Engineering filamentous fungi to improve bio-reaction performance in fermentation systems: application to the production of food/technical enzymes

by

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Statutory Declaration

I hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

I confirm that no rights of third parties will be infringed by the publication of this thesis.

Rodrigo Mora-Lugo

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Signature

Bremen, September 1st, 2015
Rather than getting to know more, we do research to be less blind eye.

- Adapted from Sor Juana Ines de la Cruz
Abstract

Solid-state fermentation (SSF) processes with the filamentous fungus *Aspergillus sojae* ATCC 20235 (*A. sojae*) are known to yield good levels of pectinolytic enzymes which are valuable biocatalysts in the food industry. However, until now, there was scarce knowledge about rational strain improvement strategies for *A. sojae* in SSF. Particularly, in fungal-based SSF systems there is a poor availability of oxygen in inner regions of the fermented mass, leading to limited cell growth and protein production. Previous studies showed that the heterologous expression of the bacterial *Vitreoscilla* hemoglobin (VHb), commonly referred to as the “vgb/VHb technology”, can favor respiratory metabolism and cell growth of prokaryotes and eukaryotes under oxygen-limited conditions. The main purpose of this thesis was to genetically engineer *A. sojae* with VHb to enhance its cell growth and protein production under conditions of SSF.

To enable genetic modification of *A. sojae*, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) was successfully developed for *A. sojae*: Firstly, the successful integration of the transformed gene cassette, including the egfp gene encoding the green fluorescence protein (EGFP) into the genome of *A. sojae* was shown by polymerase chain reaction analyses. Secondly, expression of EGFP by fluorescence microscopy and Western blot was confirmed. A maximum of 32 mitotically-stable fungal transformants per $10^5$ conidia were obtained.

In a second study, the VHb gene (*vgb*) was successfully integrated into the genome of *A. sojae* using the established ATMT protocol. The transformed *A. sojae*
strain harboring vgb (A. sojae vgb+) yielded up to 33% more biomass and up to 60% more enzymatic units (U) of exo-pectinases and protease per gram of fermented mass (g) in comparison to its parental strain (A. sojae wt) in solid-state cultures in Erlenmeyer flasks.

In a follow-up study, fermentations were scaled-up (100 times) in a rotatory-drum reactor. The recombinant fungus produced up to 38% more pectinases and 33% more protease in comparison to its wild-type. For instance, out of three pectinase activities measured, exo-polygalacturonase yielded the maximum content of 726.2 U/g after six days (d) of fermentation in the A. sojae vgb+ cultures at reactor scale, which corresponds to a productivity of 121 U/g/d and specific activity of 204.6 U/mg. A technological application study demonstrated that the enzymatic extracts of A. sojae vgb+ clarify apple and blood orange juice with up to 37% and 50% more efficiency, respectively in comparison to the enzymatic extracts of A. sojae wt.

Overall, the results of this dissertation show that the vgb/VHb technology can improve cell growth and protein production of A. sojae in SSF, and that the protein complex of the recombinant A. sojae vgb+ was substantially more efficient in the clarification of fruit-based beverages compared to A. sojae wt. The novel strain improvement strategy developed for A. sojae is a good basis for further biotechnological developments with this filamentous fungus.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AS</td>
<td>acetosyringone</td>
</tr>
<tr>
<td>A. sojae</td>
<td>Aspergillus sojae ATCC 20235</td>
</tr>
<tr>
<td>A. sojae vgb+</td>
<td>Aspergillus sojae ATCC 20235 harboring the hemoglobin vgb gene</td>
</tr>
<tr>
<td>A. sojae wt</td>
<td>Aspergillus sojae ATCC 20235 (parental or wild-type strain)</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type of Culture collection</td>
</tr>
<tr>
<td>ATMT</td>
<td>Agrobacterium tumefaciens-mediated transformation</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine -5' triphosphate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNS</td>
<td>3,5-dinitrosalicylic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>GA</td>
<td>galacturonic acid</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GlcN</td>
<td>glucosamine</td>
</tr>
<tr>
<td>gpdA</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally recognized as safe</td>
</tr>
<tr>
<td>hph</td>
<td>hygromycin resistance gene</td>
</tr>
<tr>
<td>HPH</td>
<td>hygromycin B phosphotransferase</td>
</tr>
<tr>
<td>IM</td>
<td>induction medium</td>
</tr>
<tr>
<td>kanR</td>
<td>kanamycin resistance gene</td>
</tr>
<tr>
<td>LB</td>
<td>left border of T-DNA</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media</td>
</tr>
<tr>
<td>MC&lt;sub&gt;wb&lt;/sub&gt;</td>
<td>moisture content on wet basis</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)-ethanesulfonic acid</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>oriV</td>
<td>origin of replication</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>polygalacturonase</td>
</tr>
<tr>
<td>PMG</td>
<td>polymethylgalacturonase</td>
</tr>
</tbody>
</table>
List of abbreviations

