BESCHWERDEKAMMERN DES EUROPÄISCHEN PATENTAMTS

BOARDS OF APPEAL OF THE EUROPEAN PATENT OFFICE

CHAMBRES DE RECOURS DE L'OFFICE EUROPEEN DES BREVETS

Internal distribution code:
(A) [ ] Publication in OJ
(B) [ ] To Chairmen and Members
(C) [X] To Chairmen
(D) [ ] No distribution

D E C I S I O N
of 7 December 2004

Case Number: T 0875/02 - 3.3.8

Application Number: 90202565.9

Publication Number: 0420358

IPC: C12N 15/55

Language of the proceedings: EN

Title of invention:
Cloning and expression of microbial phytase

Patentee:
BASF AKTIENGESELLSCHAFT

Opponents:
(01) F. HOFFMANN-LA ROCHE & CO. Aktiengesellschaft
(02) NOVOZYMES A/S
(03) AB Enzymes GmbH
(04) DANISCO A/S
(05) Aventis Animal Nutrition S.A.

Headword:
Microbial phytase/BASF

Relevant legal provisions:
EPC Art. 56

Keyword:
"Main request, auxiliary requests I to XI - inventive step (no)"

Decisions cited:
T 0207/94

Catchword:
-

Europäisches Patentamt | European Patent Office | Office européen des brevets

Beschwerdekammern | Boards of Appeal | Chambres de recours

Case Number: T 0875/02 - 3.3.8

D E C I S I O N
of the Technical Board of Appeal 3.3.8
of 7 December 2004

Appellant: (Proprietor of the patent)
BASF AKTIENGESELLSCHAFT
D-67056 Ludwigshafen (DE)

Representative:
Jaenichen, Hans-Reiner, Dr.
VOSSIUS & PARTNER
Postfach 86 07 67
D-81634 München (DE)

Respondent: (Opponent 03)
AB Enzymes GmbH
Postfach 10 12 39
D-64212 Darmstadt (DE)

Representative:
Hiebl, Inge, Dr.
Kraus & Weisert
Patent- und Rechtsanwälte
Thomas-Wimmer-Ring 15
D-80539 München (DE)

Respondent: (Opponent 04)
DANISCO A/S
Langebrogade 1
DK-1411 Copenhagen K (DK)

Representative:
Lethem, David J.
Hoffman Eitle,
Patent- und Rechtsanwälte
Arabellastrasse 4
D-81925 München (DE)

Respondent: (Opponent 05)
Aventis Animal Nutrition S.A.
16, Av. de l'Europe
Espace Européen de l'Entreprise
F-67300 Schiltigheim (FR)

Representative:
Hinterberg, Katherine
Cabinet Germain et Maureau
BP 6153
F-69466 Lyon Cedex 06 (FR)

Decision under appeal:
Decision of the Opposition Division of the European Patent Office posted 26 June 2002 revoking European patent No. 0420358 pursuant to Article 102(1) EPC.

Composition of the Board:

Chairman: L. Galligani
Members: P. Julia
M. B. Günzel

Summary of Facts and Submissions

I. European patent No. 0 420 358 with the title "Cloning and expression of microbial phytase" was granted with 17 claims in two versions (one for all the designated Contracting States except GR and ES, the other for GR and ES) based on European patent application No. 90 202 565.9. Five notices of opposition were filed on the grounds of Articles 100(a),(b) and (c) EPC. Opponents 01 and 02 withdrew their opposition when the case was still pending before the opposition division.

II. The patent was revoked by the opposition division for lack of inventive step of the main request (claims as granted) and of auxiliary requests I to XI. The opposition division further found that claims 1(a) and 2 of the main request did not fulfil the requirements of Article 123(2) EPC, whereas claim 1(c) of that request did not meet the requirements of Article 83 EPC.

III. An appeal against the decision of the opposition division was lodged by the patentee (appellant). The main request and auxiliary requests I to XI before the opposition division were filed again with the statement of grounds of appeal on 30 October 2002.

IV. Opponents 03, 04 and 05 (respondents I, II and III, respectively) filed observations on the appellant's statement of grounds of appeal.

V. The parties were summoned to oral proceedings and, in a communication pursuant to Article 11(1) of the Rules of

Procedure of the Boards of Appeal, they were informed of the board's preliminary opinion on the case.

VI. Oral proceedings took place on 7 December 2004.

VII. Claim 1 of the main request (claims as granted) for all designated Contracting States except GR and ES read as follows:

"1. A DNA sequence encoding a fungal phytase which catalyses the liberation of at least one inorganic phosphate from a myoinositol phosphate, said DNA sequence being selected from the group consisting of

(a) DNA sequences comprising a nucleotide sequence encoding the amino acid sequence as depicted in Figure 8 from position -23 to 444, or from position +1 to 444;
(b) DNA sequences comprising the nucleotide sequence as depicted in Figure 6 or Figure 8; and
(c) DNA sequences hybridizing at low stringency conditions (6 x SSC; 50° C; overnight; washing in 6 x SSC at room temperature) with a DNA fragment corresponding to a cDNA of the nucleotide sequence depicted in Figure 6 from position 210 to 1129."

