

***abfB-1 gene of Penicillium funiculosum***

The invention relates to the *abfB-1* gene isolated from *Penicillium funiculosum* and the ABFB-1 polypeptide encoded by this gene having an  $\alpha$ -L-arabinofuranosidase B activity.

*Penicillium funiculosum* is a *Talaromyces* belonging to the *Aspergilleae* family. The isolation of this microorganism from numerous organic substrates which are subject to aerial or aqueous contamination shows that this fungus possesses a range of hydrolytic enzymes of a surprising richness. The use of this enzymatic cocktail in animal feed contributes towards the depolymerization of the natural organic substances and makes it possible to improve their digestibility. WO 99/57325 thus describes a *Penicillium funiculosum* strain called IMI378536 which produces a mixture of enzymes which is particularly suitable as animal feed. However, the enzymatic cocktails produced by *Penicillium funiculosum* have not been biochemically characterized to any great extent. Indeed, only a limited number of enzymatic activities, such as xylanases and  $\beta$ -glucanases are generally measured on the fermentation broths obtained. These activities reflect only a fraction of the enzymatic population present in the cocktail.

Hemicellulolytic compounds derived from agriculture constitute the second polysaccharide reserve after cellulose in plant tissues. This group is characterized by a wide variety of heteropolysaccharides, of which the principle representatives are xylans, arabinans, galactans, glucans and mannans. Arabinose, in its furfural form, is widely represented among the heteropolysaccharides such as arabinans and arabinoxylans. Arabinan is a polymer with arabinofuranose residues linked by  $\alpha$ -1-5 bonds and it



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may be substituted with 1 or 2 arabinose residues at the O-2 or O-3 position. As regards the arabinoxylans, the  $\alpha$ -L-arabinofuranosyl residues are linked to the principle  $\beta$ -1-4-xylopyranosyl chain by  $\alpha$ -1-3 and  $\alpha$ -1-2  
5 bonds. The presence of arabinose residues on these side chains can restrict the enzymatic hydrolysis of hemicellulolytic compounds in numerous industrial applications such as the enhancement of the digestibility of animal feed. The enzymes cleaving the  
10  $\alpha$ -L-arabinofuranoside bonds can act in synergy with xylanases to allow the hydrolysis of arabinoxylans and arabinans.

The arabinase activities (endo-, exo-arabinase and, 15 predominantly, the  $\alpha$ -L-arabinofuranosidase activities) can therefore actively and synergistically contribute, with the xylanases, to the depolymerization of the hemicellulolytic compounds. The hemicellulolytic and pectic compounds may represent up to 50% of the total  
20 carbohydrates present in plants and they constitute a major source of energy for animals. The enhancement of the digestibility of these compounds is correlated with the decrease in the degree of substitution of the arabinosyl residues within the hemicellulolytic  
25 compounds (Brice, R.E., Morrison, I.M. 1982, Carbohydr. Res. 101: 93-100).

The enzymes which hydrolyse the bonds between L-arabinose residues have been isolated from  
30 microorganisms such as bacteria or filamentous fungi. Arabinosidases consist mainly of  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55) which are capable of hydrolysing the non-reducing  $\alpha$ -L-arabinofuranosyl residues derived from L-arabinoxylan or compounds such  
35 as arabinans and arabinogalactans.

The  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55) have been classified into two families of Glycoside Hydrolases

(GH 51 and GH 54) according to their protein sequence similarities. These two families differ by virtue of their specificity for substrate contained in polysaccharides. The first group (GH 51) contains type  
5 A arabinofuranosidases which act only on small linear structures of  $\alpha$ -1-5 linked arabinofuranosyl oligosaccharides. The second group consists of type B arabinofuranosidases (GH 54) which catalyse the hydrolysis of the  $\alpha$ -1,5,  $\alpha$ -1,3 and  $\alpha$ -1,2 bonds of the  
10 side chains contained in the arabinofuranosyl-oligosaccharide compounds.

The B arabinofuranosidases (ABFB) have been isolated from numerous bacteria, but also from filamentous  
15 fungi. The genus *Aspergillus* is the most widely represented, but they have also been isolated from the genera *Trichoderma*, *Penicillium* and *Fusarium*.

WO 96/29416, WO 96/06935, WO 2004/018662 and  
20 US 5,989,887 describe *Aspergillus niger* arabinofuranosidase genes. The protein sequence alignment indicates that the *A. niger* abfB protein is 72.4% identical to the *P. funiculosum* ABFB-1 protein. None of the characteristics essential for the use of the  
25 polypeptide in animal nutrition is described in these applications.

Clinche et al. (J. Agric. Food Chem., 45, 2379-2383, 1997) have described three  $\alpha$ -L-arabinofuranosidases  
30 derived from *Aspergillus terreus* having a potential application in oenology.

Gielkens et al. (Microbiology, 145, 735-741, 1999) have described the *Aspergillus nidulans* abfB gene.

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The *Aspergillus kawachii* and *Aspergillus awamori* abfB genes have been described by Koseki et al. (J. of Bioscience and Bioengineering, Vol. 96, No. 3, 232-241,

2003). These enzymes have applications in the fermentation of the Japanese liquor *shochu*.

5 The *abfB* gene from the filamentous fungus *Trichoderma reesei* has been described by Margolles-Clark et al. (Applied and Environmental Microbiology, 3840-3846, 1996).

10 Panagiotou et al. have also described two extracellular alpha-L-arabinofuranosidases derived from *Fusarium oxysporum* (Can J Microbiol. 2003: 49(10): 639-4).

15 Carvallo et al. (Mycol. Res., 107 (4), 388-394, 2003) have described the B  $\alpha$ -L-arabinofuranosidase from *Penicillium purpurogenum*. The protein sequence alignment indicates that the *P. purpurogenum* abf-1 protein is 85.6% identical to the *P. funiculosum* ABFB-1 protein. None of the characteristics essential for the use of the polypeptide in animal nutrition is described  
20 in this article.