PgdA \textit{Aspergillus nidulants} gpdA promoter
pRM-eGFP Donor vector for ATMT of \textit{A. sojae}
pRM-vgb Donor vector for ATMT of \textit{A. sojae}
PtrpC \textit{Aspergillus nidulants} trpC promoter
RB right border of T-DNA
RT room temperature
SD standard deviation
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
\textit{Sh ble} \textit{Streptalloteicus hindustanus} bleomycin gene (phleomycin resistance)
SmF submerged fermentation
SSF solid-state fermentation
T-DNA transfer DNA
TFA trifluoro acetic acid
TtrpC \textit{Aspergillus nidulants} trpC terminator
TrfA trans-acting gene trfA
UV ultraviolet
vgb \textit{Vitreoscilla} hemoglobin gene
VHb \textit{Vitreoscilla} hemoglobin protein
wt wild type

Units

\begin{tabular}{llll}
\textbf{d} & day & \textbf{mM} & millimolar \\
\textbf{Da} & dalton & \textbf{nm} & nanometer \\
\textbf{g} & gram & \textbf{rpm} & revolutions per minute \\
\textbf{h} & hour & \textbf{sec} & second \\
\textbf{kDa} & kilodalton & \textbf{U} & unit of enzymatic activity \\
\textbf{l} & liter & \textbf{v/v} & volume/volume \\
\textbf{M} & molar & \textbf{w/v} & weight/volume \\
\textbf{mg} & milligram & \textbf{°C} & degree Celsius \\
\textbf{min} & minute & \textbf{µg} & microgram \\
\textbf{ml} & milliliter & \textbf{µl} & microliter \\
\textbf{mm} & millimeter & & \\
\end{tabular}
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CHAPTER 1

General introduction

& Scope of this thesis
Abstract

This introductory Chapter is about the fundamentals and recent applications of solid-state fermentation (SSF) processes with filamentous fungi. The focus lies principally on SSF for production of pectinolytic enzymes with the fungus *Aspergillus sojae* ATCC 20235 (*A. sojae*). For instance, pectinolytic enzymes or pectinases are incredibly valuable in biotechnology due to their various applications in industrial processes. Genetic engineering strategies to enhance bioprocesses of microbial strains are also reviewed in this initial Chapter, and recent applications of these approaches are presented, including applications for filamentous fungi. It is pointed out particularly how the *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique and the “vgb/VHb technology” could enhance the SSF of *A. sojae*. The fundamentals and recent advances on these technologies are reviewed. On one hand, ATMT has been developed as a reliable molecular tool for genetic transformation of a wide variety of fungal species, but its application in *A. sojae* has not been explored. On the other hand, the vgb/VHb technology, consisting of heterologous expression of the bacterial *Vitreoscilla* hemoglobin (VHb) gene (*vgb*), has been shown to enhance cell growth and protein production in a wide variety of bacterial and yeast strains. However, the vgb/VHb technology has remained poorly explored in filamentous fungi, especially in combination with SSF. A detailed outline of this thesis is described in the Section “scope of this thesis” and consisted –in short- in the development and evaluation of a rational strain improvement strategy for *A. sojae* in SSF based on the ATMT and vgb/VHb technologies. The research presented forms part of the larger project entitled “PGSYS EXCHANGE” which represents an international cooperation between several partners, including academic research groups and private companies, all aiming to explore the potential of *A. sojae* for commercially production of pectinases.
1.1 General introduction

