ABSTRACT

"TALAROMYCES TRANSFORMANTS"

The invention relates to a Talaromyces transformant comprising one or more recombinant gene, capable of producing cellulase in the absence of cellulase inducer in a glucose medium, having a cellulase activity of 2 WSU/ml or more, in 16 times or more diluted supernatant or broth.

CLAIMS

1. Process for production of a *Talaromyces* transformant comprising the steps of:
   (a) providing one or more expression cassettes capable of producing one or more polypeptides of interest and comprising one or more polynucleotide of interest coding for cellulase, hemicellulase and/or pectinase and at least one promoter for expression of the polynucleotide;
   (b) providing a selection marker included in the expression cassette of (a) or included in a dedicated selection marker polynucleotide;
   (c) transfecting a *Talaromyces* host with the one or more expression cassette from (a) and/or the selection marker from (b);
   (d) selecting a *Talaromyces* transformant which contains one or more polynucleotides encoding cellulase, hemicellulase and/or pectinase and
   (e) isolating the *Talaromyces* transformant.

2. *Talaromyces* transformant comprising one or more recombinant gene, capable of producing cellulase in the absence of cellulase inducer in a glucose medium, having a cellulase activity of 2 WSU/ml or more in 16 times or more diluted supernatant or broth, obtainable according to claim 1.

3. *Talaromyces* transformant according to claim 2, having a cellulase activity of 3 WSU/ml or more, or 5 WSU/ml or more, in 16 times or more diluted supernatant or broth.

4. *Talaromyces* transformant according to any of claims 2 or 3, having an endoglucanase activity of 50 WBCU/ml or more.

5. *Talaromyces* transformants according to any of claims 2 to 4, harbouring two or more genes capable of expressing cellulase.

6. *Talaromyces* transformants according to claim 5, wherein the two or more genes capable of expressing cellulase include cellobiohydrolase, endoglucanase and/or beta-glucosidase gene.

7. *Talaromyces* transformant according to claim 6, wherein the cellobiohydrolyse gene is cellobiohydrolase I and/or cellobiohydrolase II.

8. *Talaromyces* transformant according to any of claims 2 to 7, wherein one of more genes is integrated into the genome of the *Talaromyces* transformant.

9. *Talaromyces* transformant according to any of claims 2 to 8, wherein the *Talaromyces* transformant is marker-free.

10. Process for production of a polypeptide composition comprising one or more cellulases, hemicellulases and/or pectinases comprising the steps of:
(a) providing one or more expression cassettes capable of producing one or more polypeptides of interest and comprising one or more polynucleotide of interest coding for cellulase, hemicellulase and/or pectinase and at least one promoter for expression of the polynucleotide;
(b) providing a selection marker included in the expression cassette of (a) or included in a dedicated selection marker polynucleotide;
(c) transfecting a *Talaromyces* host with the one or more expression cassette from (a) and/or the selection marker from (b);
(d) selecting a *Talaromyces* transformant which contains one or more polynucleotides encoding cellulase, hemicellulase and/or pectinase;
(e) producing the polypeptide composition by culturing the *Talaromyces* transformant in a suitable culture medium in which a cellulase inducer is substantially absent; and
(f) optionally recovering the polypeptide composition.

11. Process according to claim 10, wherein in stap (a) two or more expression cassettes, three or more expression cassettes, or four or more expression cassettes are provided.

12. Process according to claim 10 or 11, wherein the promoter is chosen from from the group consisting of: promoters of *A.niger glaA*, and *A. nidulans gpd* promoters or functional parts thereof optionally preceded by upstream activating sequences.

13. Process according to claim 12, wherein the promoter is *A.niger gla*A promoter or functional parts thereof optionally preceded by upstream activating sequences.

14. Process according to any of claims 10 to 13, wherein the selection marker gene is chosen from the group consisting of: *amdS* (acetamidase), *hygB* (hygromycin phosphotransferase), and *ble* (phleomycin resistance), preferably *ble*.

15. Polypeptide composition produced by the *Talaromyces* transformant of any of claims 2 to 9 and/or by the process according to any of claims 10 to 14.

16. Polypeptide composition according to claim 15, comprising *CBH I*, *CBH II*, *EG* and/or *BG*.

17. Polypeptide composition according to claim 16, comprising *CBH I*, *CBH II*, *EG* and *BG*.

18. Polypeptide composition according to any of claims 15 to 17, comprising a polypeptide chosen from the group including: catalase, laccase, phenoloxidase, oxidase, oxidoreductases, xylanase, peroxidase, lipase, hydrolase, esterase, cutinase, protease and other proteolytic polypeptides, aminopeptidase, carboxypeptidase, phytase, lyase, and other pectinolytic enzymes, amylase, glucoamylase, alpha-galactosidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase, mannosidase, isomerase, invertase, transferase, ribonuclease, chitinase, mutanase and deoxyribonuclease.

19. Process for saccharification of lignocellulosic material, wherein lignocellulosic material that has been optionally pretreated, is contacted with a *Talaromyces*

transformant according to claim any of claims 2 to 9; an polypeptide composition according any one of claims 15 to 18, and wherein one or more sugars are produced.

20. Process for the preparation of a fermentation product, including amino acids, vitamins, pharmaceuticals, animal feed supplements, specialty chemicals, chemical feedstocks, plastics, solvents, fuels, or other organic polymers, lactic acid, ethanol, fuel ethanol or chemicals, plastics, such as for instance succinic acid and (bio) fuels, including ethanol, methanol, butanol, synthetic liquid fuels and biogas, wherein one or more sugar produced according to the process of claim 19, is fermented with a fermenting microorganism, preferably yeast, to produce the fermentation product.

