

Chrysosporium lucknowense, a new fungal host for protein production

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A number of micro-organisms have the capability of producing cellulases. Most cellulases in commercial production, e.g. those from *Trichoderma*, have optimum activities in the acidic pH range. In a search for new neutral cellulase enzymes Dyadic International, Inc. screened a large collection of fungal strains for improved enzyme production. Here we report the isolation of a neutral cellulase producing strain of *Chrysosporium* and its development and optimisation as a new host for protein production.

Identification of a cellulase producing strain

Screening thousands of micro-organisms, isolated from very diverse environments, resulted in the identification of a fungal isolate that naturally produces a neutral cellulase activity. This strain was isolated from alkaline soil from the Far East of the Russian Federation. Taxonomic determination of this isolate revealed that it was a *Chrysosporium* species, specifically *Chrysosporium lucknowense*. We refer to this isolate as C1 strain. *Chrysosporium* is a fungal genus of mesophilic and keratinolytic fungi only distantly related to known fungi like *Aspergillus*, *Neurospora* and *Trichoderma* (Figure 1).

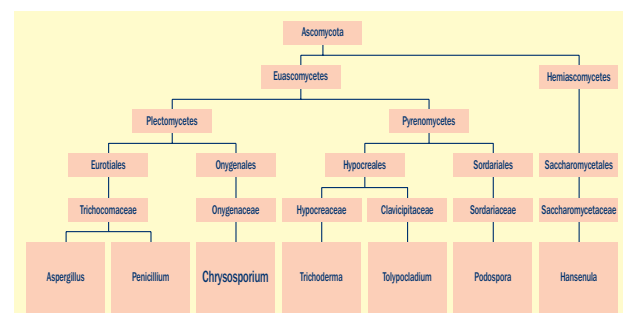


Figure 1. Fungal taxonomy

Strain and process development

Research was carried out to improve the cellulase productivity of the isolated (wild type) strain of *Chrysosporium lucknowense*.

Mutagenesis

- Mutant strains were isolated, after mutagenesis with UV irradiation or N-methyl-N'-nitro-N-nitrosoguanidine. Screening was carried out for:
 - higher neutral cellulase production (see also Table 1);
 - Improved fermentation characteristics: lower fermentor viscosity;
 - Protease deficient mutant strains (Figure 2 and 3).

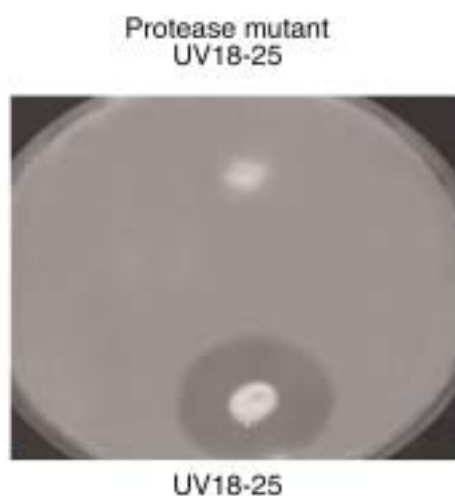


Figure 2. Halo on skim milk plate

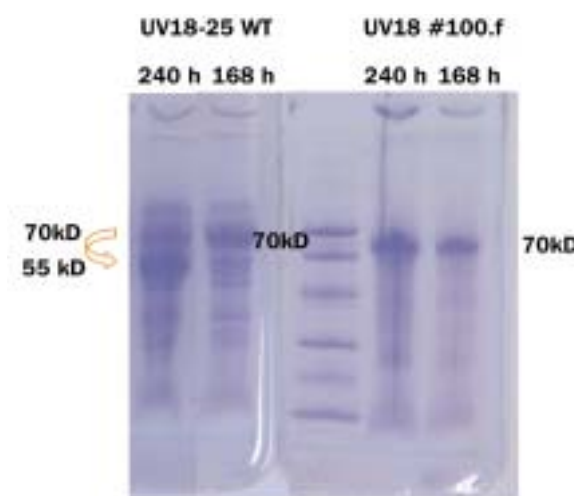


Figure 3. Medium samples of UV18-25 wildtype and protease mutant UV18#100.f after 168 hours and 240 hours cultivation

Fermentation

- Growth conditions of the isolated *Chrysosporium* strain are very versatile. It can be cultivated at temperatures from 25 to 43°C and at acidic to alkaline pH, allowing optimal cultivation at human physiological conditions;
- Large scale fermentations were developed up to 150.000 litre scale on relatively cheap and simple media;
- Strain and process development led to increases in cellulase production of about 200-fold in controlled fermentations.