Sakamoto et al. (FEBS Letters 560, 199-204, 2004) have described the *Penicillium chrysogenum* *abnx* gene encoding nevertheless an arabinase activity distinct  
25 from the ABFB activity.

However, these ABFB enzymes do not have the optimum qualities required for application in animal feed. Indeed, to be utilizable in animal feed, the ABFBs must  
30 possess properties compatible with the treatments to which the feedingstuffs intended for this feed are subjected. In particular, the activity of the enzymes used must be stable under the process temperature and pH conditions, and, if possible, be optimum in the  
35 preparation of these feedingstuffs and under the conditions which exist in the digestive system of the animals ingesting these feedingstuffs.

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Furthermore, these enzymes must have a broad spectrum of action (debranching) on the heteropolysaccharides (arabinans, arabinoxylans and arabinogalactans) to allow effective enhancement of the digestibility of the feedingstuffs by the animals. This enhancement of the digestibility of the feedingstuffs makes it possible to increase their nutritional value. Accordingly, the enzymes having enhanced specificity (stereospecificity, enantioselectivity), activity or affinity towards the natural substrates arabinoxylans and arabinans are of great interest as animal feed.

The present invention describes a *Penicillium funiculosum* L-arabinofuranosidase B (ABFB-1) suitable for application in animal nutrition and the gene encoding this enzyme. The invention also relates to the homologues, variants and fragments of ABFB-1 preserving the same catalytic properties.

Advantageously, the ABFB enzymes according to the invention have a high optimum temperature.

Another advantage of the present invention is that the expression of the *Penicillium funiculosum* ABFB-1 is naturally highly induced in this fungus under conditions for the induction of cellulolytic and hemicellulolytic enzymes (industrial-type culture medium for the production of cellulolytic and hemicellulolytic enzymes).

The enzymes according to the invention also have other industrial or agroindustrial applications. There may be mentioned in particular the treatment of fruit juices, the manufacture of paper, the conversion of hemicellulolytic biomass to fuel or chemical products, the preparation of alcoholic drinks by fermentation.

**Description of the sequences**

SEQ ID No. 1: Genomic sequence of the *Penicillium funiculosum* *abfB-1* gene.

- 5 SEQ ID No. 2: Sequence of the *Penicillium funiculosum* ABFB-1 polypeptide having type B  $\alpha$ -L-arabinofuranosidase activity.

SEQ ID No. 3: XbaI-*abfB* primer.

SEQ ID No. 4: HindIII-*abfB* primer.

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**Description of the invention**

The present invention relates to a polypeptide suitable for use in animal nutrition comprising a polypeptide  
15 chosen from the following polypeptides:

- the polypeptide of SEQ ID No. 2,
- the polypeptide whose sequence is between position 28 and position 507 of SEQ ID No. 2,
- a fragment of the polypeptide of SEQ ID No. 2  
20 having an  $\alpha$ -L-arabinofuranosidase B activity,
- a polypeptide having an  $\alpha$ -L-arabinofuranosidase B activity and exhibiting at least 90% identity with the polypeptide of SEQ ID No. 2.

25 The invention also relates to a polynucleotide, encoding an  $\alpha$ -L-arabinofuranosidase B activity, chosen from the following polynucleotides:

- the polynucleotide whose sequence is between position 845 and position 2368 of SEQ ID No. 1,
- 30 - the polynucleotide whose sequence is between position 927 and position 2368 of SEQ ID No. 1,
- a polynucleotide encoding a polypeptide as defined above.

35 Another subject of the present invention is a polynucleotide having the sequence represented by SEQ ID No. 1 or the sequence complementary to SEQ ID No. 1.

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The invention also relates to expression cassettes comprising, in the direction of transcription:

- a promoter that is functional in a host organism;
- 5       - a polynucleotide according to the invention; and
- a terminator sequence that is functional in the same host organism.

10    Another subject of the invention is a vector comprising a polynucleotide according to the invention and/or an expression cassette according to the invention.

The invention also relates to a host organism  
15   transformed with a polynucleotide according to the invention, an expression cassette according to the invention and/or a vector according to the invention.

In one embodiment of the invention, the host organism  
20   is chosen from yeasts and filamentous fungi.

Preferably, the host organism is a *Penicillium funiculosum* strain.

25   The invention also relates to a nutritional additive for animals, comprising a polypeptide according to the invention, a host organism according to the invention or a fermentation broth of a host organism according to the invention.

30   Preferably, this nutritional additive is in liquid form or in powdered form.

Another aspect of the invention is a feedingstuff  
35   comprising a nutritional base for animals and a nutritional additive for animals according to the invention.

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The invention also relates to the use of an ABFB polypeptide according to the invention or a host organism according to the invention for the manufacture of a nutritional additive for animals or of a feedingstuff.

Another subject of the invention is the use of an ABFB polypeptide according to the invention or of a host organism according to the invention for hydrolysing the  $\alpha$ -L-arabinofuranosyl bonds of arabinofuranosyl-oligosaccharide compounds.

#### Polypeptides

The present invention therefore relates to ABFB polypeptides having an  $\alpha$ -L-arabinofuranosidase B activity. Preferably, these polypeptides are isolated from *Penicillium funiculosum*.

The expression " $\alpha$ -L-arabinofuranosidase B" is understood to mean  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55) type B (GH 54) which catalyse the hydrolysis of  $\alpha$ -1,5,  $\alpha$ -1,3 and  $\alpha$ -1,2 bonds of the side chains contained in arabinofuranosyl-oligosaccharide compounds.

The  $\alpha$ -L-arabinofuranosidase B of the *Penicillium funiculosum* strain IMI378536 is represented in SEQ ID No. 2.