21. Process for production of a *Talaromyces* multiple transformant, wherein in a first transformation according to claim 1, the isolated *Talaromyces* transformant isolated in step (e) of a first transformation is used as *Talaromyces* host and is transformed in a second transformation according to claim 1 and in step (e) of the second transformation a *Talaromyces* multiple transformant is isolated.

22. Process according to claim 21, wherein in the first transformation a different selection marker is used than in the second transformation.

23. Talaromyces transformant having a total cellulase content as determined by APEX of 38% or more, 40% or more, and/or 45% or more.

Dated this 12th day of April, 2012.

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AGENT FOR THE APPLICANTS

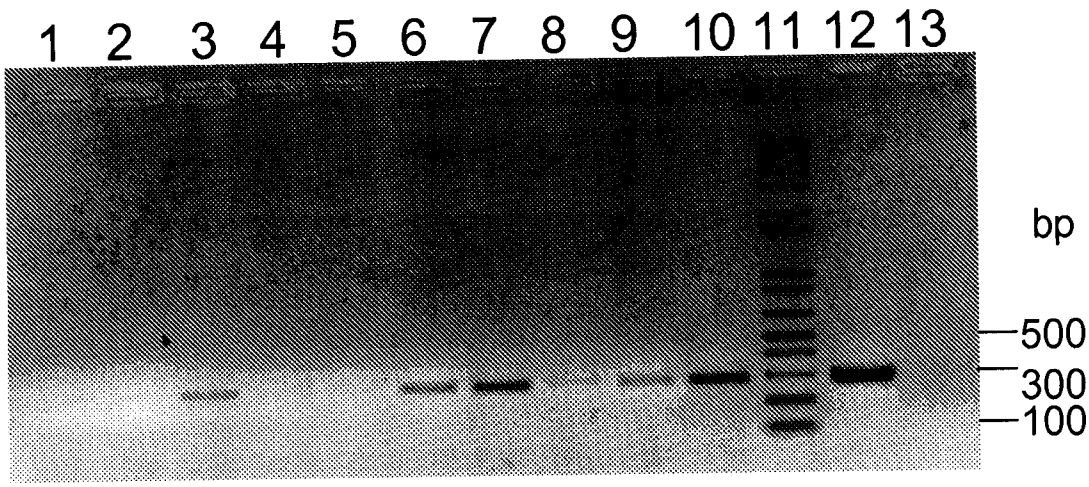

Fig. 1

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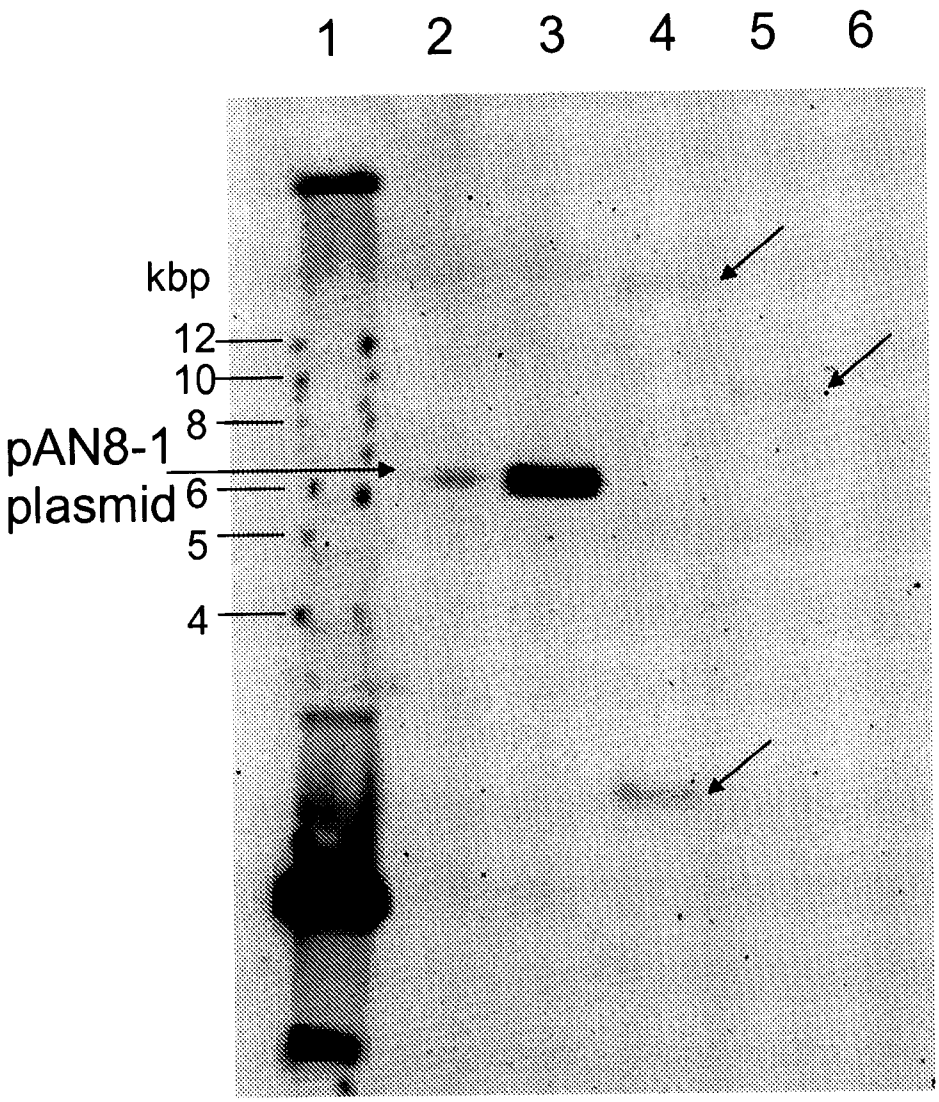

