Penicillium Hosts as the Platform for the Development of New Recombinant Strains Producers of Carbohydrases and Related Enzymes

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Value added from biomass

- Pharma & Cosmetics
- Food & Additives
- Bioplastics
- Bulk chemicals
- Liquid fuels
- Energy & Heat

High value

Low value
Penicillium host strains. Stage 1

- UV mutagenesis of the wild type *Penicillium* strains

<table>
<thead>
<tr>
<th></th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted protein</td>
<td>4-8 fold</td>
</tr>
<tr>
<td>Xylanase activity (total)</td>
<td>5-8 fold</td>
</tr>
<tr>
<td>β-galactosidase activity (total)</td>
<td>~5 fold</td>
</tr>
<tr>
<td>Cellulase (MCC) activity (total)</td>
<td>4-6 fold</td>
</tr>
</tbody>
</table>

Wild type
(e.g. RCIM F178) → UV → Mutant strains
(e.g. RCIM F178* PCA) → Select. media
NaClO3 + NaNO3- NaNO2 - NH4Cl+ HX+ Pro+ Arg+ → Host strain
(e.g. PCA10 (niaD-))
**Penicillium as the host. Stage 2**

- Cloning of xylanase gene transcription activator XlnR

Gene promoter site *xylA*

**GGCTAA**

Gene *xylA*  

Increasing the number of targeted gene transcripts  

Multicopied producer strain

Inducers: arabinose, xylose

**PCA10**  
Xylanase activity  
200 U/ml

**RN3-11**  
Xylanase activity  
350 U/ml

Xylanase activator *XlnR*

*xlnR*  
*P. canescens*

**pXlnRP53**

**pUC57**

**niAD A.niger**

**pSTA10**

**pUC57**
### Penicillium as the recipient strain. Stage 3

- Cloning the regulatory gene fragments of major enzymes

<table>
<thead>
<tr>
<th>Regulatory elements</th>
<th>The structural part of the encoding gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter 1 (~2200 bp)</td>
<td>β-galactosidase (bgal)</td>
</tr>
<tr>
<td>Promoter 2 (~1700 bp)</td>
<td>xylanase A (xylA)</td>
</tr>
<tr>
<td>Promoter 3 (~889 bp)</td>
<td>arabinofuranosidase A (abfA)</td>
</tr>
<tr>
<td>Terminator 1 (~2000 bp)</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>Terminator 2 (~</td>
<td>xylanase A</td>
</tr>
</tbody>
</table>

Promoter X  | Gene Y  | Terminator Z

- α-L-arabinofuranosidase A 70
- β-galactosidase 112
- Xylanase A 31
- PCA RN3-11-(niaD-)
Enzymes for Industrial Biotechnology

**Pulp-and-paper:**
- Non-chlorine paper bleaching
  - endo-1,4-β-glucanase
  - endo-1,4-β-xylanase
- endo-1,4-β-glucanase
- Cellulases, β-glucosidase, hemicellulases, α-amylase, glucoamylase

**Textile:**
- Fabric treatment, Denim wash
  - endo-1,4-β-glucanase
  - endo-1,4-β-xylanase
- phytase, pectinase
  - pectinase,
  - celllobiohydrolase,
  - endo-1,4-β-glucanase
  - β-glucosidase,
  - α-galactosidase,
  - β-galactosidase,
  - inulinases
- Cellulases, β-glucosidase, hemicellulases, α-amylase, glucoamylase

**Agriculture/Animal feeds:**
- Feeds additives
  - phytase, pectinase
  - endo-1,4-β-glucanase
- β-glucosidase, α-galactosidase, β-galactosidase

**Food Industry and Processing:**
- Clarification of fruit juices,
- Food Industry by-products treatment
  - cellulases, β-glucosidase, hemicellulases, α-amylase, glucoamylase

**Textile:**
- Fabric treatment, Denim wash

**Bioalcohols / Biofuels:**
- Ethanol and butanol from Starch and Lignocellulose
The Main Goal:

Strain development and obtaining of enzyme preparations with attractive biotechnological properties on base of *Penicillium* recombinant strains

**Targets:**

- Targeted genes cloning into *Penicillium* (ΔniaD) host strains.
- Screening and selection of transformants with targeted activities.
- Optimization of fermentation schemes and conditions (joint cultivations, inducers, batch/feed-batch et al) and enzyme preparation production for further evaluations.
### Targeted Genes, Expressed in the *Pen.canescens* strain