VIII. Claim 1 for the designated Contracting States GR and ES was directed to a process for the preparation of a DNA sequence as defined in claim 1 above, wherein said process further comprised:

"(d) isolating RNA from suitable source, preparing the corresponding mRNA, constructing a cDNA library; or
(e) isolating genomic DNA from a suitable source, constructing a genomic DNA library; and

(f) screening for and isolating the desired DNA."

IX. Claim 1 of all auxiliary requests in the two versions was similarly formulated by reference to the specific sequence as depicted in Figure 8.

X. The following documents are referred to in the present decision:

D4: A.H.J. Ullah and D.M. Gibson, Preparative Biochemistry, 1987, Vol. 17(1), pages 63 to 91;

D8: A.H.J. Ullah, Preparative Biochemistry, 1988, Vol. 18(4), pages 459 to 471;

D9: D.M. Gibson et al., Proceedings UJNR Protein Resources Panel 16th Annual Meeting, Tsukuba, Japan, October 13 to 17, 1987, pages 27 to 39;

D10: Affidavit of R.M. Berka and S.A. Thompson, 6 September 1994;

D12: J. Sambrook et al., "Molecular Cloning. A Laboratory Manual", 2nd Edition, 1989, Cold Spring Harbor Laboratory Press, Section 11;

D14: C.S. Piddington et al., Gene, 1993, Vol. 133, pages 55 to 62;

D26: Affidavit of Dr J. Rambosek, 18 November 1999;

D28: EP-A-0 215 594 (publication date 25 March 1987);

D34: E.J. Mullaney et al., Appl. Microbiol. Biotechnol., 1991, Vol. 35, pages 611 to 614;

D48: Affidavit of Dr G.C.M. Selten, 28 August 1997;

D49: WO-A-94/03612 (publication date 17 February 1994);

D68: W.I. Wood, Methods in Enzymology, 1987, Vol. 152, pages 443 to 447;

D81: I.E. Mattern et al., Fungal Genetics Newsletter, 1988, Vol. 35, page 25.

XI. The arguments of the appellant in writing and during oral proceedings, insofar as they are relevant to the present decision, may be summarized as follows:

Article 56 EPC

Document D9, the closest prior art, referred to the purification and characterization of a phytase derived from *Aspergillus ficuum*. The document disclosed the amino acid composition of this phytase – based on about 600 residues - as well as the amino acid sequence of the N-terminal peptide and of three internal peptides obtained by cyanogen bromide (CNBr) cleavage. A first approach for cloning the phytase gene based on antibody probes and immunoscreeening of an expression genomic library was said to have failed. Presumptive positive clones were reported for a second cloning approach using an oligonucleotide probe most probably based on peptide II (lowest degree of degeneracy). However, a second independent tool - the antibody probes – was still necessary for selecting the positive clones.

Starting from this closest prior art, the technical problem underlying the patent in suit was seen as the provision of a nucleic acid sequence encoding the phytase from *Aspergillus ficuum*.

The use of a single oligonucleotide probe was not selective enough for a successful isolation of positive phytase clones as shown by the affidavit of Dr Selten (document D48). There was no evidence for the presence of a single band in Southern blot and, since the target sequence was unknown, the relevance of such a band could only be assessed with hindsight. As stated in document D9, a second independent tool was required. However, the second tool proposed in document D9 – the antibody probes - failed in the first cloning approach and the said probes were useless in the second strategy since they were polyclonal antibodies raised against a mixture of proteins and not against homogeneous phytase. The production of specific anti-phytase antibody probes required first to set up a new purification method for isolating homogeneous phytase as done in the patent in suit. Thus, as a second independent tool for cloning the phytase gene, a second independent, non-overlapping oligonucleotide probe was necessary. The two-probe approach was taught in the most up-to-date laboratory handbook (document D12) and corroborated by several declarations on file. This was in fact the cloning approach that would have been taken by the skilled person as defined in the case law (a cautions bench molecular biologist). However, oligonucleotide probes based on peptides III and IV of document D9 (the only ones available since the N-terminal was said not to be useful for generating a probe) were bound to fail since

both peptides were contaminants not derived from phytase. Document D14 as well as the affidavit of Dr. Rambosek (document D26) relied on a single probe approach for cloning the phytase gene but they were contradictory in the probe used (17-mer or 29-mer) and dubious when looked at in the light of the later document D49 from the same authors that used a completely different (nested-PCR) cloning approach. Similarly, the affidavit of R.M. Berka and S.A. Thompson (document D10) did not provide any valid experimental evidence. All relevant evidence relied on the nucleic and amino acid sequences disclosed in the patent in suit and thus, they could not avoid hindsight. In contrast, the patent in suit disclosed and used three different probes for screening the genomic library as well as a monoclonal antibody specific for the *A. ficuum* phytase enzyme. These tools were not available to the authors of document D9 and they were missing in the prior art.

