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SOLID STATE FERMENTATION AS A TOOL FOR PRODUCTION OF THREE INDUSTRIALLY VALUABLE MICROBIAL ENZYMES: CHITINASE, LIPASE / ESTERASE AND TRANSGLUTAMINAS

Ph.D thesis book

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1. INTRODUCTION

„God did not create filamentous fungi to grow in fermentors". This famous and frequently cited quote from Prof. A.P.J. Trinci expresses that submerged fermentation (SF) represents artificial conditions for propagation of fungi. Vast majority of microorganisms (especially filamentous fungi and many actinomycetes) primarily live and grow in nature in solid-state fermentation (SSF) conditions. SSF is generally defined as the growth of the microorganisms on (moist) solid materials in absence or near absence of free water.

Though SSF has a long tradition (especially in the Oriental countries) dating back to thousands of years (for example, production of fermented food and alcoholics in Far East), its application could not compete with SF after World War II in the antibiotic and enzyme era. However, some microbial enzymes are still produced by SSF in Japan, India and China. SSF gained attention again a few years ago when Biocon India successfully started to produce some microbial drugs such as lovastatin and mycophenolic acid on large scale. SSF requires less processing and down-streaming stages, utilizes less power and generates less effluent (sewage).

Another method I applied during my research was a selection way of valuable microbes which is often called „screening". Nowadays screening is a very condemned and neglected method by many researchers, but without having the proper isolate in hand, a lot of effort might be invested vainly, into a less productive starting biological material.

Therefore throughout my PhD work SSF and screening have been used in the belief that this fermentation and selection method will play more important role in future than today. Screening method was applied in order to find good or – so far - undescribed producer microorganisms of three industrially valuable enzymes, namely chitinases, lipases/esterases and transglutaminases. Screening for these enzymes was motivated by actual industrial projects (financial support) and collaborative partners outside Hungary.

2. NEW SCIENTIFIC RESULTS OF THE THESIS

2.1. Based on the extracellular chitinase production of fifty *Trichoderma harzianum* strains in solid state fermentation and the ITS1/ITS2 and *tef1* sequences of these isolates it was concluded that *T. harzianum* isolates with similar ITS1/ITS2 and *tef1* sequences to ex-type CBS 226.95 isolate (*sensu stricto* group) showed a statistically supported overproduction of extracellular chitinases in solid state fermentation (II. Nagy *et al*, 2007).

The aim of the research collaboration with the Technical University of Vienna was to determine whether good chitinase enzyme production could be predicted via DNA barcodes in different *Trichoderma harzianum* isolates. Currently, selecting a certain strain for a specific industrial purpose is largely a random procedure therefore screening requires a lot of effort. One must note that nowadays DNA barcodes of *Trichoderma* species can be easily determined therefore our results point toward a less complicated selection way of industrially interesting enzyme producers.

Extracellular chitinase production of 50 *T. harzianum* isolates was investigated in SSF on a wheat bran-crude chitin mixture, while the sequence analysis of two phylogenetic marker loci (internal transcribed spacer 1 and 2 (ITS1, ITS2) of the rRNA-encoding gene cluster) and the large intron of the elongation factor 1-alpha (*tef1*) was performed by the Austrian partner.

The tested isolates were divided into two major groups, based on ITS1, ITS2 and *tef1* sequences. *Sensu stricto* group contained strains carrying the same ITS1, ITS2 and *tef1* sequences as the reference strain ('type-strain'), *T. harzianum* CBS 226.95, while *sensu lato T. harzianum* strains had several polymorphic positions in both ITS1 and ITS2 loci. Altogether 50 *T. harzianum* isolates were tested, from which 21 belonged to *sensu stricto* group.