Fig. 2

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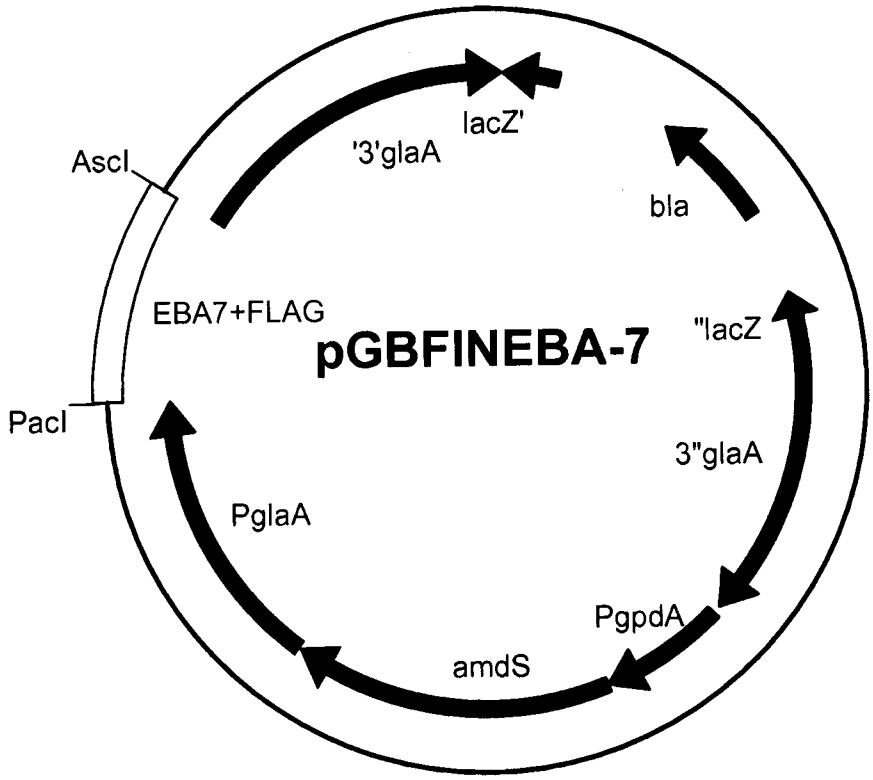

Fig. 3

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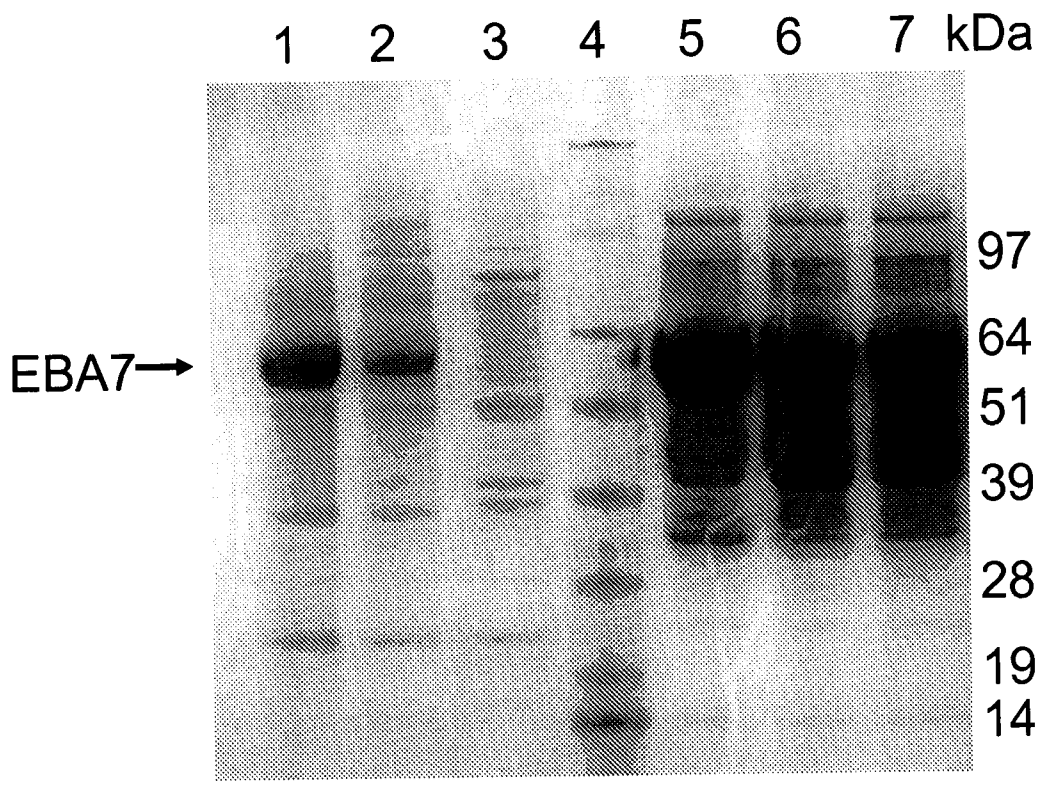