Even if, for the sake of argument, positive clones were to be identified, the criteria for selecting and isolating those positive clones – the criteria for success - were completely misleading and wrong. As shown for the soybean phytase in document D9, once positive clones were identified, they were sized and sequenced. However, for the phytase gene derived from *A. ficuum*, the size of the gene would have been wrongly expected to correspond to a gene encoding a protein of about 600 residues. The nucleic acid inserts of positive clones would also have been wrongly expected to encode a protein with phytase activity comprising all four peptides disclosed in document D9, including the contaminant peptides III and IV. Thus, clones

fulfilling the expected criteria for success could never be isolated.

Document D9 did not disclose a host system suitable for expression of the phytase gene. In fact, the skilled person would not have used an expression system for verifying the cloning of the phytase gene. Firstly, clones tested in such expression systems had to be first sized and sequenced (restriction mapping, sequence analysis, length of open-reading frame, etc.) since expression analysis was only feasible if the complete coding sequence and adjacent regulatory sequences were available. Secondly, *Escherichia coli* was not a useful expression system since it was not capable of glycosylating proteins (necessary for phytase activity) and recognizing fungal regulatory sequences (promoter, introns). Thirdly, most fungal organisms suitable for expression analysis, such as the ones indicated in document D9, contained endogenous phytase. Thus, they were not suitable for detecting the activity of a recombinant phytase since several uncertainties could arise (upregulation or activation of endogenous gene, integration into the genome and strong expression variability, etc.) and there was no tool (eg. specific monoclonal antibodies) available to distinguish the endogenous phytase from the recombinant one. There was no reference in document D9 to suitable expression systems for overcoming these uncertainties, such as *Aspergillus* host cells deficient in phytase activity, or to the cloning of fragments to be tested with a promoter regulated in a different way than the native phytase promoter. The analysis for phytase activity was not a quick-and-easy method but a laborious, time-consuming approach the outcome of which

was uncertain and which involved a lot of risks and difficulties.

The authors of document D9 had not been able to obtain the phytase gene from the "presumptive positive clones" referred to in that document given the fact that document D34, from the same authors and published four years later, described the isolation of a clone containing only part of the phytase gene (expected to encode a protein of about 600 residues) and using an antibody screening approach similar to the first cloning approach said to have failed in document D9. There was no publication showing the sequence of the "presumptive positive clones" identified by the second cloning strategy of document D9.

XII. The respondents' arguments in writing and during oral proceedings, insofar as they are relevant to the present decision, may be summarized as follows:

Article 56 EPC

Document D9, the closest prior art, identified the source of the phytase gene, Aspergillus ficuum NRRL 3135, and referred to the purification, structural properties and enzymatic activity of the phytase enzyme. The amino acid composition of this enzyme and the partial amino acid sequences of peptides I to IV were disclosed. Document D9 referred to two strategies for cloning the phytase gene. A first cloning strategy based on antibody probes and immunoscreening, which was said to have failed, and a second cloning strategy based on the use of an oligonucleotide probe for

screening. Presumptive positive results were reported for this second strategy.

Hence, the technical problem underlying the patent in suit was seen as the provision of the full-length nucleic acid sequence encoding the phytase derived from *A. ficuum*, i.e. the phytase gene.

At the priority date, the preferred method for isolating and cloning a gene encoding a purified protein was the use of an oligonucleotide probe, usually about 15 to 20 nucleotides long. As shown in handbooks on file (*inter alia* document D12), the second-probe approach was advantageously used only for genomic libraries of great size (such as mammalian) but not necessarily for fungi libraries. Two steps were, however, critical: (i) the quality of the probe for screening the library and identifying the positive clones, and (ii) the selection or validation of the positive clones, which could be difficult and complex if the properties of the gene were unknown.

Document D9 referred to a single band in Southern blot demonstrating that the probe used was specific. The document further referred to presumptive positive clones obtained after several screenings. Thus, there was no need to use a second oligonucleotide probe. Due to the exceptionally low degeneracy of peptide II, this peptide was the most suitable candidate for synthesizing a probe. There was evidence on file showing that the one-probe approach was successfully applied with both 17-mer and 29-mer probes derived from peptide II, *inter alia* document D14 and the affidavits of R.M. Berka and S.A. Thompson and of Dr Rambosek

(documents D10 and D26, respectively). The results shown in the affidavit of Dr Selten (document D48) were not reliable since the experiment was intended to fail. Moreover, the results shown in Figure 1 (17-mer probe based on peptide II) and Figure 3 (probe comprising the complete phytase gene) were inconsistent.

