal tract, indicates the unraveled and unsuspected opportunities of these special, exceptional, but not easily graspable fungi with excellent enzymatic properties.

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