Fig. 4A

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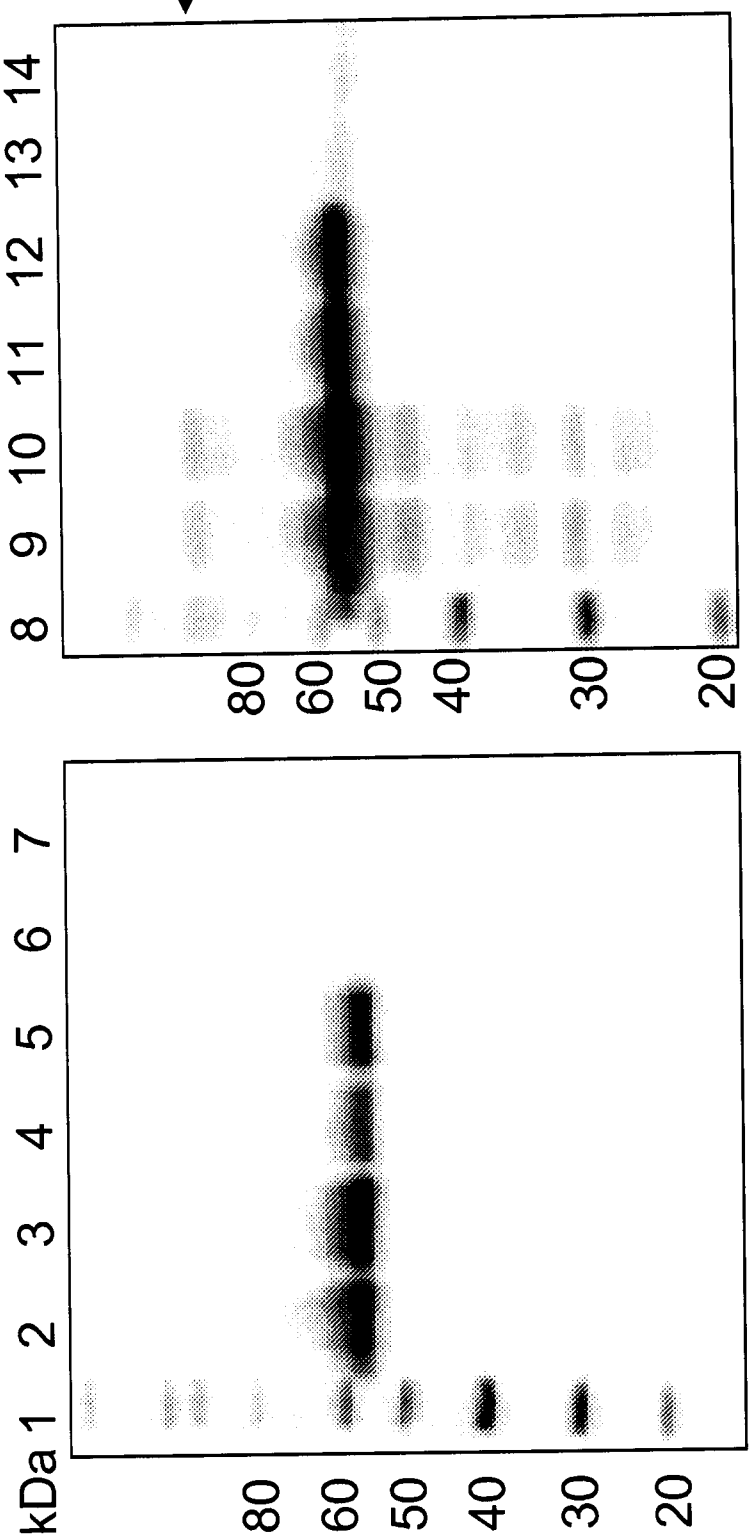