The expression "polypeptide suitable for use in animal nutrition" is understood to mean a polypeptide whose characteristics are such that it is suitable for animal nutrition. The characteristics essential for use in animal nutrition are in particular the pH and the temperature at which the enzyme is active. Indeed, the pH of the digestive system of the animals is acidic and it is therefore essential that the enzyme remains active at this pH, this being in order to preserve its

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activity in the hydrolysis of the L-arabinose residues. In addition, conditioning the enzyme in a nutritional additive or in the animal feed involves treatments and a temperature greater than room temperature. The activity of the enzymes used must therefore be stable under the process conditions, and in particular the temperature conditions.

According to one embodiment of the present invention, the polypeptide exhibits an  $\alpha$ -L-arabinofuranosidase B activity at an acidic pH, for example less than 5, preferably less than 4. Also, according to one embodiment of the present invention, the polypeptide exhibits an optimum  $\alpha$ -L-arabinofuranosidase B activity between pH 2 and pH 3.5.

According to a preferred embodiment of the present invention, the polypeptide exhibits an  $\alpha$ -L-arabinofuranosidase B activity at temperatures greater than room temperature. Preferably, the polypeptide of the present invention has an optimum  $\alpha$ -L-arabinofuranosidase B activity at a temperature of between 40°C and 70°C, more preferably between 50°C and 65°C.

In a preferred embodiment, the polypeptides according to the invention are glycosylated. The polypeptide of SEQ ID No. 2 possesses in particular N-glycosylation sites at amino acid 92 and at amino acid 376. In a preferred embodiment, the asparagin residues at position 92 and 376 of the polypeptide of SEQ ID No. 2 are glycosylated.

The  $\alpha$ -L-arabinofuranosidase B of *Penicillium funiculosum* is an enzyme secreted by the fungus into its extracellular environment. The polypeptide of SEQ ID No. 2 thus comprises a signal peptide of 27 amino acids. The subject of the invention is also the mature polypeptide obtained after cleaving the signal peptide.

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In particular, the invention relates to the polypeptide whose sequence is between position 28 and position 507 of SEQ ID No. 2. In another embodiment, the signal peptide of the polypeptide of SEQ ID No. 2 may be  
5 replaced by a heterologous signal peptide for the expression and the secretion of the polypeptide of SEQ ID No. 2 by a heterologous host organism.

The invention also relates to fragments of the  
10 polypeptide of SEQ ID No. 2 having an  $\alpha$ -L-arabinofuranosidase B activity.

The term "fragment" of a polypeptide denotes a polypeptide comprising a portion but not the entire  
15 polypeptide from which it is derived. The invention thus relates to a polypeptide comprising a fragment of at least 100, 200, 300, 400 or 500 amino acids of the polypeptide of SEQ ID No. 2.

20 This fragment of the polypeptide of SEQ ID No. 2 preserves its  $\alpha$ -L-arabinofuranosidase B activity. The invention therefore relates to the biologically active fragments of the polypeptide of SEQ ID No. 2. The term "biologically active fragment" denotes a fragment of a  
25 polypeptide preserving the function of the polypeptide from which it is derived. The biologically active fragments of the polypeptide of SEQ ID No. 2 thus preserve the function of the *Penicillium funiculosum* ABFB-1 polypeptide. These biologically active fragments  
30 have an  $\alpha$ -L-arabinofuranosidase B activity.

The methods for preparing fragments of a polypeptide and the techniques for measuring the  $\alpha$ -L-arabinofuranosidase B activity are well known to a  
35 person skilled in the art.

The subject of the invention is also polypeptides having an L-arabinofuranosidase B activity and

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exhibiting at least 90% identity with the polypeptide of SEQ ID No. 2. Preferably, these polypeptides have the same properties and in particular the same catalytic properties as the polypeptides of SEQ ID No. 2. Preferably, these polypeptides are isolated from other strains of *Penicillium funiculosum* or from other filamentous fungi. Alternatively, these polypeptides may be obtained by site-directed mutagenesis techniques for example.

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The subject of the invention is polypeptides having at least 90%, 95%, 98% and preferably at least 99% of amino acids that are identical with the polypeptide of SEQ ID No. 2.

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The expression identical amino acids is understood to mean amino acids that are invariant or unchanged between two sequences. These polypeptides may exhibit a deletion, an addition or a substitution of at least one amino acid compared with the polypeptide of SEQ ID No. 2.

The subject of the invention is also polypeptides exhibiting at least 90%, 95%, 98% and preferably at least 99% similarity with the polypeptide of SEQ ID No. 2.

The expression similarity is understood to mean the measurement of the resemblance between proteic or nucleic sequences. These polypeptides may exhibit a deletion, an addition or a substitution of at least one amino acid compared with the polypeptide of SEQ ID No. 2. The degree of similarity between two sequences, quantified by a score, is based on the percentage sequence identity and/or sequence-preserving substitutions.

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Methods for measuring and identifying the degree of identity and the degree of similarity between polypeptides are known to persons skilled in the art. It is possible to use for example Vector NTi 9.1.0, the  
5 alignment programme AlignX (Clustal W algorithm) (Invitrogen INFORMAX, <http://www.invitrogen.com>). Preferably, the default parameters are used.

The polypeptides according to the invention are  
10 isolated or purified from their natural environment. The polypeptides may be prepared by means of various methods. These methods are in particular purification from natural sources such as cells naturally expressing these polypeptides, the production of recombinant  
15 polypeptides by appropriate host cells and their subsequent purification, production by chemical synthesis or, finally, a combination of these various approaches. These various methods of production are well known to persons skilled in the art. Thus, the  
20 ABFB polypeptides of the present invention may be isolated from *Penicillium funiculosum*. In another embodiment, the ABFB polypeptides of the present invention are isolated from recombinant host organisms expressing an ABFB polypeptide according to the  
25 invention.

The subject of the invention is also fusion proteins, recombinant proteins or chimeric proteins comprising the polypeptides according to the invention. The term  
30 "polypeptide" also denotes modified proteins and polypeptides.

The polypeptides according to the invention have an ABFB activity and preferably preserve the catalytic  
35 properties of the *Penicillium funiculosum* ABFB-1 enzyme. In particular, these polypeptides have an optimum activity at 60°C and at pH 3.4.