1.1.1 Solid state fermentation of filamentous fungi

Solid state fermentation (SSF) is a well-known process for the production of proteins and other biomolecules with filamentous fungi (Table 1.1). Solid-state cultures can be defined as the growth of microorganisms on moist, water-insoluble solid substrates in the absence or near-absence of free liquid (Krishna 2005). On the contrary, submerged fermentation (SmF) which is still the most used by enzyme manufactures, is defined by the microorganism growth under the surface of the liquid medium or nutrient broth. Filamentous fungi are referred as favored for SSF applications, as they hold a distinctive capacity to adhere and assimilate complex solid substrates. Some bacteria are also capable to grow under SSF conditions, but filamentous fungi are better adapted to solid substrates because hyphal growth allows them to penetrate the substrates better (Figure 1.1). In addition, filamentous fungi possess a good tolerance to low water activity ($A_w$) and high osmotic pressure (Krishna 2005). These characteristics, among others, have placed SSF with filamentous fungi as attractive alternatives to SmF processes.

There has been an increased number of studies about SSF with filamentous fungi in the last years, citing various advantages of these bioprocesses over the more traditional SmF (Aguilar et al. 2008; De la Cruz Quiroz et al. 2014; Viniegra-Gonzalez et al. 2003). These are listed as higher productivity per reactor volume, lower capital costs, less space requirements, simpler equipment, and easier downstream processing. Another very important advantage is the possibility to use agricultural and agro-industrial residues as substrates in solid-state cultures, which are converted into bulk chemicals and into products with high commercial value such as organic acids, alcohol and enzymes (Table...
1.1. Utilization of agro-industrial residues as substrates helps also in solving pollution, which otherwise cause their disposal. Some of the applications of SSF with filamentous fungi in the last years have been reviewed by Singhania et al. (2009) and Martins et al. (2011).

Limitations or challenges associated to SSF processes with filamentous fungi had, to some extent, reduced their exploitation. For instance, an important challenge associated is the downstream processing. Separation of product from the solids is a technically and economically challenging process, and although there is much advent in biochemical engineering, SSF is generally used only for the production of less-purity-needed metabolites. Moreover, the design of the bioreactor for large-scale production represents a challenge. Factors such as fungal strain, substrate, type of product, etc. have to be taken into consideration for the bioreactor design. In addition to this, a major drawback in a SSF process with an aerobic microorganism is the lower diffusion of

Figure 1.1. Conceptual representation of filamentous fungal growth on a solid substrate. The penetrative hyphae and part of the anaerobic wet hyphal layer are in direct contact with the substrate that serves as a carbon (C) and nitrogen (N) source. The aerial hyphae are mainly responsible for oxygen (O2) uptake. The polygonal shapes illustrate the gradients in oxygen and substrate concentrations. Adapted from te Biesebeke (2005).
oxygen in the biomass, which in turn limits the production of proteins (Stark et al. 2011; te Biesebeke et al. 2006; Wei and Chen 2008). The low availability of oxygen affects considerably the viability of the fungal host during its culturing. In this respect, mechanisms to improve the oxygen uptake in the fungal host and thus alleviate the hypoxic conditions found in a typical SSF process are considered of great interest. For instance, co-expression of globins has shown to be a reliable strain improvement strategy in several aerobic hosts to alleviate the hypoxic conditions found in a typical SSF process.

Table 1.1. Examples of solid-state fermentation of filamentous fungi.