Fig. 4B

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ORIGINAL

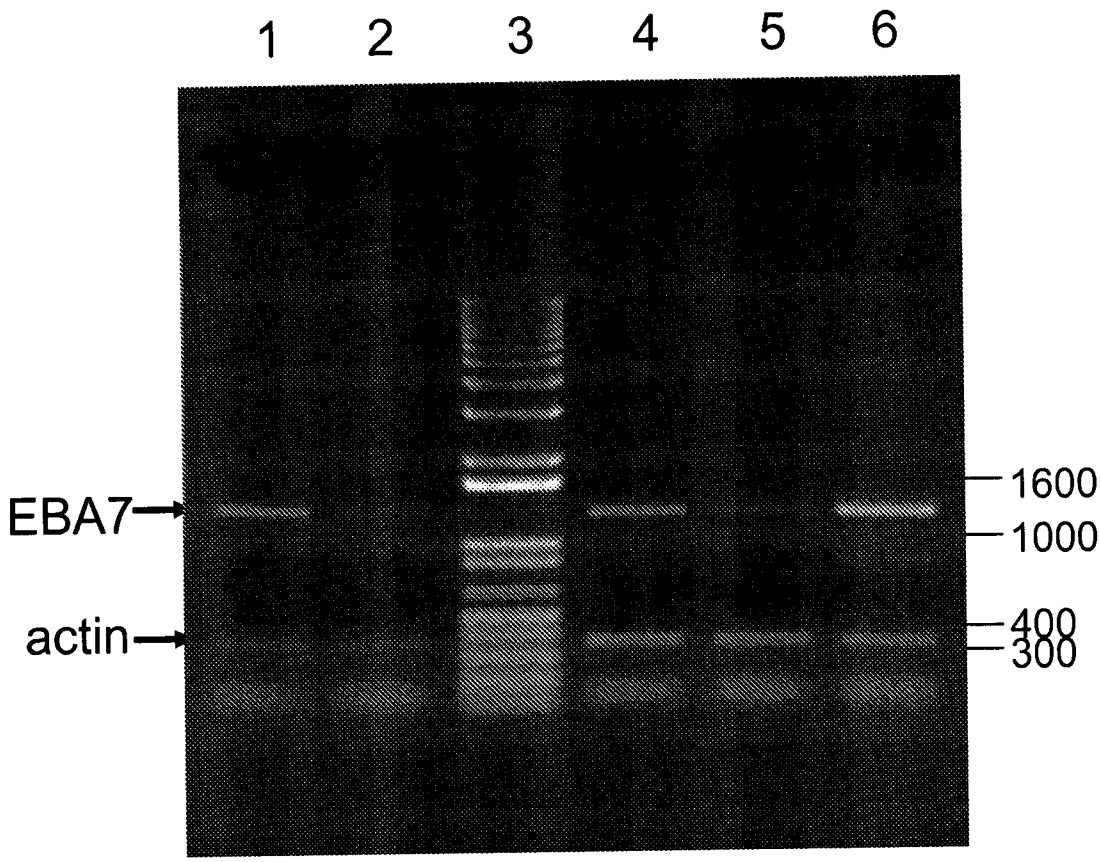

Fig. 4C

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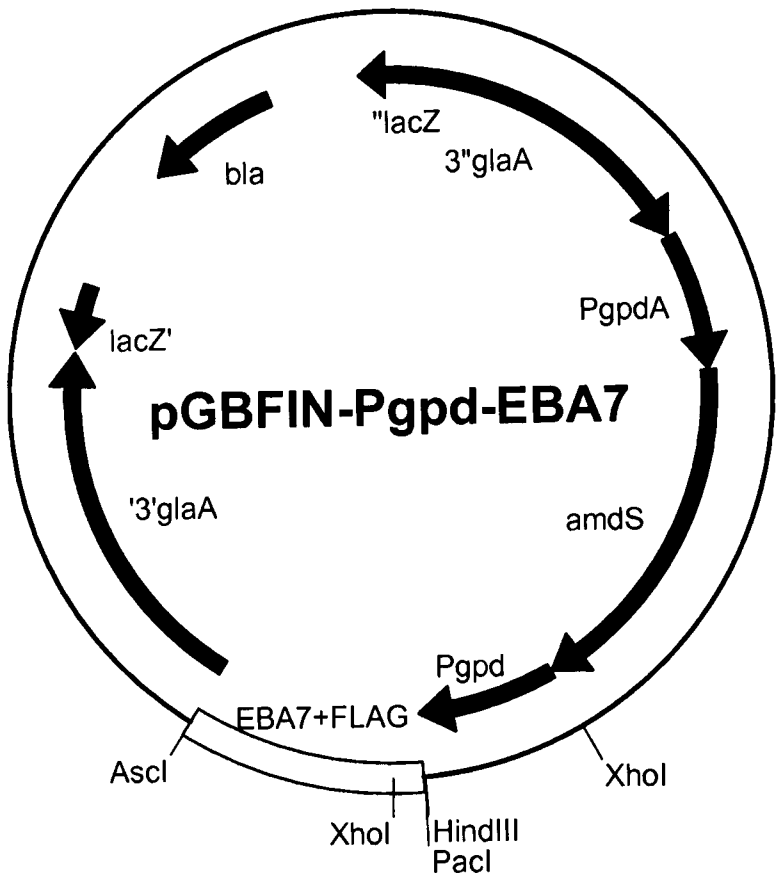

Fig. 5

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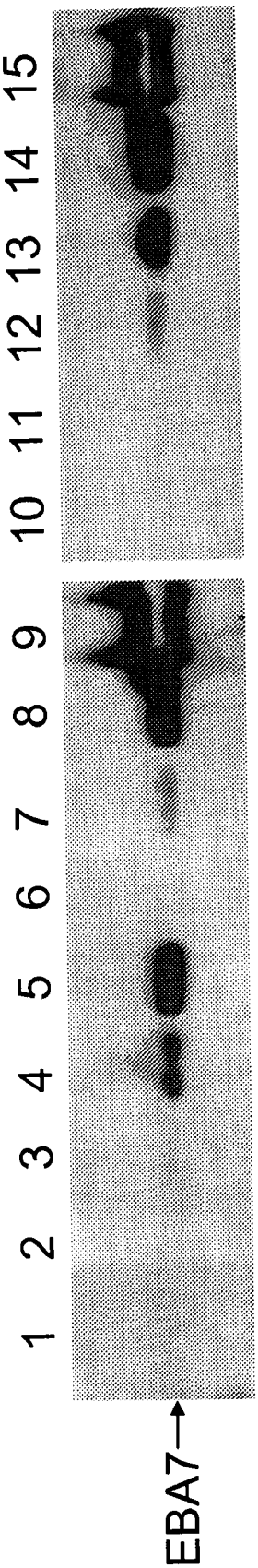