Polynucleotides

The invention also relates to polynucleotides encoding an  $\alpha$ -L-arabinofuranosidase B activity. Preferably, these polynucleotides encode a *Penicillium funiculosum*  $\alpha$ -L-arabinofuranosidase B.

According to the present invention, the expression "polynucleotide" is understood to mean a single-stranded nucleotide chain or its complementary strand which may be of the DNA or RNA type, or a double-stranded nucleotide chain which may be of the complementary or genomic DNA type. Preferably, the polynucleotides of the invention are of the DNA type, in particular double-stranded DNA. The term "polynucleotide" also denotes the modified polynucleotides.

The polynucleotides of the present invention are isolated or purified from their natural environment. Preferably, the polynucleotides of the present invention may be prepared by conventional molecular biology techniques as described by Sambrook et al. (Molecular Cloning: A Laboratory Manual, 1989) or by chemical synthesis.

In a first embodiment, the invention relates to the polynucleotide whose sequence is between position 845 and position 2368 of SEQ ID No. 1. This polynucleotide encodes the *Penicillium funiculosum* ABFB-1 enzyme of SEQ ID No. 2.

In a second embodiment, the invention relates to the polynucleotide whose sequence is between position 927 and position 2368 of SEQ ID No. 1. This polynucleotide encodes the *Penicillium funiculosum* mature ABFB polypeptide after cleavage of the signal peptide.

The invention also relates to polynucleotides having at least 70%, 75%, 80%, 85%, 90%, 95%, 98% and preferably at least 99% identity with the polynucleotide whose sequence is between position 845 and position 2368 of SEQ ID No. 1 and/or with the polynucleotide whose sequence is between position 927 and position 2368 of SEQ ID No. 1. These polynucleotides encode an  $\alpha$ -L-arabinofuranosidase B activity. Preferably, these polynucleotides encode a *Penicillium funiculosum*  $\alpha$ -L-arabinofuranosidase B.

The expression identical nucleotides is understood to mean nucleotides that are invariant or unchanged between two sequences. These polynucleotides may exhibit a deletion, an addition or a substitution of at least one nucleotide compared with the reference polynucleotide.

The invention also relates to polynucleotides exhibiting at least 70%, 75%, 80%, 85%, 90%, 95%, 98% and preferably at least 99% similarity with the polynucleotide whose sequence is between position 845 and position 2368 of SEQ ID No. 1 and/or with the polynucleotide whose sequence is between position 927 and position 2368 of SEQ ID No. 1. These polynucleotides encode an  $\alpha$ -L-arabinofuranosidase B activity. Preferably, these polynucleotides encode a *Penicillium funiculosum*  $\alpha$ -L-arabinofuranosidase B.

The expression similarity is understood to mean the measurement of the resemblance between protein or nucleic sequences. These polynucleotides may exhibit a deletion, an addition or a substitution of at least one nucleotide compared with the reference polynucleotide. The degree of similarity between two sequences, quantified by a score, is based on the percentage sequence identity and/or sequence-preserving substitution.

The methods for measuring and identifying the degree of identity and the degree of similarity between nucleic acid sequences are well known to persons skilled in the art. It is possible to use for example Vector NTi Vector NTi 9.1.0, an alignment programme AlignX (Clustal W algorithm) (Invitrogen INFORMAX, <http://www.invitrogen.com>). Preferably, the default parameters are used.

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Preferably, the polynucleotides exhibiting a degree of similarity with a reference polynucleotide preserve the function of the reference sequence. In the present case, the polynucleotides encode an  $\alpha$ -L-arabinofuranosidase B activity.

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The invention also relates to polynucleotides capable of selectively hybridizing with the polynucleotide whose sequence is between position 845 and position 2368 of SEQ ID No. 1 and/or with the polynucleotide whose sequence is between position 927 and position 2368 of SEQ ID No. 1. Preferably, the selective hybridization is carried out under conditions of average stringency and preferably under conditions of high stringency. These polynucleotides encode an  $\alpha$ -L-arabinofuranosidase B activity. Preferably, these polynucleotides encode a *Penicillium funiculosum*  $\alpha$ -L-arabinofuranosidase B.

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The expression "sequence capable of selectively hybridizing" is understood to mean, according to the invention, the sequences which hybridize with the reference sequence at a level significantly above the background noise. The level of the signal generated by the interaction between the sequence capable of selectively hybridizing and the reference sequences is generally 10 times, preferably 100 times more intense than that of the interaction of the other DNA sequences

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generating the background noise. Stringent hybridization conditions allowing selective hybridization are well known to persons skilled in the art. In general, the hybridization and washing temperature is at least 5°C less than the T<sub>m</sub> of the reference sequence at a given pH and for a given ionic strength. Typically, the hybridization temperature is at least 30°C for a polynucleotide of 15 to 50 nucleotides and at least 60°C for a polynucleotide of more than 50 nucleotides. By way of example, the hybridization is carried out in the following buffer: 6× SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, 500 µg/ml denatured salmon sperm DNA. The washings are for example carried out successively at low stringency in a 2× SSC, 0.1% SDS buffer, at average stringency in a 0.5× SSC, 0.1% SDS buffer and at high stringency in a 0.1× SSC, 0.1% SDS buffer. The hybridization may of course be carried out according to other customary methods well known to persons skilled in the art (see in particular Sambrook et al., Molecular Cloning: A Laboratory Manual, 1989). Preferably, the polynucleotides selectively hybridizing with a reference polynucleotide preserving the function of the reference sequence. In the present case, the polynucleotides, which selectively hybridize with the polynucleotide whose sequence is between position 845 and position 2368 of SEQ ID No. 1 and/or with the polynucleotide whose sequence is between position 927 and position 2368 of SEQ ID No. 1, encode an α-L-arabinofuranosidase B activity.

The invention generally relates to the polynucleotides encoding the polypeptides according to the invention. Because of the degeneracy of the genetic code, various polynucleotides can encode the same polypeptide.

