Discovery of a novel thermophile β-galactosidase, TtbGal1, for the production of prebiotic oligosaccharides from acid whey

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β- galactosidases (E.C. 3.2.1.23)

- glycosyl hydrolase → hydrolysis of lactose to glucose and galactose

β-Galactosidases in dairy industry
- lactose hydrolysis in dairy products
- production of lactose-free products, for consumers with lactose intolerance

- β-galactosidases also catalyze the transgalactosylation reaction, producing galactooligosaccharides (GOS)

GOS are significant prebiotics
- improve the gut health
- promote the growth of the probiotic intestinal bacterial flora.
What about the use of a low-cost material as substrate, which would not compete with food and feed raw materials?

Whey, a liquid byproduct of the dairy industry

**Acid whey**  
- pH 4.5  
- From cottage cheese and Greek yoghurt manufacturing

**Sweet whey**  
- pH 6.5  
- From cheese manufacturing

Commercial β-galactosidases for GOS production
- *Aspergillus oryzae*
- *Kluyveromyces lactis*
- *Bacillus circulans*

High transgalactosylation activity, high GOS yield
Whey as a waste material

**Disposal methods**
- Spraying in fields
- Discharge in water bodies
- Municipal sewage system
- Animal feed

**Issues with current disposal methods**
- Smell, salt and heavy polluting load
- High BOD (30,000-35,000 ppm) and COD (60,000-80,000 ppm)

- 100 L of milk used for cheese → 80-90 L of whey
- Annual production of whey: 160 million tons, sweet whey is 22.5 million tons
- Acid whey production is increasing steadily, due to increasing popularity of the Greek strained yoghurt worldwide

**Production of GOS from whey**
- Good yield with β-galactosidases from *A. oryzae* and *K. lactis* in sweet whey (32.5%)
- Most known β-galactosidases are active in neutral pH
- For valorization of acid whey, *thermophile, acidic* β-galactosidases are needed
Discovery and characterization of novel enzymes

1. Database mining of genes with desired properties
2. Cloning in proper vectors
3. Transformation in the yeast *Pichia pastoris*
4. Screening and selection of recombinant clones
5. Production of heterologous enzymes
6. Purification of recombinant enzymes with chromatographic methods
7. Biochemical and physicochemical characterization of enzymes
8. Biocatalysis applications
Molecular Phylogenetic analysis by Maximum Likelihood method
Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

*Thielavia terrestris* 2047729 structure prediction based on beta-galactosidase from *Aspergillus oryzae* (61.22% identity) with SWISS-MODEL.
Screening of recombinant clones

Screening of *P. pastoris* clones for β-galactosidase activity in plate assays with X-GAL as the substrate.

Screening of *P. pastoris* clones for β-galactosidase activity in liquid media.

‘Jackpot’ clones
Characterization of purified TtbGal1

- T optimum: 60 °C
- pH optimum: 4
- M.W.: 110 kDa
## Characterization of purified TtbGal1

### Kinetic parameters

<table>
<thead>
<tr>
<th></th>
<th>Km (mM)</th>
<th>Kcat (min(^{-1}))</th>
<th>k(_{cat}/K_m) (mM min(^{-1}))</th>
<th>Specific activity (U mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>oNPhG</td>
<td>0.18 ± 0.02</td>
<td>275280 ± 7932</td>
<td>1522566 ± 187443</td>
<td>1956.5 ± 117.7</td>
</tr>
<tr>
<td>lactose</td>
<td>12.4 ± 1.4</td>
<td>24636 ± 759</td>
<td>1981 ± 233</td>
<td>95.3 ± 10.6</td>
</tr>
</tbody>
</table>

### Effect of salts on the activity of TtbGal1.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Residual activity (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>79.8 ± 13.7</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>93.4 ± 5.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>86.5 ± 7.2</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>96.2 ± 2.1</td>
</tr>
<tr>
<td>KCl</td>
<td>86.8 ± 3.1</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>94.0 ± 8.8</td>
</tr>
<tr>
<td>NaN(_3)</td>
<td>94.8 ± 10.5</td>
</tr>
</tbody>
</table>

Very satisfactory activity in the presence of a variety of salts → promising property for application in untreated acid whey.
Optimization of GOS production in defined lactose solutions

**Enzyme load**

Maximum GOS concentration: $1.46 \pm 0.02 \% \text{ (w/v)}$

**Substrate concentration**

Maximum GOS concentration: $3.26 \pm 0.04 \% \text{ (w/v)}$
TtbGal1-mediated GOS synthesis from acid whey

**Untreated whey**
3.4% (w/v) lactose

Maximum GOS concentration: 0.35 ± 0.05 % (w/v)

**Concentrated whey**
9.28% (w/v) lactose

Maximum GOS concentration: 1.49 ± 0.08 % (w/v)
TtbGal1-mediated GOS synthesis from acid whey

Monosaccharides (glucose, galactose)

Lactose

GOS disaccharide

GOS trisaccharide

High-Performance Anion-Exchange Chromatography Coupled with Pulsed Amperometric Detection (HPAEC-PAD)
Conclusions

- A novel fungal β-galactosidase, *TtbGal1*, was heterologously expressed, purified and characterized.
- *TtbGal1* is thermostable and is optimally active in acidic pH.
- Satisfactory activity in the presence of salts.
- GOS production with yields up to 19.4%.
- Valorization of acid whey as a substrate to produce GOS with prebiotic activity.

*Work in progress...*

- Further optimization is needed.
- LC-MS analyses to determine the chemical nature of the produced GOS.
- Scale-up of the process.
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Thank you for your attention!