Document D9 referred to the possible selection of the identified presumptive positive clones by antibody probes, such as the ones successfully used in document D34. This selection step was different from the screening step used in the first cloning strategy and therefore, the negative results obtained in the first strategy were not relevant for the selection step in the second cloning strategy. In fact, the said antibody probes were not even necessary since the positive clones could be sized and sequenced, or alternatively, used in a straightforward manner in expression system assays and known activity tests.

As regards the criteria for success, the length of the expected coding region (about 600 residues) was not a reliable criterion for the size of possible positive clones since the true molecular weight of glycosylated proteins was known not to be simple to assess with accuracy. Moreover, genes from genomic libraries were also expected to comprise non-coding regions such as introns. The absence of peptides III and IV in the deduced amino acid sequence was irrelevant once the complete sequence and the open reading frame of the phytase gene were determined and known. In fact, due to the evident low quality of these two peptides III and IV, the presence of the N-terminal peptide and peptide

II in the complete sequence of the phytase gene were the most reliable criteria.

It was also known (cf. *inter alia* document D12) that for an enzyme the most reliable identification was the presence of its enzymatic activity. Thus, it was obvious for the skilled person to express the positive clones. There was no reason to expect any problems when expressing the phytase gene in eukaryotic cells and, more particularly, when using *Aspergillus* cells as host cells (so as to obtain an advantageous glycosylation, recognition of fungi promoter, etc.), as also proposed in document D9 and shown in document D10.

The reasons for failing to report the nucleic acid sequences of the "presumptive positive clones" of document D9 were unknown and only speculations could be made about it. Therefore, this failure could not be taken into account when assessing the inventive step in the light of document D9.

XIII. The appellant (patentee) requested that the decision under appeal be set aside and that the patent be maintained as granted or, in the alternative, on the basis of one of the Auxiliary requests I to XI filed with the statement of grounds of appeal on 30 October 2002, all in two versions for all the designated Contracting States except ES and GR, and for ES and GR.

XIV. The respondents (opponents) requested that the appeal be dismissed.

Reasons for the Decision

Procedural matters

1. Common to all requests on file are claims centred on DNA sequences comprising a specific nucleotide sequence depicted in Figure 8 or a nucleotide sequence encoding the specific amino acid sequence depicted in Figure 8. In view of this, the board found it expedient to deal with the key question whether said specific sequences involve an inventive step in the light of the prior art, and to leave aside the issue of compliance of the requests with Articles 123 and 83 EPC.

Inventive step - Article 56 EPC
Closest prior art

2. Document D9, considered to be the closest prior art, discloses an *Aspergillus* isolate, namely *A. ficuum* NRRL 1335, which is a high producer of the phytase enzyme - 10 times greater levels of activity than other isolates (cf. page 29, last full paragraph). Three different chromatographic columns are used for purifying the phytase enzyme, which shows two broadly diffused bands of 85 KDa and 100 kDa on SDS-PAGE. The purified phytase is a glycoprotein, which, upon partial deglycosylation, has a molecular weight of about 76 kDa (cf. page 30). The amino acid composition of the purified enzyme (cf. page 37) and the amino acid sequences of four peptides – the N-terminal peptide (peptide I) and three internal peptides (peptides II, III and IV) – are also disclosed (cf. page 38). The document further refers to the kinetic characterization of the purified phytase (cf.

page 30, last two paragraphs and paragraph bridging pages 30 to 31).

3. Two cloning strategies for the fungal phytase are outlined in document D9. A first strategy is based on the immunoscreening of a genomic library, whereas the second strategy is based on an oligonucleotide probe for screening. For the immunoscreening, polyclonal antibodies are first produced and shown to react with all forms - native and deglycosylated - of the phytase enzyme. These polyclonal antibodies are purified, so as to remove any possible immunological reactivity with E. coli, and used to screen a lambda gt11 genomic library. The average size of the *A. ficuum* DNA insert is 7 Kb and, if in proper reading frame, the insert ultimately produces a fusion protein with the beta-galactosidase encoded by the *E. coli* lacZ gene present in the lambda gt11 library. Document D9 states that "*although multiple attempts have been made to use the antibody probes to pull out the phytase gene, we have been unsuccessful at the present time*" and that "*another strategy for making antibodies is in progress*". In this context, reference is made to the growth of *Aspergillus* in presence of glycosylation inhibitors and production of antibodies against deglycosylated and denaturated phytase (cf. paragraph bridging pages 31 to 32).