<table>
<thead>
<tr>
<th>Fungal specie</th>
<th>Substrate</th>
<th>Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus awamori</em></td>
<td>Babassu cake</td>
<td>Hydrolases</td>
<td>Castro et al. (2015)</td>
</tr>
<tr>
<td><em>Aspergillus japonicus</em></td>
<td>Waste from castor bean</td>
<td>Cellulase</td>
<td>Herculano et al. (2011)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>Cassava bagasse</td>
<td>Citric acid</td>
<td>Ramachandran et al. (2008)</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em> S2</td>
<td>Soybean meal, fish powder waste and wheat gluten waste</td>
<td>Alpha-amylase</td>
<td>Sahnoun et al. (2015)</td>
</tr>
<tr>
<td><em>Monascus purpureus</em></td>
<td>Jack fruit seed</td>
<td>Pigment</td>
<td>Babitha et al. (2007)</td>
</tr>
<tr>
<td><em>Penicillium simplicissimum</em></td>
<td>Soy cake</td>
<td>Lipase</td>
<td>Di Luccio et al. (2004)</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em> NRRL 1891</td>
<td>na</td>
<td>Phytase</td>
<td>Hama et al. (2007)</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td>Wheat bran</td>
<td>Cellulase</td>
<td>Singhania et al. (2007)</td>
</tr>
</tbody>
</table>
(Stark et al. 2011; Wei and Chen 2008). Interest in SSF of filamentous fungi is expected to substantially grow, as novel strain improvement strategies overcome typical limitations of SSF processes with these hosts.

SSF with fungal hosts are well suited for the production of various enzymatic complexes composed of multiple enzymes. Pectinases complexes are among some of the most common enzymes produced in fermentation processes with fungi (Gottumukkala et al. 2011).

1.1.2 Pectinases and their biotechnological importance

In the enzymes industry, pectinases or pectinolytic enzymes strongly contribute to the global enzyme market with increasing demand. They are one of the highly sold enzymes and alone contribute to 40% share of all food enzymes (Adapa et al. 2014). Pectinases are a heterogeneous group of related enzymes that hydrolyze pectic substances or pectins. Pectins are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) that are present in the plant kingdom. Apart from cellulose, pectin is the second most abundant carbohydrate in a plant tissue, accounting for 0.5–4.0% of the fresh weight of plant material (Jayani et al. 2005), and is responsible for conferring the rigidity to the fruits. It acts as a “mortar” or “cement” between the cellular “bricks” of cellulose (Figure 1.2), which is broken down by naturally occurring pectinase, when the fruit ripens making it soft (Madden 2000). Contrary to the proteins, lipids and nucleic acids, pectic substances do not have a defined molecular weight. The relative molecular masses of pectic substances range from 25 to 360 kDa.
The breaking down of pectin substances requires the combined action of several pectinolytic enzymes. Among the most extensively studied pectinases are exo-polygalacturonases (exo-PG; EC 3.2.1.67), polymethylgalacturonases (PMG), endo-polygalacturonases (endo-PG; EC 3.2.1.15), pectate lyases (EC 4.2.2.2), pectin lyases (PL; EC 4.2.2.10) and pectin methyl esterase (PME; EC 3.1.1.11) with different mode of action (Figure 1.3). For instance, PGs hydrolyze the O-glycosyl bonds in pectin’s polygalacturonan network, resulting in alpha-1,4-polygalacturonic residues. These enzymes have also different hydrolytic modes of action known as Exo- and EndoPGs. Exo-PGs hydrolyze at the non-reducing end of the polymer, generating a monosaccharide galacturonic acid. Endo-PGs hydrolyze in a random fashion along the polygalacturonan network, generating oligogalacturonides (Ferrari et al. 2013).

The production of pectinases in a cost effective and productive way is of big interest for the industrial enzymes market. Commonly used as enzymatic extracts...
powders (Figure 1.4), pectinases are widely used in the beverage industry due to their ability to improve pressing, clarification and filtration of concentrated fruit juices. Pectinases have also application in paper, pulp, waste management, animal feed and textile industry (Silva et al. 2002). These enzymes are commonly used in processing of fruits and vegetables, in the production of wine, in the extraction of olive oil and fermentation of tea and coffee (Castilho et al. 2000; Silva et al. 2005). As for all industrial applications known for pectinolytic enzymes, enzymatic commercial