Development of C. lucknowense-based expression system

An initial transformation system was developed based on the dominant phleomycine resistance marker. For the construction of C1 expression vectors, promoters of highly expressed cellulase and xylanase genes were isolated from an available gene library. Table 1 shows relative expression levels of a few of the cloned genes in C1 wildtype and various improved mutant strains in shake flasks. Expression vectors were constructed with (among others) the *cbh1* promoter.

Table 1. Results of Northern analysis of some mutant strains of *Chrysosporium lucknowense* (cultivation in shake flasks). After 2 days, RNA was analysed, after 5 days the total amount of protein was determined in the culture medium.

strain/ probe	pharmamedia/cellulose/lactose				pharmamedia/glucose				
	relative protein levels	EG5	EG6	XYL1 CBH1	relative protein levels	EG5	EG6	XYL1 CBH1	
C1 WT	1	+	+/-	++	0.4	-	-	-	+/-
UV13-6	1	++	+/-	+++	0.8	-	-	-	+
NG7C-19	4	+	-	++	2	++++	++++	++++	++++
UV18-25	7	++	-	+++	5	+++	++++	+	+++

Protein production

Using the developed expression system several fungal and nonfungal genes are expressed in UV18-25.

Fungal genes

Improved cellulase and xylanase strains were generated

by introducing extra gene copies from fungal origin (e.g. *Trichoderma*, *Chrysosporium*) (results not shown).

Non-fungal genes

Table 2 and Figure 4 show results of expression of phleomycine-human lysozyme and glucoamylase-interleukin6 fusion proteins in shake flasks. Significant levels of active human lysozyme could be detected in shake flasks, indicating proper formation of SS bridges, required for HLZ activity. The glucoamylase carrier protein is also efficiently produced. Western analysis indicates appropriate glycosylation of this protein. Interleukin6 was also detected, but at lower levels than glucoamylase. Some residual proteolytic degradation was observed, but the level of degradation could be modulated under controlled fermentation conditions. These observations are based on preliminary experiments regarding interleukin6 expression, as the work on it is ongoing.

Table 2. Active Human Lysozyme levels in UV18-25 transformants after cultivation in shake flasks

	Active Human Lysozyme in culture media
UV18-25	0 mg/l
transformant 1	8 mg/l
transformant 2	4 mg/l
transformant 3	2 mg/l
transformant 4	2.5 mg/l

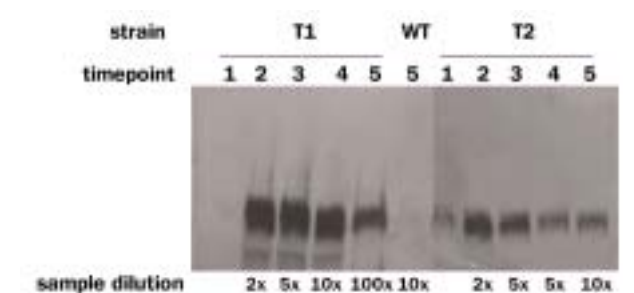


Figure 4. Western analysis (α -gla) on medium of 2 different Gla-Il6 transformants of UV18#100.f (different timepoints of shake flask cultures)

Conclusions

- New fungal expression host is isolated
 - Distantly related to known expression hosts like *Trichoderma*, *Aspergillus* or *Penicillium*
- Mutant production strains developed
 - Improved fermentation characteristics
 - Protease deficient mutants
- Versatile fermentation properties
 - Broad pH / temperature range
 - Large scale fermentation developed
- Gene expression system developed
 - Gene transfer system
 - Expression vectors
 - Efficient gene expression

For further information

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