Fig 6

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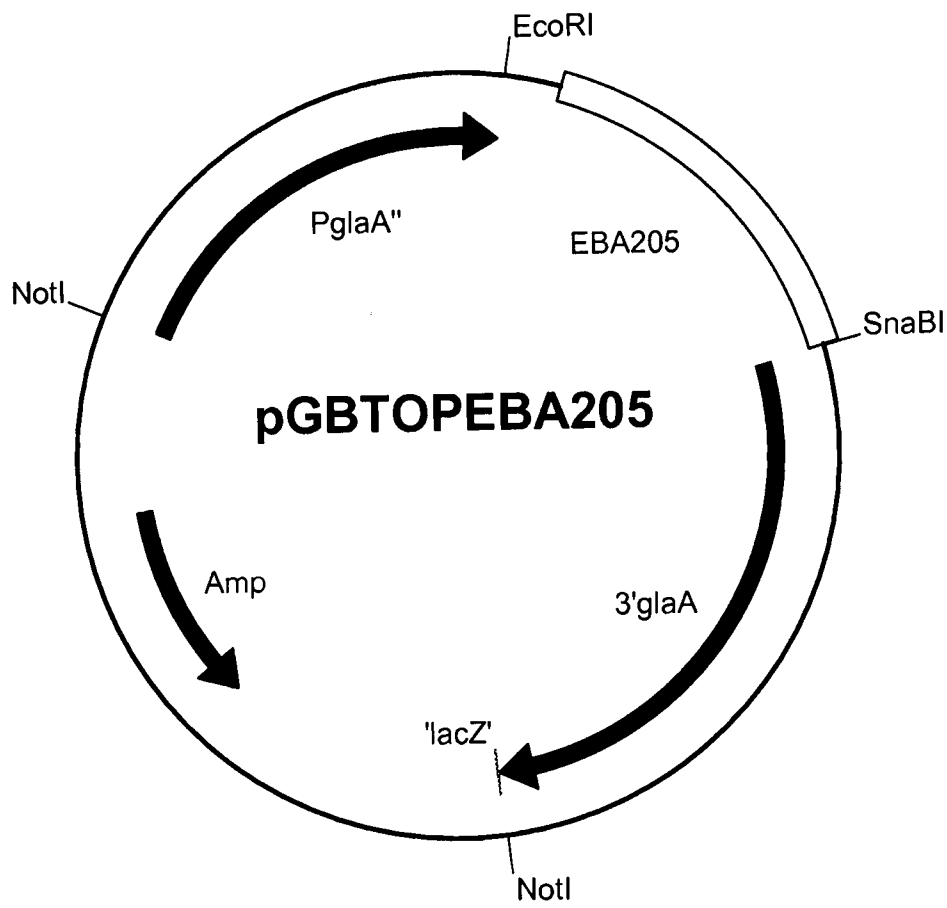

Fig. 7

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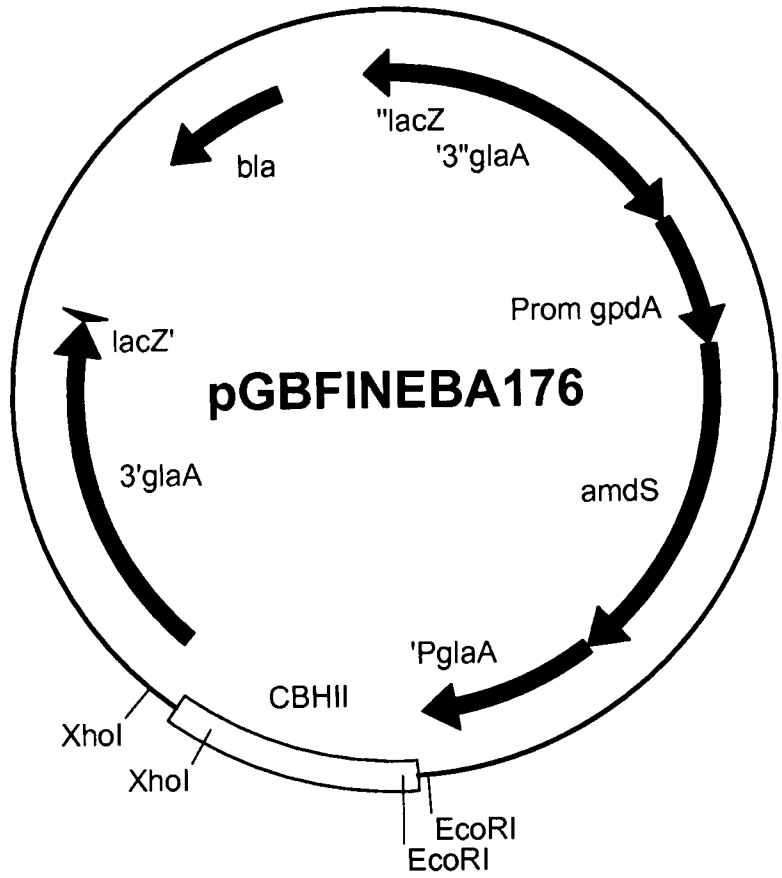