Figure 1.3. Mode of action of pectinases a) PMG, polymethylgalacturonases; PG, polygalacturonases (EC 3.2.1.15). R = H for PG and CH3 for PMG; b) PE, pectinesterase (EC 3.1.1.11); c) PL, pectin lyase (EC 4.2.2.10); PGL, polygalacturonase lyase. R = H for PGL and CH3 for PL. The arrow indicates the place where the pectinase reacts with the pectic substances. Adapted from Gummadi & Panda (2003).
preparations derived from filamentous fungi are recognized for their high biotechnological value (Lara-Marquez et al. 2011). The filamentous fungi most frequently used for the production of pectinases are *Trichoderma reesei* and a number of strains of *Penicillium* and *Aspergillus* (Aro et al. 2005; Cardoso et al. 2007). For instance, production of polygalacturonases (PGs) by *Aspergillus niger* is possibly the best studied bioprocess with commercial significance. PGs are among the most studied and widely used commercial pectinases (Castilho et al. 2000; Couto and Sanroman 2006). The growing interest for PGs and other pectinases has led to the study of filamentous fungi, such as various *Aspergillus* species, as potential biomanufactories for pectinases production (Blandino et al. 2002; Ustok et al. 2007).

1.1.3 Filamentous fungi as cell factories for biomolecules production

Besides their common use for food preparation or preservation, filamentous fungi have been developed as great biofactories for the production of proteins and other

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**Figure 1.4. Pectinases application.** Example of a commercial pectinase complex (enzymatic extract) used for fruit and vegetable clarification. Reference: NeoSources Inc. and Chembond Chemicals.
biomolecules with industrial value. In ancient times, humans used filamentous fungi, often unknowingly, as food sources, including in the preparation of leavened bread and fermented juices. However, the recent knowledge and understanding gained about these eukaryotic microorganisms have led us further in their utilization. As a result, the role of filamentous fungi for human needs is steadily growing. A great number of biomolecules with commercial value are produced in bioprocesses with filamentous fungi (Nevalainen and Peterson 2014). For example, in the biotechnology industry, filamentous fungi are used to produce in the large-scale a wide variety of products such as human therapeutics (e.g. vitamins, antibacterial and antifungal agents) and specialty chemicals (e.g. commercial enzymes and organic acids), which together represent billions of euros in annual sales (Punt et al. 2002).

Filamentous fungi are attractive as production hosts because of their natural ability to secrete large amounts of proteins (mainly hydrolytic enzymes) into the growth medium. Eukaryotic post-translational protein processing machinery is an added bonus, of using fungi as expression hosts for proteins requiring elaborate posttranslational modification -for example, in the form of protein glycosylation, proteolytic cleavage or formation of multiple disulfide bonds (Nevalainen, Te'o et al. 2005). Moreover, the particular morphological growth phenotypes of filamentous fungi on solid substrates have been considered an advantage over other microorganisms systems to increase protein production in diverse fermentation processes (Viniegra-Gonzalez et al. 2003).
1.1.3.1 Morphology and protein secretion in filamentous fungi

Filamentous fungi grow as hyphae, which are cylindrical, thread-like structures 2–10 µm in diameter and up to several centimeters in length. A typical lifecycle for an industrially applied filamentous fungus (Figure 1.5) includes vegetative hyphae and asexual conidia (or spores) that germinate forming new hyphae, or more rarely, production of sexual spores that undergo meiosis (Nevalainen and Peterson 2014).

The cellular morphology of filamentous fungi is the result of growth by the extension of existing hyphae and the formation of branches along the existing hyphae that may contain multiple nuclei and extend at their tips. As reviewed by Dynesen and

![Figure 1.5. Filamentous fungi lifecycle. Hyphae are main mode of vegetative growth and are collectively called mycelium. Conidia are borne on specialized stalks called conidiophores. Under specific conditions, conidia germinates to form the hyphae and fungal mycelia.](image)
Nielsen (2003) and te Biesebeke (2005), the grow of hyphal tips includes actin cytoskeleton, signal transduction pathways, cell wall formation, membrane biosynthesis and the secretion pathway. There are various molecular mechanisms that control the growth of filamentous fungi. The secretion of proteins in fungi is regulated by a vesicular transport system composed of secretory vesicles. These vesicles play also a role in the transportation of proteins that maintain cellular morphology (cell wall and membrane assembly, cell signaling). An extraordinary feature of filamentous fungi is a specialized organelle located at the hyphal apex called the spitzenkorper that has been proposed to serve as a vesicle supply center. Besides its role in protein secretion, the microtubule-dependent movement and positioning of vesicles of the spitzenkorper are essential for generation of shape of the hyphae. A schematic drawing that illustrates the relation between protein secretion and cellular morphogenesis of filamentous fungi is presented in Figure 1.6.