<table>
<thead>
<tr>
<th>Promoter X</th>
<th>Gene Y</th>
<th>Secreted Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bgaS</em></td>
<td><em>abfA</em></td>
<td>$\alpha$-L-arabinofuranosidase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>aglA</em></td>
<td>$\alpha$-galactosidase A</td>
</tr>
<tr>
<td><em>xylA</em></td>
<td><em>faeA</em></td>
<td>ferruloil esterase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>xegA</em></td>
<td>xyloglucanase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>xylA</em></td>
<td>xylanase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>pelA</em></td>
<td>pectinlyase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>phyA</em></td>
<td>phytase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>rglA</em></td>
<td>rhamnogalacturonanlyase A</td>
</tr>
<tr>
<td><em>bgaS</em></td>
<td><em>abfB</em></td>
<td>arabinofuranosidase B</td>
</tr>
<tr>
<td><em>abfA</em></td>
<td><em>bgal</em></td>
<td>$\beta$-galactosidase</td>
</tr>
</tbody>
</table>
Targeted Genes, Expressed in the *Penicillium* strains

**Penicillium sp.**
- *egl2* - endo-1,4-β-glucanase II
- *egl3* - endo-1,4-β-glucanase III
- *cbhI* - cellobiohydrolase I
- *cbhII* - cellobiohydrolase II

**Aspergillus sp.**
- *aglC* - α-galactosidase C
- *inu1* - exo-inulinase
- *inuA* - endo-inulinase
- *bgl1* - β-glucosidase
- *phyA* - phytase

**Trichoderma sp.**
- *manB* - mannanase B
- *xyl3* - xylanase III

Heterologous genes

Lipases, esterases, oxidases and more…
Targeted Genes, Expressed in the *Pen. canescens*

- **α-galactosidase** *P. can* (60 kDa)
- **Pectinlyase** *P. can* (40 kDa)
- **Cellobiohydrolase I** *P. verruculosum* (66 kDa)

![Diagram showing protein bands and molecular weights](image)
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Substrate</th>
<th>Targ. activity, U/ml</th>
<th>Increase, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abf6</td>
<td>p-NPh-(\alpha)-L-arabinofuranoside</td>
<td>50-60</td>
<td>&gt;10</td>
</tr>
<tr>
<td>AglA33</td>
<td>p-NPh - (\alpha)-Gal</td>
<td>1200-1400</td>
<td>&gt;500</td>
</tr>
<tr>
<td>AglC4</td>
<td>p-NPh - (\alpha)-Gal</td>
<td>140-160</td>
<td>&gt;15</td>
</tr>
<tr>
<td>FAE9</td>
<td>p-NPh – butyrate</td>
<td>50-70</td>
<td>&gt;30</td>
</tr>
<tr>
<td>XG9</td>
<td>Xyloglucan</td>
<td>60-80</td>
<td>&gt;20</td>
</tr>
<tr>
<td>PhPl29</td>
<td>Phytin, pectin</td>
<td>220, 180</td>
<td>&gt;10, &gt;90</td>
</tr>
<tr>
<td>PhPlAgI9</td>
<td>Phytin, pectin, p-NPh -(\alpha)-Gal</td>
<td>280, 120, 325</td>
<td>&gt;15, &gt;60, &gt;400</td>
</tr>
<tr>
<td>Phy215</td>
<td>Phytin</td>
<td>400</td>
<td>&gt;150</td>
</tr>
<tr>
<td>PEC23</td>
<td>Pectin from citrus</td>
<td>190</td>
<td>~100</td>
</tr>
<tr>
<td>Eg2</td>
<td>Carbohymethylcellulose</td>
<td>1200-1500</td>
<td>&gt;200</td>
</tr>
<tr>
<td>pBGL-32</td>
<td>p-NPh -(\beta)-Glc</td>
<td>800-1000</td>
<td>&gt;500</td>
</tr>
<tr>
<td>CBHI</td>
<td>Avicel</td>
<td>7-10</td>
<td>&gt;5</td>
</tr>
<tr>
<td>CBHI2</td>
<td>Avicel</td>
<td>7-10</td>
<td>&gt;5</td>
</tr>
<tr>
<td>INU1</td>
<td>Inulin</td>
<td>2500-3000</td>
<td>&gt;800</td>
</tr>
<tr>
<td>MANB</td>
<td>Galactomannan</td>
<td>35</td>
<td>&gt;30</td>
</tr>
<tr>
<td>XYLIII</td>
<td>Birch xylan</td>
<td>500-600</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>
Enzymes in Animal Feeds