Fig. 8

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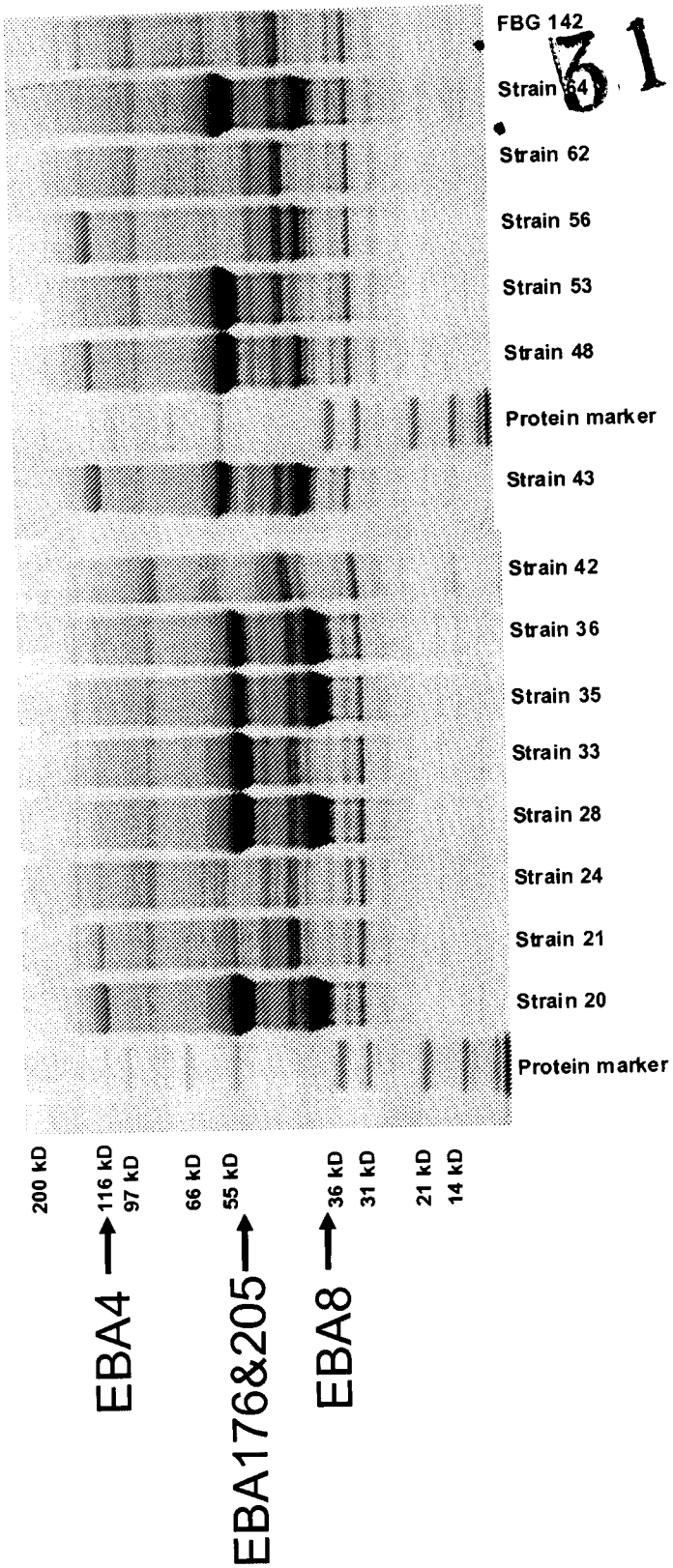

Fig 9A

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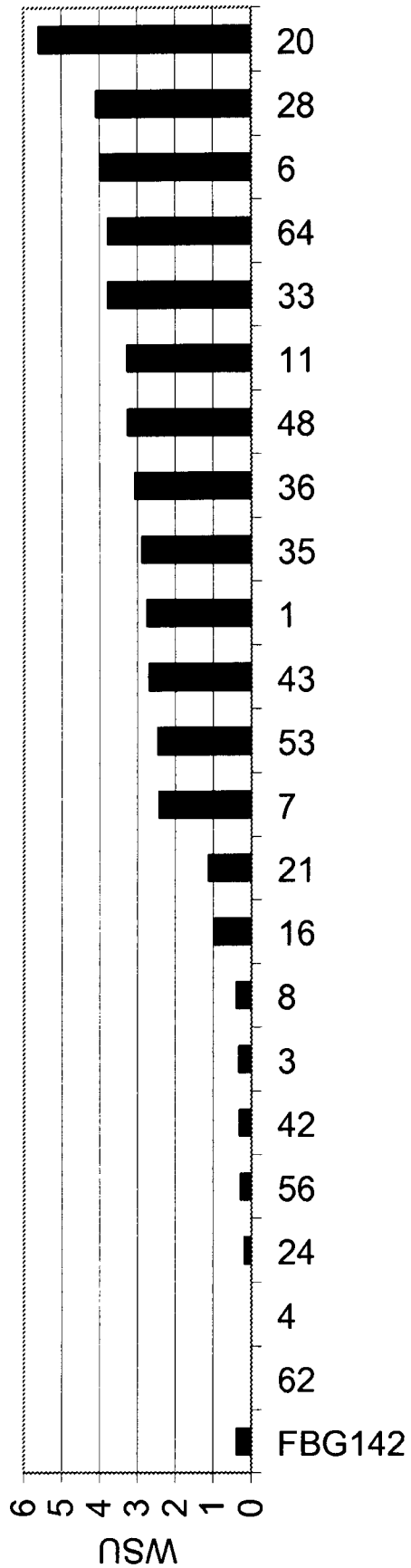

Fig 9B

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TALAROMYCES TRANSFORMANTS

Field of the invention

The invention relates to a process for the production of *Talaromyces* transformants, to *Talaromyces* transformants and to a process for production of polypeptide using the *Talaromyces* transformants. The invention also relates to a process for saccharification of lignocellulosic material, wherein the lignocellulosic material is contacted with the transformant or a cellulase, hemicellulase and/or pectinase produced by the transformant, and sugars are produced. Further the invention relates to a process for the preparation of a fermentation product, for instance ethanol, wherein those sugars are fermented with a fermenting microorganism, preferably yeast, to produce the fermentation product.