4. With regard to the second cloning strategy, the N-terminal peptide (peptide I) is said not to be useful for generating an oligonucleotide probe. One of the other three internal peptides is identified as having a useful amino acid sequence for generating a probe. Neither the peptide nor the probe derived therefrom are specified in terms of the structure (cf. however

point 8 *infra*). Preliminary results using the said probe show that it binds to a restriction fragment from genomic DNA on Southern blots and that "*presumptive positive clones have been selected following several screenings*" of a lambda EMBL4 library with this radiolabelled probe. Document D9 states that "*the antibody probes will be used to select from these positive clones. Using both the antibody and the oligonucleotide probes, we should be able to unequivocally identify the phytase gene from Aspergillus ficuum*" (cf. page 32, first two paragraphs). Reference is also made to transformation systems for *Aspergillus ficuum* so as to "*achieve enhanced secretion of phytase*" as well as to the cotransformation of *A. ficuum*, wherein "*the second gene ... in our case will be phytase ... after we have verified the cloning of this gene*" (cf. page 33, last full paragraph).

Technical problem underlying the patent in suit

5. Starting from the closest prior art, the technical problem to be solved is regarded as being the provision of the complete genomic nucleotide sequence encoding the phytase enzyme derived from *Aspergillus ficuum* NRRL 31335, i.e. the phytase gene. The solution is provided by the specific nucleotide sequence depicted in Figure 6, with the coding sequence and derived amino acid sequence shown in Figure 8, of the patent in suit, these sequences being the term of reference used in claim 1 of all requests in order to define the invention (cf. Sections VII to IX *supra*).

6. In the board's view, it would have been obvious for the skilled person to try to follow the teachings of

document D9 in order to solve said problem, attain similar positive clones and select the ones comprising the phytase gene. In doing so, no particular problems would have been expected and, in the light of document D9 alone, a reasonable expectation of success was fully justified. The appellant, however, has referred to several difficulties and problems, which, allegedly, would have been encountered by the skilled person when attempting to follow the teachings of document D9 and which would have prevented him or her from obtaining the phytase gene. Those problems are examined hereinafter in order to establish whether they would have indeed had an impact on the expectation of success by the skilled person (cf. T 207/94, OJ EPO 1999, 273).

Alleged technical difficulties

The selection of the oligonucleotide probe: one-probe or two-probe cloning approach?

7. The appellant submits that, starting from document D9 and in the light of common general knowledge, the skilled person would not have considered the one-probe cloning approach since the two-probe cloning approach was generally seen as being more advantageous for screening genomic libraries. However, in their view, for the construction of a second probe, there was not enough reliable information available in document D9 (cf. Section XI *supra*).

8. According to document D9, the amino acid sequence of the N-terminal peptide is not useful for generating a probe, whereas "***one*** *peptide was identified as having a useful amino acid sequence for generating an*

oligonucleotide probe" (cf. page 32, first full paragraph). Although the "one" peptide is not expressly identified in structural terms, it is beyond dispute that this peptide is peptide II, which, due to its exceptionally low degeneracy, has been described by all parties as a "molecular biologist's dream".

9. The length of the probe, however, is not disclosed in document D9. From the prior art on file, which reflects the common general knowledge at the time of the invention (cf. *inter alia* documents D12 and D68), short probes are typically 11-20 nucleotides in length (pools of 8-32 or more degenerate sequences). Probes of 17 nucleotides or longer are used to screen high-complexity (mammalian) genomic libraries. For longer probes, beyond 20 and up to 30 bases (the shortest length for a long probe), the degeneracy of the pool must be as low as possible so as to avoid unspecific hybridization (cf. pages 11.7 and 11.8, document D12 and page 443, document D68). The stretch of amino acid residues of peptide II having an exceptionally low degeneracy extends to the first 9-10 residues. Only an Ala residue at position 6 has a high degeneracy. Other neighbouring residues are not to be considered since their degeneracy is too high. Thus, the length for a probe based on peptide II varies from a short 17-mer (pool of 8 degenerate sequences), based on the first 5-6 residues without the Ala at position 6, to a long 29-mer based on the complete stretch of 9-10 residues. For this longer probe, following the recommendations of the prior art, the high degeneracy of the Ala residue might be reduced by using a neutral base such as inosine (pool of 64 degenerate sequences) (cf. document D12, page 11.17). The provision of probes

within this specific length range does not require any inventive ingenuity due to the exceptional properties - low degeneracy - of peptide II disclosed in document D9.

10. In principle, the number of unspecific hybridizations increases with the complexity of the targeted library and, the longer a probe the more likely it is to be unique among all the sequences present in a targeted genome library. Thus, when possible, it is useful to determine first the specificity of the probe by using, for example, the probe in a Southern blot. In document D9 such a determination results in the probe binding to "***a** restriction fragment from genomic DNA on Southern blots*", i.e. to a single genomic fragment. This shows that the probe is specific. In fact, Southern blots might be performed under several stringency conditions so as to select those probes that are specific under high stringency conditions. This allows the use of these stringency conditions in the screening of the genomic library and the achievement of a higher specificity (cf. page 446, last paragraph, document D68).