Another subject of the present invention is a polynucleotide whose sequence is represented in SEQ ID

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No. 1. The polynucleotide of SEQ ID No. 1 comprises sequences flanking the open reading frame (ORF) of the *Penicillium funiculosum abfB-1* gene. They are in particular promoter and terminator sequences of the *abfB-1* gene. The *abfB* gene may be expressed from its homologous regulatory sequences, in particular for overexpression in *Penicillium funiculosum* or in other filamentous fungi.

In another embodiment, the *abfB* gene may be expressed in various host organisms such as bacteria, yeasts and fungi for example. The *abfB* gene may be expressed in a host organism under the control of the promoter of SEQ ID No. 1 of the present invention or under the control of a heterologous promoter.

#### Expression cassettes

According to one embodiment of the invention, a polynucleotide encoding a polypeptide according to the invention is inserted into an expression cassette using cloning techniques well known to persons skilled in the art. This expression cassette comprises the elements necessary for the transcription and the translation of the sequences encoding the polypeptides according to the invention.

Advantageously, this expression cassette comprises both elements which make it possible to cause a host cell to produce a polypeptide and elements necessary for the regulation of this expression.

These expression cassettes comprise, in the direction of transcription:

- a promoter that is functional in a host organism;
- a polynucleotide according to the invention;
- a terminator sequence that is functional in the same host organism.

Any type of promoter sequence may be used in the expression cassettes according to the invention. The choice of the promoter will depend in particular on the host organism chosen for the expression of the gene of interest. Some promoters allow a constitutive expression whereas other promoters are on the contrary inducible. Among the promoters that are functional in fungi, there may be mentioned in particular that for *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (Roberts et al., Current Genet. 15: 177-180, 1989). Among the promoters that are functional in bacteria, there may be mentioned in particular that for the T7 bacteriophage RNA polymerase (Studier et al., Methods in enzymology 185: 60-89, 1990). Among the promoters that are functional in yeasts, there may be mentioned the promoter for the *GAL1* gene (Elledge et al., Proc Natl Acad Sciences, USA. 88: 1731-1735, 1991) or the *S. cerevisiae* *GAL4* and *ADH* promoters. All these promoters are described in the literature and are well known to persons skilled in the art.

For expression in *Penicillium funiculosum*, expression cassettes will be chosen for example that comprise a histone H4.B promoter, an aspartyl acid protease promoter or a *cs113* promoter (WO 00/68401).

The expression cassettes according to the present invention may additionally include any other sequence necessary for the expression of the polypeptides or polynucleotides, such as for example regulatory elements or signal sequences allowing the secretion of the polypeptides produced by the host organism. It is possible to use in particular any regulatory sequence that makes it possible to increase the level of expression of the coding sequence inserted into the expression cassette. According to the invention, it is possible to use in particular, in combination with the

regulatory promoter sequence, other regulatory sequences, which are located between the promoter and the coding sequence, such as transcription activators ("enhancer").

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A wide variety of terminator sequences can be used in the expression cassettes according to the invention, these sequences allow the termination of transcription and the polyadenylation of the mRNA. Any terminator  
10 sequence that is functional in the selected host organism may be used.

For expression in *Penicillium funiculosum*, expression cassettes will be chosen for example that comprise a  
15 histone H4.B terminator, an aspartyl acid protease terminator or a csl13 terminator (WO 00/68401).

The subject of the present invention is also a polynucleotide comprising an expression cassette  
20 according to the invention, advantageously the expression cassettes according to the present invention are inserted into a vector.

#### Vectors

25 The present invention therefore also relates to replicating or expression vectors for transforming a host organism comprising at least one polynucleotide or one expression cassette according to the present invention. This vector may correspond in particular to  
30 a plasmid, a cosmid, a bacteriophage or a virus into which a polynucleotide or an expression cassette according to the invention has been inserted. The techniques for constructing these vectors and for inserting a polynucleotide of the invention into these  
35 vectors are well known to persons skilled in the art. In general, it is possible to use any vector capable of maintaining itself, self-replicating or propagating in a host cell in order to induce in particular the

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expression of a polynucleotide or of a polypeptide. Persons skilled in the art will choose the appropriate vectors according to the host organism to be transformed, and according to the transformation  
5 technique used.

The vectors of the present invention are used in particular to transform a host organism for replication of the vector and/or the expression of a polypeptide  
10 according to the invention in the host organism.

The invention also relates to a method for preparing a polypeptide according to the invention comprising the following steps:

- 15       - a host organism is transformed with an expression vector comprising an expression cassette according to the invention and/or with a polynucleotide according to the invention,
- the polypeptides produced by the host organism  
20       are isolated.

#### Host organisms

The subject of the present invention is also a method for transforming a host organism by integrating into  
25 the said host organism at least one polynucleotide or an expression cassette or a vector according to the invention. The polynucleotide may be integrated into the genome of the host organism or can stably replicate in the host organism. Methods for transforming the host  
30 organisms are well known to persons skilled in the art and are well described in the literature.

The present invention also relates to a host organism transformed with a polynucleotide, an expression  
35 cassette or a vector according to the invention. The expression host organism is understood to mean in particular according to the invention any mono- or pluricellular, lower or higher, organism, chosen from

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bacteria, yeasts and fungi. The expression host organism is understood to mean a non-human organism. Advantageously, the yeasts are chosen from *Pichia pastoris*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Schwanniomyces occidentalis*. The fungi are chosen from *Aspergillus* and *Penicillium*, preferably from *Penicillium funiculosum*, *Trichoderma reesei*, *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus kawachii* and *Trichoderma koningii*. In a preferred embodiment, the host organism is a *Penicillium funiculosum* strain in which an ABFB polypeptide according to the invention is expressed or overexpressed.

The techniques for constructing vectors, transforming host organisms and expressing heterologous proteins in these organisms are widely described in the literature (Ausubel F.M. et al., "Current Protocols in Molecular Biology" Volumes 1 and 2, Greene Publishing Associates and Wiley Interscience, 1989; T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning A laboratory Handbook, 1982).