Enzymes that are produced by filamentous fungi are transported to the exterior of the cell via the secretory pathway. As reviewed by Punt et al. (1994), Archer and Peberdy (1997) and Conesa et al. (2001), compared to the knowledge of other eukaryotic organisms, little is known about the filamentous fungal secretory pathway through which secreted proteins are transported to the exterior of the cell. However, it is generally accepted that the secretory pathway is similar to those of yeasts and higher eukaryotes (Conesa et al. 2001; Moir and Mao 1990).
Figure 1.6. Schematic drawing depicting the general morphology of filamentous fungi (top) and proposed pathways of protein synthesis and secretion of a hyphal tip (insert below). Proteins are produced by the ribosomal complex, translocated into the endoplasmic reticulum (ER) where protein folding takes place, and travel in secretory vesicles (sv) to the Golgi-complex. Secretory proteins might be post-translationally modified in the ER or in the Golgi-complex. Secretory vesicles carry the modified proteins to the growing hyphal tip for apical secretion, or possibly to the septa in an alternative secretory pathway. The horizontal arrow indicates the direction of hyphal growth. Adapted from Nevalainen and Peterson (2014).
1.1.3 Recombinant protein production in filamentous fungi

The study of filamentous fungi as host for the production of heterologous proteins has been actively explored in the last years (Nevalainen and Peterson 2014; Su et al. 2012). There are a growing number of successful foreign proteins, either from prokaryotic or eukaryotic sources expressed in various fungal hosts. Additionally, it is well-known that filamentous fungi have a better capacity to secrete proteins compared to higher eukaryotes and yeasts, such as *Saccharomyces cerevisiae* (Conesa et al. 2001; Moir and Mao 1990). For instance, a yield of 30 g/l of extracellular protein can be obtained with specific *Aspergillus* and *Trichoderma* strains (Durand et al. 1988; Finkelstein et al. 1989). In contrast, yields in the grams-per-liter range are only obtained with a few yeast strains (*Pichia, Hansenula*) used for extracellular protein production (Werten et al. 1999; Wyss et al. 1999). The particular hyphal growth in fungal morphology, which is not found in either yeast such as *S. cerevisiae*, *Pichia* or in higher eukaryotic organisms, had been considered a contributor factor to the higher protein secretion capacity in filamentous fungi.

Some examples of successfully heterologous gene products expressed in filamentous fungi are listed in Table 1.2. However, this list goes far beyond as a great number of studies describing heterologous expression in filamentous fungi are continuously published. As observed in this table, the expression level of a certain biomolecule is commonly variable in each fungal strain. The suitability of each fungal strain for expression of heterologous products is regarded as specific in this sense. Nevertheless, it has been shown that different fungal strains may also have similar protein yields. For example, amyloglucosidase (EC 3.2.1.3), a major fungal enzyme in
terms of bulk, is secreted at similar levels in *A. niger* and *T. reesei*, over 20 g/l in the culture media (Jeenes et al. 1991). As new successful studies of successful recombinant proteins expressed in filamentous fungi appear, new ways to approach the fungal recombinant protein technology are facilitated (Ward 2012). In this respect, modern