11. There is evidence on file showing that a 17-mer (PHY-1) used to probe a genomic DNA from *Aspergillus* under high stringency conditions results indeed in a unique band (cf. *inter alia* page 56, right-hand column, first full paragraph and Figure 1 of document D14, cited as expert opinion). Similar results are also disclosed for longer probes, in particular a 29-mer (PHY-2) probe (cf. points 3.1 and 3.2 of the affidavit of Dr J. Rambosek, document D26, cited as expert opinion). Thus, in the board's judgment, following the teachings of document D9 in combination with the common general knowledge, no

particular skill is considered to be required for selecting an oligonucleotide probe (based on peptide II) with a suitable length - within the range indicated above (cf. point 9 *supra*) – for achieving a high specificity under high stringency conditions.

12. It might well be, as argued by the appellant (cf. point XI *supra*), that under certain conditions the use of a two-probe cloning strategy is more advantageous. However, in the light of the unique properties – low degeneracy and high specificity - of **the** probe referred to in document D9, this strategy does not necessarily come into consideration in the present case. There is no indication in document D9 as to the need for a second probe and thus, the only cloning strategy considered is the one-probe cloning strategy.

Aspergillus genomic library and screening of this library

13. The appellant submits, based in particular on the affidavit of Dr G.C.M. Selten (document D48), that the use of only one-probe would not have been selective enough for screening a genomic library of Aspergillus with success (cf. Section XI *supra*).

14. Document D9 refers to the construction of a lambda EMBL4 genomic library of *Aspergillus ficuum* which is screened with the radiolabelled oligonucleotide probe and it further states that *"presumptive positive clones have been selected following **several** screenings"*, i.e. the lambda EMBL4 genomic library is screened several times with the specific oligonucleotide probe so as to eliminate unspecific hybridizations and identify the positive clones. There is also evidence on file showing

that, in a single screening step, few positive clones are identified using a 17-mer (PHY-1) probe (cf. point 2 of the affidavit of R.M. Berka and S.A. Thompson, document D10 and paragraph bridging pages 56 to 57, document D14) or a 29-mer (PHY-2) probe (cf. point 3.3 of the affidavit of Dr J. Rambosek, document D26) (both documents cited as expert opinions).

15. Different results are, however, disclosed in the affidavit of Dr G.C.M. Selten (document D48, relied upon by the appellant as expert opinion), which, repeating the conditions indicated in the affidavit of R.M. Berka and S.A. Thompson (document D10) and using a 17-mer probe 100% complementary to the phytase gene or a pool of 8 degenerate 17-mer probes, shows a high background and a low resolution in a (single) screening of an *A. ficuum* genomic library. Nevertheless, the conditions used therein are said to be of "*very low hybridization stringency*". In the board's view, similar poor results obtained in an initial Southern blot (cf. point 10 *supra*) would have prompted the skilled person to use higher stringency conditions and/or a longer oligonucleotide (29-mer) probe so as to perform the "***several*** *screenings*" indicated in document D9. Thus, the skilled person could easily overcome the alleged technical problem indicated in the affidavit of Dr G.C.M. Selten (document D48) using only common general knowledge. Therefore, this alleged technical problem is not considered to be relevant.

Selection of the identified "presumptive positive clones"

16. The appellant submits that, as shown for the first cloning approach disclosed in document D9, the antibody

probes would not have been useful in the second cloning approach. The production of useful antibody probes required first to set up a new purification method for isolating homogenous phytase (cf. Section XI *supra*).

17. Document D9 indicates that "*the antibody probes will be used to select from these positive clones*" (cf. page 32, second full paragraph). Thus, although antibody probes are said to have failed in the first cloning strategy based on immunoscreening, they are, however, expected by the authors to be useful in the second cloning strategy for further selecting (not screening) the "*presumptive positive clones*" identified by "*several screenings*" with the oligonucleotide probe. The positive expectations expressed can possibly be explained by the fact that the skilled person based on common general knowledge foresaw some benefits resulting from the elimination of other genes coding for cross-reactive proteins (such as phosphatases), from an improved titre of the polyclonal antiserum, from a lower contamination by neighbouring phage particles, etc.