#### Food additives and feedingstuffs

The present invention therefore relates to food additives providing an  $\alpha$ -L-arabinofuranosidase B activity. The intake of this type of enzymatic activity makes it possible to enhance the digestibility of the food and to increase its nutritional value.

The expression nutritional additive is understood to mean a substance that is intentionally added to a food, generally in small quantities, in order to improve its nutritional characteristics or its digestibility. The nutritional additives for animals may contain for example vitamins, mineral salts, amino acids and enzymes.

Typically, the nutritional additives for animals comprise a polypeptide according to the invention, a host organism according to the invention or a fermentation broth of a host organism according to the invention. Thus, the polypeptides having an  $\alpha$ -L-arabinofuranosidase B activity according to the invention can be purified or isolated from a *Penicillium funiculosum* strain or from a recombinant host organism for the manufacture of a nutritional additive for animals. Alternatively, a *Penicillium funiculosum* strain or a host organism producing AbfB polypeptides may be used directly for the manufacture of a nutritional additive for animals. In a preferred embodiment of the invention, the culture supernatant or fermentation broth of a *Penicillium funiculosum* strain or of a host organism according to the invention is used for the manufacture of nutritional additives for animals. This embodiment is particularly advantageous when the ABFB polypeptides are secreted by the *Penicillium funiculosum* strain or the host organism. Usually, this culture supernatant is concentrated or freeze-dried for the manufacture of the nutritional additive.

Accordingly, the invention also relates to a method for preparing an ABFB enzyme comprising the following steps:

- a) culturing a *Penicillium funiculosum* strain or a transformed host organism according to the invention under conditions for inducing the expression of ABFBs,
- b) separating the culture supernatant comprising the ABFB enzyme.

This culture supernatant or fermentation broth may then be concentrated or freeze-dried for the formulation of a food additive or of a feedingstuff.

If the host organism does not secrete the ABFB enzyme in the culture medium, an additional step of opening the cells and purifying the cellular extract may be necessary.

5

The nutritional additives of the present invention comprise an  $\alpha$ -L-arabinofuranosidase B activity but may also comprise other nutritional substances such as vitamins, amino acids or mineral salts.

10

The additives according to the invention increase the digestibility of the feedingstuffs, thus contributing to a better enhancement of the nutritional value of diets based on cereals (wheat, barley, maize, oat, rye and the like) and on oilcakes (soybean, sunflower, rapeseed and the like) in particular.

15

The present invention also relates to the feedingstuffs comprising a nutritional base and a nutritional additive according to the invention. These feedingstuffs are usually provided in the form of meals or granules into which the additives according to the invention are incorporated.

20

The expression feedingstuff is understood to mean anything that can serve as food for animals.

25

The feedingstuffs comprise a polypeptide according to the invention, a host organism according to the invention or a fermentation broth of a host organism according to the invention.

30

For intensive animal breeding, these feedingstuffs usually comprise a nutritional base and nutritional additives.

35

The expression nutritional base is understood to mean what constitutes the main part of the animal feed

ration, consisting by way of example of a mixture of cereals, proteins and fat of animal and/or plant origin.

- 5 The nutritional bases for animals are suitable as feed for these animals and are well known to persons skilled in the art. Usually, these nutritional bases comprise, for example, maize, wheat, pea and soybean. These nutritional bases are suitable for the needs of the various animal species for which they are intended. These nutritional bases may already contain nutritional additives such as vitamins, mineral salts and amino acids.
- 10
- 15 In a preferred embodiment, the invention relates to feedingstuffs for monogastric animals and in particular for poultry and pigs. Poultry comprises in particular laying hens, broilers, turkeys and ducks. Pigs comprise in particular growing-finishing pigs and piglets.

20

#### **Description of the figures**

Figure 1: Determination of the optimum pH of the ABFB-1 enzyme in a McIlvaine buffer series (pH 2.2 to 8) at 40°C in the presence of 5 mM PNPAF.

Figure 2: Determination of the optimum temperature for the ABFB-1 enzyme at its optimum pH in the presence of 5 mM PNPAF.

Figure 3: Determination of the kinetic constants  $K_m$  and  $V_m$  ( $1/V_i = f(1/S)$ ) for ABFB-1 for a PNPAF range from 0.5 mM to 5 mM at pH 3.4 and 60°C.

Figure 4: Values of relative quantitative differential expression of the *abfB-1* and *abfB-2* genes according to the *P. funiculosus* growth conditions.

35

### Examples

#### Development of the assay of the L-arabinofuranosidase B activity

5

The L-arabinofuranosidase activity was measured from a *P. funiculosum* culture on M2 medium with a mixed addition composed of 0.15% provasoy and 0.3% cellulose after 40 h. Samples were collected at 48 h and 72 h of culture. The culture was performed in a 200 ml Erlenmeyer flask with a useful volume of 50 ml. The activity was determined by hydrolysing 5 mM para-nitrophenyl-(L-arabinofuranoside (PNPAF) in a 50 mM sodium acetate buffer, pH 5. 50  $\mu$ l of culture supernatant were incubated with 250  $\mu$ l of substrate preheated at 50°C for 15 min. The reaction was stopped by adding 500  $\mu$ l of 0.5 M NaOH. The release of p-nitrophenyl (PNP) is measured at 405 nm with a molar extinction coefficient of 17 000 M<sup>-1</sup>.cm<sup>-1</sup>. An enzyme unit is defined as the quantity of enzyme which hydrolyses 1  $\mu$ mol of PNPAF per minute under the conditions described above. For the culture of *P. funiculosum*, we obtained 20 mU.ml<sup>-1</sup> after 48 h and 112 mU.ml<sup>-1</sup> after 72 h of culture. These results are in agreement with the literature, indeed for *Aspergillus niger*, activities of the order of 100 to 600 mU.ml<sup>-1</sup> were observed according to the inducer used in the culture.