The average chitinase production for *sensu stricto* isolates was 2.3 and 4.1 IU g^{-1} DM in a 3 and a 5 day SSF, respectively. For *sensu lato T. harzianum* group, the average chitinase productions were 1.2

and 1.7 IU g^{-1} DM in a 3 and 5 day SSF. Based on the results, it was concluded that isolates from *sensu stricto* group were significantly more efficient extracellular chitinase producers than *sensu lato* isolates.

2.2. The overproduction of extracellular chitinase by *sensu stricto* isolates was neither due to faster growth and metabolism on certain chitin monomers (N-acetyl-β-D-glucosamine or glucosamine), nor due to increased secreting ability of chitinase isoenzymes. *T. harzianum sensu stricto* group did not show an increased extracellular cellulase production (II. Nagy *et al*, 2007).

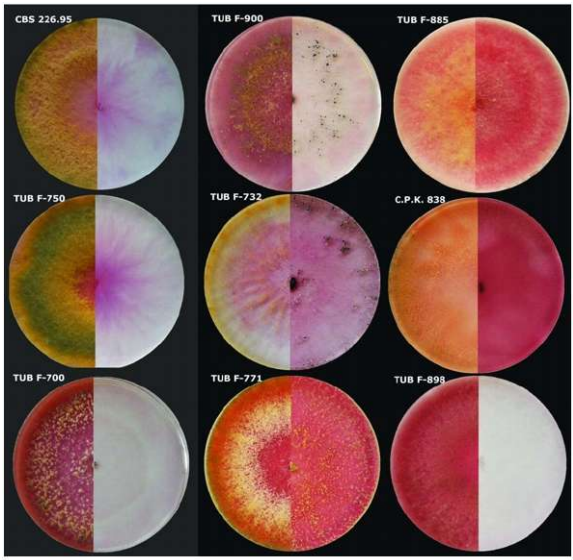
The fact that *sensu stricto* strains showed an increased extracellular chitinase production in SSF, prompted us to investigate the physiological background of the increased chitinase production. Therefore it was studied whether the increased extracellular chitinase production of *sensu stricto* isolates is originated from a faster growth and faster metabolism of certain chitin monomers, such as N-acetyl glucosamine or glucosamine. Based on the results, no correlation was obtained between the increased extracellular production and faster assimilation of chitin monomers.

The total extracellular chitinase production of a particular isolate can be originated from the activity of several isoenzymes, therefore the overproduction of certain chitinase isoenzymes could explain why *sensu stricto* strains show an increased chitinase production. In order to find out whether *sensu stricto* strains have an increased formation of one or more of the chitinases, native and SDS PAGE was performed. It was concluded that the increased extracellular chitinase production is not due to the fact that *sensu stricto* strains overproduce certain chitinase isoenzymes.

Overproduction of extracellular chitinase may also be due to an improved secreting capability of certain hydrolytic enzymes, such as cellulases. Therefore the extracellular cellulase production of few isolates was also determined in SSF conditions. The results indicated that *T. harzianum sensu stricto* strains do not show an increased cellulase production.

2.3. While *T. harzianum sensu stricto* strains could not metabolize N-acetyl mannosamine according to the Biolog analysis, a plate test was developed for the quick identification of *T. harzianum sensu stricto* strains (II. Nagy *et al*, 2007).

The metabolism of 95 different carbon sources by *T. harzianum* isolates can be determined in the Biolog Phenotype Microarray assay. Previous results of the Biolog analysis revealed that *T. harzianum sensu stricto* strains could not metabolize N-acetyl mannosamine. Therefore a plate test was developed for the quick identification of *sensu stricto* isolates.



A **B**

Fig. 9. Plate assay for screening superior chitinase producer *T. harzianum* isolates, with 9 randomly selected *sensu stricto* (A) and *sensu lato T. harzianum* (B) strains. The left side of each plate shows the growth on Glucose, while the right side

represents the growth on N-acetyl-mannosamine. Red color indicates microbial metabolic activity.