18. Moreover, whereas the lambda EMBL4 library has some disadvantages in comparison to the lambda gt11 library (such as the absence of a promoter specific for expression in *E. coli*), it has, however, other important advantages. In particular, it is known that the average insert size in this lambda EMBL4 library is about 9-20 kb (cf. point 2 of the affidavit of R.M. Berka and S.A. Thompson, document D10), which is higher than the one in the expression lambda gt11 library (7 kb) (cf. page 31, second full paragraph, document D9). Thus, the lambda EMBL4 genomic library

has a higher probability of having an insert which comprises the complete phytase gene, including the target sequence to be hybridized with the oligonucleotide probe, i.e. it has a higher probability of cloning the complete gene with associated regulatory sequences which (if functional in *E. coli*) results in the expression of all protein epitopes. Furthermore, proteins expressed from this lambda EMBL4 library are not fused with an *E. coli* protein (beta-galactosidase) that may in certain cases destabilize and modify native epitopes. All these considerations justify the positive expectations conferred by document D9.

19. Document D34 (cited as expert opinion) also confirms that the polyclonal antibody probes raised against the purified phytase – following the same purification method as in document D9 – and isolated as described in document D9 (cf. document D34 from page 611, right-hand column, last full paragraph to page 612, left-hand column, first full paragraph and page 612, left-hand column, fourth full paragraph), are specific enough to detect the phytase (as expected by the skilled person when reading document D9), if expression of the phytase gene actually occurs.

20. In spite of all these positive considerations, the skilled person is well aware that the promoter of the phytase gene might not be functional in *E. coli* and that problems associated with the processing of fungi introns in *E. coli* might also arise (a drawback shared by both lambda gt11 and EMBL4 genomic libraries). Thus, the skilled person knows that the possible absence of positive results in the selection of clones by antibody probes (as proposed in document D9) does not directly

indicate the absence of clones comprising the phytase gene. In that case, a straightforward selection of the identified positive clones by expression of the phytase gene in fungal host cells constitutes an obvious consideration for the skilled person as in fact such fungal expression systems are explicitly referred to in document D9 itself (cf. pages 32 and 33, document D9).

Expression of the phytase gene in fungi host cells

21. The appellant submits that no suitable host systems were known to the skilled person since *Escherichia coli* was fully inappropriate and fungal organisms contained endogenous phytase, which was not to be distinguished from the recombinant phytase. Thus, for a suitable expression system, it was necessary first to obtain *Aspergillus* host cells deficient in phytase activity (cf. Section XI *supra*).

22. Document D9 refers to the development of two selectable (gene) marker systems for transformation of *A. ficuum* which allow the cotransformation with a second plasmid comprising a second gene, such as the phytase gene (cf. pages 32 to 33, document D9). References to appropriate fungal plasmids, which allow the introduction of (large) nucleotide inserts, are on file (cf. *inter alia* documents D28 and D81) and, if necessary, methods for sizing the cloning insert were also known in the prior art (cf. points 26 to 27 *infra*).

23. As suggested in document D9, the advantages of (over)expressing the phytase gene in fungal host cells, particularly in *A. ficuum* NRRL 3135 the source of the phytase gene (functional promoter, properly processed

introns and secretion, etc.), compensate for the possible hypothetical problems referred to by the appellant, in particular the inability to distinguish the recombinant phytase from the endogenous one (cf. Section XI *supra*). Moreover, whereas *A. ficuum* NRRL 3135 is known to be a high phytase producer (cf. point 2 *supra*) and the development of phytase-deficient fungi might well be advantageous, other available taxonomically related fungi (*A. niger*) with lower phytase production and known transformation systems, would have been taken into consideration by the skilled person as suitable host cells too.

24. The board further notes, that none of the hypothetical problems referred to by the appellant has actually been found in the patent in suit, which also exemplifies the straightforward overexpression of the phytase gene in *A. ficumm* host cells (cf. Table 4, phytase production of untransformed and transformed *A. ficuum* NRRL 3135).

Sizing and sequencing of positive clones

25. The appellant submits that the criteria to be used, when sizing and sequencing positive clones, for identifying those inserts comprising the phytase gene, were misleading. In particular, the appellant refers to the expectation of a larger molecular weight for the phytase and to the absence of peptides III and IV in the sequence of the phytase enzyme. These difficulties, in its view, lower the expectations of the skilled person and support an inventive step. Moreover, the fact that the authors of document D9 never arrived at the complete sequence of the phytase gene by following the allegedly promising second cloning approach

discussed in document D9 but by other routes, shows, in the appellant's view, that the said strategy was not feasible and could not give raise to any positive expectations (cf. Section XI *supra*).

26. In principle, the selection of positive clones by expression of the phytase gene in fungi hosts (cf. points 22 to 24 *supra*) does not necessarily require the sizing and sequencing of the cloning inserts, since vectors accepting large inserts – the minimal average size of lambda EMBL4 inserts - are available in the art (cf. point 22 *supra*). Large size inserts are also usually preferred due to the possible presence of introns and the advantageous presence of regulatory regions in the lambda EMBL4 genomic library of *Aspergillus*. If desired, however, sizing by digestion of cloning inserts by restriction endonucleases and probing of the fragments with the oligonucleotide probe specific for the phytase gene are both known routine steps (cf. point 10 *supra*).