#### 30 Cloning of *P. funiculosum* abfB ORF into *Saccharomyces cerevisiae*

Starting with genomic DNA from *P. funiculosum*, the *abfB* gene was amplified by PCR with the aid of the pair of primers (HindIII-*abfB*/XbaI-*abfB*) under the following conditions (94°C 30 sec; 62°C 30 sec; 1 min 30 sec at 72°C) for 30 cycles. The PCR product was cloned into a commercial vector pGEM-T(tm)easy.

Sequence of the PCR primer pair

XbaI-abfB-1: >5'-TCTAGAATGTTTCCAAGAATAAAACCAG-3'<

HindIII-abfB-1: >5'-AAGCTTTCATGCAAAGGCAGTCT-3'<

5

The HindIII/XbaI fragment of 1534 bp was excised from the vector pGEM-T and subcloned at the HindIII/XbaI sites into a shuttle vector pJL 52 (plac195-PGK/CYC1). For heterologous expression, the *abfB* gene is therefore  
10 under the control of the constitutive PGK promoter of the gene encoding phosphoglycerate kinase (*S. cerevisiae*) and the CYC1 terminator (*S. cerevisiae*) of the gene encoding a cytochrome C oxidase activity. The new expression cassette is called pOT-01.

15

The *S. cerevisiae* strain JF #1194 (CEN.PK113-5D), a clone derived from the strain CEN.PK 122 carrying the *ura 3-52* auxotrophy, was transformed (lithium acetate/heat shock method) with the expression vector  
20 pOT-01. The transformant strains were selected by phenotype complementation on uracil-free selective plates (*URA3* marker).

Six transformants were selected in order to test for  
25 the presence of an arabinofuranosidase B activity in the culture supernatant. The transformants were cultured in 50 ml of uracil-free YNB medium (except the wild-type control strain) for 24 hours. The arabinofuranosidase activity was assayed on the culture  
30 supernatants with the aid of the method described in the preceding paragraph.

#### Determination of the optimum pH

35 The *abfB-1* gene encoding an arabinofuranosidase B activity derived from *P. funiculosus* was cloned into *S. cerevisiae*. After checking for the presence of an arabinofuranosidase B activity in several

transformants, a transformant was chosen and the ABFB activity was assayed on the culture supernatant after 24 h of growth. The cultures were carried out in a 200 ml Erlenmeyer flask (working volume 50 ml). The activity was determined in the presence of 5 mM p-nitrophenyl- $\alpha$ -L-arabinofuranoside (PNPAF) in a McIlvaine buffer series (pH 2.2 to 8.0). 80  $\mu$ l of culture supernatant were incubated with 320  $\mu$ l of substrate preheated at 40°C for 10 min. The reaction was stopped by adding 1 ml of 1M Na<sub>2</sub>CO<sub>3</sub>. The release of p-nitrophenyl is measured at 405 nm<sup>-1</sup>. One enzyme unit is defined as the quantity of enzyme which hydrolyses 1  $\mu$ mol of PNPAF per minute under the conditions defined above. The activity curve is represented in **Figure 1**. For ABFB-1, the activity optimum is at pH 3.4 and the enzyme preserves 65% activity at pH 5.

#### Determination of the optimum temperature

Using the same protocol, we determined the optimum temperature for the activity of ABFB-1. The enzyme was incubated for 10 min at each of the temperatures in a McIlvaine buffer at pH 3.4. The activity curve is presented in **Figure 2**. The *P. funiculosum* ABFB-1 has an activity optimum at 60°C. ABFB-1 therefore has a temperature optimum higher than the ABFBs described. If the optimum pH and temperature of the ABFB-1 enzyme are selected (pH 3.4 and 60°C), it is observed that the activity for ABFB-1 is 4 times as high as the activity determined in an acetate buffer pH 5 and 40°C (424 mU vs 102 mU).

#### Determination of K<sub>m</sub> and V<sub>m</sub>

The kinetic constants (K<sub>m</sub> and V<sub>m</sub>) for ABFB-1 were determined by measuring the hydrolysis of PNPAF over time, under the optimum conditions determined above.

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The substrate (PNPAF) concentration ranges were established between 0.5 and 5 mM in a pH 3.4 buffer. The kinetics of hydrolysis was monitored for 10 minutes at 60°C. The results were treated according to the double inverse method (Lineweaver and Burk) and presented in **Figure 3**.

The  $K_m$  value is 1 mM for ABFB-1. By comparison, in the literature, the  $K_m$  values for this type of enzyme varies from 0.05 to 1.2 mM according to the genus and the fungal species studied. ABFB-1 has a maximum speed of hydrolysis ( $V_m$ ) of 521 mol PNPAF/mol of enzyme/min under the conditions described above.

#### 15 Determination of the molecular weight of the ABFB-1 enzyme

In order to determine the molecular weight of the ABFB-1 enzyme, the culture supernatant, derived from the growth of a mutant (*S. cerevisiae*) in a minimum medium, was concentrated 200-fold, denatured by boiling at 100°C for 5 min, and then deposited in an SDS-polyacrylamide gel.

25 It is observed that the quantity of extracellular proteins is extremely low in the wild-type strain. For the mutants, the ABFB-1 enzyme is secreted into the culture supernatant. It is predominant in relation to the basal level of the *S. cerevisiae* extracellular proteins.

The determination of the molecular weight was carried out with the aid of the size marker SeeBlue (Invitrogen). The results are presented in Table 1.

35

|        | Predicted MW | MW estimated on gel |
|--------|--------------|---------------------|
| ABFB-1 | 53           | 65                  |

Table 1: ABFB-1 molecular weight in KDa

We compared the molecular weight predicted by the  
5 algorithm Vector NTi and the weight obtained by  
electrophoretic migration in a denaturing SDS-PAGE gel.  
We observed an overestimation of the molecular weight  
of the enzyme in the SDS-PAGE gel. A high glycosylation  
10 on gel of a diffuse electrophoretic band (O and N  
glycosylations). The glycosylations occur during the  
processing of the proteins in the expressing organism.