Background of the invention

Carbohydrates constitute the most abundant organic compounds on earth. However, much of this carbohydrate is sequestered in complex polymers including starch (the principle storage carbohydrate in seeds and grain), and a collection of carbohydrates and lignin known as lignocellulose. The main carbohydrate components of lignocellulose are cellulose, hemicellulose, and pectins. These complex polymers are often referred to collectively as lignocellulose.

Bioconversion of renewable lignocellulosic biomass to a fermentable sugar that is subsequently fermented to produce alcohol (e.g., ethanol) as an alternative to liquid fuels has attracted an intensive attention of researchers since 1970s, when the oil crisis broke out because of decreasing the output of petroleum by OPEC. Ethanol has been widely used as a 10% blend to gasoline in the USA or as a neat fuel for vehicles in Brazil in the last two decades. More recently, the use of E85, an 85% ethanol blend has been implemented especially for clean city applications. The importance of fuel bioethanol will increase in parallel with increases in prices for oil and the gradual

depletion of its sources. Additionally, fermentable sugars are being used to produce plastics, polymers and other biobased products and this industry is expected to grow substantially therefore increasing the demand for abundant low cost fermentable sugars which can be used as a feed stock in lieu of petroleum based feedstocks.

The sequestration of such large amounts of carbohydrates in plant biomass provides a plentiful source of potential energy in the form of sugars, both five carbon and six carbon sugars that could be utilized for numerous industrial and agricultural processes. However, the enormous energy potential of these carbohydrates is currently under-utilized because the sugars are locked in complex polymers, and hence are not readily accessible for fermentation. Methods that generate sugars from plant biomass would provide plentiful, economically-competitive feedstocks for fermentation into chemicals, plastics, such as for instance succinic acid and (bio) fuels, including ethanol, methanol, butanol synthetic liquid fuels and biogas.

Regardless of the type of cellulosic feedstock, the cost and hydrolytic efficiency of enzymes are major factors that restrict the commercialization of the biomass bioconversion processes. The production costs of microbially produced enzymes are tightly connected with a productivity of the enzyme-producing strain and the final activity yield in the fermentation broth.

In spite of the continued research of the last few decades to understand enzymatic lignocellulosic biomass degradation and cellulase production, it remains desirable to discover or to engineer new highly active cellulases and hemicellulases. It would also be highly desirable to construct highly efficient enzyme compositions capable of performing rapid and efficient biodegradation of lignocellulosic materials.

Such enzyme compositions may be used to produce sugars for fermentation into chemicals, plastics, such as for instance succinic acid and (bio) fuels, including ethanol, methanol, butanol, synthetic liquid fuels and biogas, for ensiling, and also as enzyme in other industrial processes, for example in the food or feed, textile, pulp or paper or detergent industries and other industries.

One genus of microorganisms that is known to produce suitable enzymes for enzymatic lignocellulosic biomass degradation is the genus *Talaromyces*. *Talaromyces* is a filamentous fungus.

Jain, S. et al, Mol Gen Genet (1992), 234, 489-493 discloses a transformation system for the fungus *Talaromyces* sp CL240. No expression of polypeptides is disclosed.

Murray, F.R. et al, Curr Genet (1997), 32, 367-375 discloses over-expression of the glucose oxidase gene from *Talaromyces flavus* in *Talaromyces macrosporus.* The effect fungal isolates on growth inhibition of *V. dahliae* was studied.

WO200170998 discloses *Talaromyces emersonii* beta-glucanases. On page 16, it is described that the polynucleotide of beta-glucanase may be heterologously expressed in a host, e.g. a yeast cell.

WO200224926 discloses *Talaromyces emersonii* xylanase. On page 24, 5th paragraph, it is described that production of the polypeptide may be achieved by recombinant expression of the xylanase DNA sequence in a suitable homologous or heterologous host cell. In paragraph 7, it is said that the host cell may over-express the polypeptide, and techniques for engineering over-expression are well known from WO99/32617. WO99/32617 relates to expression cloning, but does not disclose cloning in *Talaromyces* host.

WO2007091231 discloses strains of *Talaromyces emersonii* which are thermostable and encode thermostable enzymes, and also discloses enzyme compositions produced by the *Talaromyces emersonii* strains. No recombinant production of homologous or heterologous polypeptides is disclosed. In table 1 shows inducing carbon sources were added in an amount of 0.2-6 %. Solka floc and glucose (2%) were included for comparative purposes. On page 78, line 28 it is said that "glucose does not completely repress exoglucosidase production by the *T. emersonii* strains (table 31A). Table 31A shows that IMI393751 produces beta-glucosidase activity of 31.90 IU with glucose as carbon source, but no other cellulase activities, e.g. glucanases or xylanases. Due to lack of such enzyme activities, the strain IMI393751 is not suitable for the production of cellulases for the conversion of lignocellulose on glucose as carbon source.

Summary of the invention

The presence of a cellulase inducer, necessary sofar in *Talaromyces* cellulase production methods, has several disadvantages. First, the inducer, such as a plant material, may have a variable composition, which is disadvantageous for the controllability of the cellulase production process. Secondly, energy is required to sterilise plant material for