2.4. Based on screening of fourteen *Penicillium* strains it was concluded that some *Penicillium* strains can produce almost as much chitinase in SSF conditions as *Trichoderma harzianum* isolates belonging to the *sensu lato* group (V. Binod *et al*, 2005).

Very few data may be found for chitinase production of *Penicillium* spp, especially in SSF conditions. The aim of this screening was to find good extracellular chitinase producer strains among members of *Penicillium* genus and to characterize its chitinase enzyme.

Among the 14 tested strains, *Penicillium aculeatum* NRRL 2129 showed the highest chitinase activity. The enzyme properties of the produced chitinase were determined. The pH optimum was 4.0, temperature optimum of the enzyme was found to be 50°C. The secreted chitinase was not thermostable. Ion exchange chromatography revealed four major chitinase peaks of molecular mass 82.7 kDa, 44.6 kDa, 28.2 kDa and 26.9 kDa. On SDS PAGE three protein bands of molecular weight 82.6 kDa, 33.9 kDa and 29.1 kDa were identified

2.5. Naturally immobilized cheap lipase and esterase enzymes were prepared by solid state fermentation and consecutive air-drying of the material at room temperature for organic chemical biotransformation and bioconversion processes (III. Tőke *et al*, 2007 and IV. Nagy *et al*, 2006).

In a collaboration with the Organic Chemistry Department, SSF was used for microbial production of lipase and esterase enzymes applying wheat bran and olive oil or sterol-ester as a carbon source and an enzyme inducer in the medium. Screening of 42 mesophilic fungi resulted in good enzyme activities for many isolates. The *in situ* produced enzymes (gently dried whole SSF cultures) were used without a prior downstream processing for testing the whole fermented materials as raw biocatalysts in organic reactions.

The majority of the air-dried lipase SSF preparations proved to be effective in enantiomer selective biocatalysis of three selected racemic secondary alcohols (1-phenylethanol, 1-cyclohexylethanol and 1-(naphth-2-yl) ethanol) while the air-dried sterol-esterase samples were also effective in the esterification of β-sitosterols.

2.6. Without any downstream processing, the SSF materials of *Gliocladium* and *Chaetomium* strains were gently dried at room temperature and successfully applied in enantiomer selective biotransformation of racemic secondary alcohols. (IV. Nagy *et al*, 2006).

Dry SSF preparations of *Gliocladium catenulatum* NRRL 1093 and *Chaetomium globosum* OKI 270 strains were successfully applied in preparative scale kinetic resolutions of the above mentioned three selected racemic secondary alcohols, indicating their usefulness as inexpensive, naturally immobilized biocatalysts.

2.7. Air dried SSF samples of *Aspergillus oryzae* and *A. sojae* strains (food-grade fungi, GRAS microbes) were successfully applied to produce biocatalysts with useful sterol-esterase activity (III. Tőke *et al*, 2007).

Food-grade *Aspergillus oryzae* and *A. sojae* strains were cultivated under SSF and successfully applied in production of sterol-esters on a preparative scale. Using 60 and 67% moisture content in the SSF conditions, 40-60% conversions were achieved with the application of the air-dried sterol-esterase containing SSF samples.

2.8. *Streptomyces mobaraensis* NRRL B-3729, *S. paucisporogenes* ATCC 12.596 and *S. platensis* NRRL 2364 strains were successfully propagated in solid state fermentation in order to produce transglutaminase enzyme (I. Nagy and Szakacs, 2008).

Transglutaminases (TGases) catalyze the acyl transfer reaction between a γ-carboxyamide group of a lysine residue in a peptide chain and a γ-amino group of a lysine residue, resulting in the formation of a lysine cross-linkage. TGases are mainly used in food industry as cross-linking agents, but their applications are

increasingly important in textile and other industries too. For industrial production of the enzyme *Streptomyces mobaraensis* mutants or recombinants are recently used.