Improved digestibility and energy value of feeds

Reduction of «antinutritional» factors in feeds

Improvement of the physiological state of animals

Growth and productivity acceleration

Feed components and enzymes used:

Wheat, rye, barley, corn – xylanases, β-glucanases, phytase
Soy, peas, lupin – α-galactosidases, protease
Sunflower – protease, hemicellulases
Protein-containing substrates (feather, trimming flour) – (endo)protease
Phytate Conversion in Animal Feeds

Soybean mill  Corn mill

Conversion of phytate,%

Load, U of phytase activity
black - 150, gray - 100, light gray - 75

PHY 215  Commercial Phytase \textit{Asp.niger}  PHY 215  Commercial Phytase \textit{Asp.niger}
Reducing sugars yield from soybean mill upon enzyme dosage
1- α-GalA *P. canescens*,
2- commercial preparation α-D-galactosidase/Amano/10

*In vitro Feed Test.*

![Graph showing p-NPh-Gal activity dosage on 1 g of soy mill](image-url)
Protein digestibility from different sources

Untreated
- Egg white: 100 (taken as 100%)
- Whole milk proteins: 91
- Beef/trimming: ~80
- Casein: 77
- Soybean: 74
- Wheat gluten: 64

Hydrolysates:
- Trimming: 99-102
- Soybean hydrolysate: 95-98

SDS-PAGE of soluble soy proteins with and w/out protease/a-galactosidase treatment
Applications in Food Industry

Fruit and berry juices and purees
Increase of juice yield from fruits and berries with the help of pectinases

Jerusalem artichoke syrup processing
Cranberries Juice Production and Clarification

Yield of cranberry juice

Viscosity of cranberry juice

PelA preparation from *Pen. can.*

Control: Commercial Preparation “Rapidase Press” (70 ml per 1 ton of berries)
Value added from food industry by-products (e.g. in biofuels production process)

- Soy solubles and by-products: stachiose, raffinose
- Milk whey (lactose)
- Sugar beat pulp
- Apple pulp
- Fermentable sugars
  - α-Galactosidase A/C
  - β-Galactosidase
- Glucose, galactose
  - β-Glucosidase
- Fermentable sugars
  - Endoglucanases
  - Pectinlyases
  - β-glucosidase
  - Cellobiohydrolase
- Ethanol
- Butanol
- Fermentation
Bioconversion of Food Industry by-products
Apple Pulp after Juice Extraction

Reducing sugars and glucose yield after the hydrolysis of apple pulp, 24 hours, 50°C,

**Control** – enzyme preparation after co-cultivation of single enzyme strains – β-G, Pel A, and EG II

**RN3-11-7** – initial *Penicillium* host strain

**Duplet**: PelA, β-G

**Triplet1**: EG II, PelA, β-G

**Triplet2**: EG II, PelA, β-G
Sugar beat pulp balance in Russia

Existing by-products can be processed to get valuable feeds and bio-based bulk chemicals.
Hydrolysis for sugarbeat pulp by laboratory and commercial enzyme preparations, 100 g/L S, 50°C, 5 mg E / 1g S, 24 hours

Sugars yield,
Textile Processing

Application of topolytic endoglucanases for biopolishing of fabric and denim wash processes

Before enzymatic treatment

After enzymatic (endoglucanases) treatment

Pulp-and-Paper

Xylanases in biobleaching of craft pulp

w/out xylanases  after xylanase treatment
The efficiency of cellulose pulp biobleaching with enzymes Xyl31-nat and Xyl31-rec and enzyme preparations. Protein load 0.005 mg/ml, 50°C, 200 rev/min
Bioconversion of (ligno)cellulosic materials

- Pretreatment
- Deep succharification
- Glucose, xylose
- Fermentation

- LCM
- Cellulases
- Xylanases
- Bulk chemicals, biofuels
- Microbiological or/and chemical processing
- Aminoacids
- Feed protein
- Drug / cosmetic components
- Biobased polymers
Hydrolysis of Cellulose-containing Substrates

Microcrystalline cellulose [S]=100 g/L, [E]=5 mg/g, 50°C pH 5.0

Delignified wheat straw [S]=100 g/L, [E]=5 mg/g, 50°C pH 5.0

Glucose yield, g/L

Hydrolysis time, hours

Celloviridin G20X
Tr. reesei
Celloviridin G20X + β-glucosidase
Pen. canescens

Delignified wheat straw
Results

- Universal gene expression systems based on fungal *Penicillium* strains were developed.
- Recombinant strains and enzyme preparations with promising properties were obtained.
- The enzyme preparations were tested for various industrial applications.
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