27. The sizing and sequencing of the cloning inserts may be carried out either directly on the positive clones identified in the initial screening with the specific oligonucleotide probe (cf. point 14 *supra*), as shown in document D9 for the soybean phytase, or else after the selection of positive clones by immunoscreening or expression in fungi host cells. As stated above, however, the digestion with restriction endonucleases, probing with the specific oligonucleotide probe, subcloning and determination of the nucleotide sequence, identification of introns, etc. although laborious and time-consuming, are all known routine steps.

28. With regard to the appellant's first argument, the difficulties to assess appropriately, in a reliable and accurate manner, the molecular weight of glycosylated proteins are well-known in the prior art. Document D9 refers to diffused SDS-PAGE bands (poorly focused banding pattern) of 85 and 100 kDa for the glycosylated phytase, whereas a molecular weight of 76 kDa is indicated for a partially deglycosylated enzyme (cf. page 30, fourth full paragraph). A much lower molecular weight – of about 61.7 kDa – is disclosed for an unglycosylated enzyme in document D8 (cf. page 463, Table II). In view of this variability in particular in case of glycosylated molecules, molecular weight values are to be considered cautiously and they certainly cannot be relied on as a strong criterion of success. In other words, in the present case the skilled person, in the board's view, is open-minded as to the actual value of the molecular weight of the phytase enzyme. Thus, contrary to appellant's allegation, molecular weight considerations do not have an influence on the skilled person's expectations, when applying the second cloning strategy indicated in document D9.

29. With regard to appellant's second argument, although document D9 states that the phytase is purified to homogeneity, the presence of other possible protein contaminants is not excluded by the skilled person. In fact, document D9 relies on the method of purification disclosed in document D4, which shows that further purification of the phytase enzyme by an additional chromatographic step is still possible (cf. page 74, last paragraph, document D4). Figure 1C of this document also shows that the 4-step purified enzyme is obtained from overlapping protein peaks (cf. page 73)

and thus, the presence of protein contaminants is not excluded. Actually, judging from the length of the peptides disclosed in document D9 and the number of gaps present in their sequences, the N-terminal peptide I (16 residues) and the peptide II (19 residues) are for the skilled person the most relevant peptides. As stated above, the skilled person would put into practice the second cloning strategy in document D9 essentially based on peptide II with a reasonable expectation of success. Peptides III and IV would **not** have any particular influence on his or her expectations. The absence of these sequence stretches in the then deduced amino acid sequence of the phytase enzyme would in any case be noticed only **after** achieving the result and would easily be attributed to the presumed presence of contaminants in the phytase preparation of document D9.

30. With regard to appellant's third argument, it is true that there is no evidence on file showing that the second cloning approach described in document D9 was successfully pursued by the authors. Indeed, document D34 (cited as expert technical opinion), a publication of the authors of document D9 disclosing only a partial phytase sequence from *A. ficuum* NRRL 3135, is based on the first (failed) cloning approach of document D9, and in document D49 (cited as expert technical opinion), the authors of document D9 followed a different (nested-PCR) cloning strategy for arriving at the phytase gene. The reasons for this are unknown and it can only be speculated about. In any case, inventive step is to be judged based on the actual technical evidence on file and on its substantive merits independently from any possible speculations about a

given occurrence. Thus, the appellant's argument in this respect is irrelevant.

Conclusion

31. In view of the above considerations, it is concluded that the manifest high quality and specificity of the oligonucleotide probe which the skilled person would have readily derived from the peptide II disclosed in document D9, the optimistic view expressed in the latter document as regards the possibility of identifying the phytase gene from *Aspergillus ficum* and the apparent absence of a priori obstacles and/or difficulties would have induced the skilled person to try to pursue the cloning path indicated in document D9 with a reasonable expectation of success. In the board's judgment, by following it the skilled person would have arrived by use of routine measures and without undue difficulties and burden at a DNA sequence encoding phytase falling under the terms of claim 1 of all requests. The fact that "in real life" cloning was then achieved by following different (or partly different) routes is immaterial as long as no valid evidence is put forward that the obvious theoretical protocol derivable from document D9 on the basis of common general knowledge is either unreasonable, given the circumstances, or bound to fail, due to excessive difficulties or burden. This is not the case here.

32. Thus, the subject-matter of claim 1 of all requests in the two versions for all designated Contracting States except GR and ES and for GR and ES lacks an inventive step and thus fails to meet the requirements of Article 56 EPC.

Order

For these reasons it is decided that:

The appeal is dismissed.

The Registrar: The Chairman:

A. Wolinski L. Galligani