Since the production of TGase by actinomycetes was previously studied only in submerged fermentation, our aim was to test *Streptomyces* strains for TGase production under SSF conditions. For this purpose *S. mobaraensis* NRRL B-3729 (patent strain of Ajinomoto Co., Japan), *S. paucisporogenes* ATCC 12596, *S. platensis* NRRL 2364 (patent strain of Novozymes Inc.) and 17 unidentified actinomycetes were applied testing 26 different agro-food substrates.

Good TGase activities were obtained on substrates with high protein content, such as beans, peas and lentils. Highest activities were reached on liver kidney beans, *S. mobaraensis* produced 0.7 IU/g DM in a 3 day SSF, *S. platensis* 5.1 IU/g DM in a 7 d SSF, while *S. paucisporogenes* 4.2 IU/g DM in a 4 d SSF. TGase production by *S. paucisporogenes* strain was not indicated formerly in any paper or patent. It was observed and described by us in this paper.

The produced TGases had temperature optima at 45-50°C, pH optima at 7-8 and their molecular weights were 37-38 kDa according to the SDS PAGE analysis.

From the non-identified actinomycetes isolates TUB B-739 showed the highest TGase activity, with 5.9 IU/g DM in a 5 d SSF. This particular isolate was identified by DSMZ as a rare subspecies of S*treptomyces hygroscopicus* and originally was isolated from a soil sample collected in the garden next to our Ch building of the Budapest University of Technology and Economics.

3. LIST OF PUBLICATIONS

3.1. Publications mentioned in the thesis:

I. Nagy, V. and Szakacs, G. (2008) Production of transglutaminase by *Streptomyces* isolates in solid state fermentation. *Letters Appl Microbiol* **47.** 122-127. IF: 1.593

II. Nagy, V.; Seidl, V.; Szakacs, G.; Komon-Zelazowska, M.; Kubicek, C.P. and Druzhinina, I. (2007) Application of DNA barcodes for screening of industrially important fungi: the haplotype of *Trichoderma harzianum* sensu stricto indicates superior chitinase formation. *Appl Environ Microbiol* **73.** 7048-7058. IF: 3.532

III. Tőke, E.R.; Nagy, V.; Recseg, K.; Szakács, G. and Poppe, L. (2007) Production and application of novel sterol esterases from *Aspergillus* strains by solid state fermentation. *JAOCS* **84.** 907-915.

IF: 0.910

IV. Nagy, V.; Tőke, E.R.; Kheong, L.C.; Szatzker, G.; Ibrahim, D.; Che Omar, I.; Szakacs, G. and Poppe, L. (2006) Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation. *J Mol Catal B: Enzymatic* **39.** 141-148. IF: 2.149

V. Binod, P.; Pusztahelyi, T.; Nagy, V.; Sandhya, C.; Szakacs, G.; Pocsi, I. and Pandey, A. (2005) Production and purification of extracellular chitinases from *Penicillium aculeatum* NRRL 2129 under solid-state fermentation. *Enzyme Microbiol Technol* **36.** 880-887. IF: 1.897

3.2. Other publications:

Rahulan, R.; Nampoothiri, K.M.; Szakacs, G.; Nagy, V. and Ashok, P. (2009) Statistical optimization of L-leucine aminopeptidase production from *Streptomyces gedanensis* IFO 13427 under submerged fermentation using response surface methodology. *Biochem Eng J* **43.** 64-71. IF: 1.872

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Szakacs, G.; Tengerdy, R.P. and Nagy, V. (2004) Cellulases. In Enzyme Technology, A. Pandey, C. Webb, C.R. Soccol and C. Larroche, Asiatech Publishers, Inc., New Delhi, Chapter 13, 246-265. ISBN 81-87680-12-1

Nampoothiri, K.M.; Tomes, G.J.; Roopesh, K.; Szakacs, G.; Nagy, V.; Soccol, C.R. and Pandey, A. (2004) Thermostable phytase production by *Thermoascus aurantiacus* in submerged fermentation. *Appl Biochem Biotechnol* **118.** 205-214. IF: 1.102