**Analysis of the profile of expression of the *abfB-1***  
15 **gene in *Penicillium funiculosum***

*Penicillium funiculosum* possesses two genes encoding B  
 $\alpha$ -L-arabinofuranosidases: the *abfB-1* and *abfB-2* genes.  
The profiles of expression of these genes under various  
20 *P. funiculosum* culture conditions were compared.

*P. funiculosum* was cultured under conditions for  
inducing cellulolytic and hemicellulolytic enzymes  
(type M2 industrial growth medium) and under non-  
25 producing conditions (minimum glucose medium M0). After  
40 h of growth, the cultures were stopped, the mycelium  
was recovered, and the total RNAs were extracted. The  
quantity and the quality of the RNAs were assessed by  
measuring the absorbance at 260 nm and at 280 nm  
30 (260/280 ratio > 1.8). The level of transcripts  
encoding the B-type arabinofuranosidase (*ABFB-1* and  
*ABFB-2*) activities were quantified under each of the  
two conditions (M0 and M2) by real-time quantitative  
PCR.

The gene encoding *P. funiculosum* tubulin (*tub-1*) was used as a control under the two conditions. This gene encodes a structural protein that is essential for the integrity of the cell. This gene is commonly used as the reference gene because it exhibits a constant level of expression regardless of the culture condition used (ubiquitous).

Specific primers for quantitative PCR were designed for each of the genes (*abfB-1*, *abfB-2* and *tub-1*). For both growth conditions (M0 and M2), 2 µg of total RNA were retrotranscribed. A series of dilutions of the complementary DNAs derived from the retrotranscription were carried out in order to determine the optimum conditions for amplification of the target genes (constraints of the quantitative PCR method and for the efficiency of these pairs of primers).

The normalized results are presented in Table 2 and **Figure 4**.

|               | M0       | M2          |
|---------------|----------|-------------|
| <b>abfb_1</b> | <b>1</b> | <b>107</b>  |
| <b>abfb_2</b> | <b>1</b> | <b>1.27</b> |

Table 2: Values for differential expression of the *abfB-1* and *abfB-2* genes as a function of the *P. funiculosum* growth conditions

The transcriptional regulations of the genes encoding cellulolytic and hemicellulolytic activities have been described. The expression of these genes is highly subject to the nature and/or to the complexity of the carbon and nitrogen source on which the microorganism is cultured. A high transcriptional repression of these genes has been reported in the presence of glucose. This regulation is performed via a catabolic repression protein CreA which specifically binds to the promoter

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of these genes and blocks their transcription. In our experiment for quantifying, by PCR, the *abfB-1* and *abfB-2* messengers, that the level of expression of these two genes under the glucose (M0) condition is very low. This is in agreement with the literature since it has been shown that these genes have a basal level of expression even under unfavourable conditions (absence of cellulolytic and/or hemicellulolytic substrates). The results obtained for the M0 condition are in agreement with the literature. As regards the expression of the *abfB-1* gene, an induction factor is observed that is 107 times as high as the basal level obtained under the M0 condition, whereas the *abfB-2* gene is not overexpressed.

15

## CLAIMS

1. Polypeptide suitable for use in animal nutrition, characterized in that it comprises a polypeptide chosen  
5 from the following polypeptides:
- the polypeptide of SEQ ID No. 2,
  - the polypeptide whose sequence is comprised between position 28 and position 507 of SEQ ID No. 2,
  - 10 - a fragment of the polypeptide of SEQ ID No. 2 having an  $\alpha$ -L-arabinofuranosidase B activity,
  - a polypeptide having an  $\alpha$ -L-arabinofuranosidase B activity and exhibiting at least 90% identity with the polypeptide of SEQ ID No. 2.
- 15
2. Polynucleotide encoding an  $\alpha$ -L-arabinofuranosidase B activity, characterized in that it is chosen from the following polynucleotides:
- the polynucleotide whose sequence is comprised  
20 between position 845 and position 2368 of SEQ ID No. 1,
  - the polynucleotide whose sequence is comprised between position 927 and position 2368 of SEQ ID No. 1,
  - 25 - a polynucleotide encoding a polypeptide according to Claim 1.
3. Polynucleotide, characterized in that it has the sequence of SEQ ID No. 1 or the sequence complementary  
30 to SEQ ID No. 1.
4. Expression cassette, characterized in that it comprises, in the direction of transcription:
- 35 - a promoter that is functional in a host organism;
  - a polynucleotide according to Claim 2; and
  - a terminator sequence in the same host organism.

- 33 -

5. Vector comprising a polynucleotide according to one of Claims 2-3 and/or an expression cassette according to Claim 4.

5 6. Host organism transformed with a polynucleotide according to one of Claims 2-3, an expression cassette according to Claim 4 and/or a vector according to Claim 5.

10 7. Host organism according to Claim 6, characterized in that the host organism is chosen from yeasts and filamentous fungi.

8. Host organism according to Claim 7, characterized  
15 in that it is a *Penicillium funiculosum* strain.

9. Nutritional additive for animals, characterized in that it comprises a polypeptide according to Claim 1.

20 10. Nutritional additive for animals, characterized in that it comprises a host organism according to one of Claims 6-8 and/or a fermentation broth of a host organism according to one of Claims 6-8.

25 11. Nutritional additive for animals according to one of Claims 9-10, characterized in that it is in liquid form or in powdered form.

12. Feedingstuff, characterized in that it comprises a  
30 nutritional base for animals and a nutritional additive for animals according to one of Claims 9-11.

13. Use of a polypeptide according to Claim 1 or of a host organism according to one of Claims 6-8 for the  
35 manufacture of a nutritional additive for animals or of a feedingstuff.

14. Use of a polypeptide according to Claim 1 or of a host organism according to one of Claims 6-8, for hydrolysing the  $\alpha$ -L-arabinofuranosyl bonds of arabinofuranosyl-oligosaccharide compounds.