Ramachandran, S.; Patel, A.K.; Nampoothiri, K.M.; Francis, F.; Nagy, V.; Szakacs, G. and Pandey, A. (2004) Coconut oil cake - a potential raw material for the production of alpha-amylase. *Bioresource Technol* **93.** 169-174. IF: 2.180

3.3. Posters and oral presentations:

Nagy, V.; Tőke, E.R.; Kheong, L.C.; Szatzker, G.; Ibrahim, D.; Omar, I.C.; Szakacs, G. and Poppe, L.: Kinetic resolutions with novel, highly enantioselective fungal lipases of *Gliocladium* and *Chaetomium* origin produced by solid state fermentation. 9th International Workshop on Trichoderma and Gliocladium, Apr. 6-8, 2006, Vienna, Austria (poster)

Nagy, V.; Kubicek, C.P.; Seidl, V.; Druzhinina, I. and Szakács, G.: Increased extracellular chitinase production in solid substrate fermentation by the *Trichoderma harzianum* ex-type clade.1st Central European Forum for Microbiology (CEFORM) and the annual meeting of the Hungarian Society for Microbiology, Oct. 26-28, 2005, Keszthely (presentation)

Nagy V.; Szakács, G. and Pandey, A.: *Trichoderma harzianum* izolátumok kitináz enzimtermelésének vizsgálata szilárd fázisú fermentáció alkalmazásával. A Magyar Mikrobiológiai Társaság 2004. évi Nagygyűlése és a X. Fermentációs Kollokvium, Oct. 7-9, 2004, Keszthely (poster)

Nagy V. and Szakács, G.: *Trichoderma* fajok alkalmazása ipari fermentációs folyamatokhoz. A Magyar Mikrobiológiai Társaság 2004. évi Nagygyűlése és a X. Fermentációs Kollokvium, Oct. 7-9, 2004, Keszthely (presentation)

Nagy, V.; Szakacs, G. and Pandey, A.: Production of chitinases with *Trichoderma harzianum* isolates using solid substrate fermentation. 8th International Workshop on *Trichoderma* and *Gliocladium*, Sept. 20-23, 2004, HangZhou, China (poster)

Nagy, V. and Kovács, K.: Biotechnology at Budapest University of Technology and Economics, Hungary. International Seminar on Biotechnology, Febr. 10, 2004, Sree Ayyappa College For Women, Chunkankadai, India (presentation)

Nagy, V.; Szakács, G. and Pandey, A.: Production of chitinolytic enzymes by selected *Penicillium* strains in solid substrate fermentation. 14th International Congress of the Hungarian Society for Microbiology, Oct. 9-11, 2003, Balatonfüred (poster)

Nagy, V.; Szakács, G. and Pandey, A.: Production of chitinolytic enzymes by *Trichoderma harzianum* using solid substrate fermentation. 14th International Congress of the Hungarian Society for Microbiology, Oct. 9-11, 2003, Balatonfüred (poster)

Nagy, V.; Linden, J.C.; Tengerdy, R.P. and Szakacs, G.: Coexistence of filamentous fungi in semi-solid and solid state fermentation. 14th International Congress of the Hungarian Society for Microbiology, Oct. 9-11, 2003, Balatonfüred (poster)

Nagy, V.; Linden, J.C.; Tengerdy, R.P. and Szakacs, G.: Coexistence of filamentous fungi in semi-solid and solid state fermentation. 2003 SIM Annual Meeting, Aug. 10-14, 2003, Minneapolis, MN, USA (poster)

Nagy, V.; Szakács, G. and Pandey, A.: *Trichoderma harzianum* isolates capable of producing chitinase in solid substrate

fermentation. International Conference on the Emerging Frontiers at the Interface of Chemistry and Biology (ICB-2003), Apr. 28-30, 2003, Trivandrum, India (poster)