**Table 1.2. Examples of heterologous gene products derived from filamentous fungi.**

<table>
<thead>
<tr>
<th>Specie</th>
<th>Biomolecule</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus awamori</em></td>
<td>Sweet protein thaumatin II</td>
<td>9.6 mg/l</td>
<td>Moralejo et al. (1999)</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>Human lactoferrin</td>
<td>5 mg/l</td>
<td>Ward et al. (1992)</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td><em>Gaeumannomyces graminis lipoygenase</em></td>
<td>n.a.</td>
<td>Heshof et al. (2014)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>IgG1, trastuzumab</td>
<td>900 mg/l</td>
<td>Ward et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>IgG1, Hu1D10</td>
<td>200 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fab antibody fragment</td>
<td>1200 mg/l</td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Human interleukin 6</td>
<td>150 mg/l</td>
<td>Punt et al. (2002)</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Amyloglucosidase</td>
<td>&gt;20 g/l</td>
<td>Jeenes et al. (1991)</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Bovine chymosin</td>
<td>150 mg/kg wheat bran</td>
<td>Tsuchiya et al. (1994)</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>Bovine RNase A</td>
<td>0.36 mg/l</td>
<td>Allgaier et al. (2010)</td>
</tr>
<tr>
<td><em>Talaromyces cellulolyticus</em></td>
<td>Hyperthermophilic cellulases</td>
<td>&gt;100 mg/l</td>
<td>Kishishita et al. (2014)</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>Serine protease</td>
<td>&gt;16 U/ml</td>
<td>Liu and Yang (2013)</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td>Barley endopeptidase B</td>
<td>500 mg/l</td>
<td>Saarelainen et al. (1997)</td>
</tr>
<tr>
<td><em>T. reesei</em></td>
<td><em>A. niger</em> lipase</td>
<td>310 mg/l</td>
<td>Qin et al. (2012)</td>
</tr>
<tr>
<td><em>T. reesei</em></td>
<td>amyloglucosidase</td>
<td>&gt;20 g/l</td>
<td>Jeenes et al. (1991)</td>
</tr>
</tbody>
</table>

n.a. = not available in the study.
biotechnologists combine experience gained from these successful studies with new scientific information being generated through genomics and proteomics research to be better prepared to extend biomanufacturing capabilities of filamentous fungi for recombinant proteins.

1.1.3.3 *Aspergillus*

Filamentous fungi that dominate the market as production hosts are the ones belonging to the genus *Aspergillus*. There are about 250 named species in the genus *Aspergillus* (Geiser et al. 2007), and as of 2015, eleven genomes of the most important industrial and medical Aspergilli have been sequenced, making this genus one of the best studied among fungi. The last *Aspergillus* genome sequenced corresponds to *A. kawachi*, a traditionally koji mold used for brewing alcoholic beverages in Japan (Futagami et al. 2011). *Aspergillus* stands out from other microbial cell factories of bacterial or yeast origin due to its ability to tolerate extreme cultivation conditions (Kis-Papo et al. 2003; Machida and Gomi 2010). For example, *Aspergillus* can be cultivated over a wide range of temperatures (10-50°C), pH (2-11), salinity (0-34%), water activity (0.6-1) and under oligotrophic or nutrient-rich conditions (Meyer et al. 2011). Due to their specific growth properties, *Aspergillus* have been used for diverse fermentations processes, and therefore, have built a long history in biotechnology as expression platforms for the production of food ingredients, pharmaceuticals and enzymes. Recent achievements and breakthroughs in the research of *Aspergillus* strains assure this genus a dominant place among other microbial cell factories.
The most industrially exploited Aspergillus are A. niger, A. oryzae and A. nidulans strains (Kniemeyer 2011; Meyer et al. 2011). Consequently, most information on heterologous protein expression has come from studies using these particular fungi. However, the study of less common Aspergillus species or filamentous fungi for the production of industrial proteins stands as an active field with more reports exploring their utility (Ward 2012).

Aspergillus sojae has been developed as a platform for protein production, in a similar manner as for several other industrially-important Aspergillus species, such as A. niger and A. oryzae (Heerikhuisen et al. 2008). One aspect that makes this filamentous fungus attractive is its generally recognized as safe (GRAS) status. A. sojae, as its close relative A. oryzae, are well-known koji molds widely used for the production of oriental food and beverage products such as soy sauce, sake (rice wine) and miso (soybean paste) (Ushijima et al. 1990). Their long history of (safe) use has been an advantageous characteristic over other toxigenic (aflatoxin-producing) filamentous fungi for many bioprocess applications (Matsushima et al. 2001). Moreover, these fungi have the ability to secrete large amounts of hydrolytic enzymes. Diverse homologous and heterologous proteins have been expressed in A. sojae (Heerikhuisen et al. 2008), and its potential for production of commercially important enzymes such as pectinases (Heerd et al. 2012 and 2014b), mannanases (Ozturk et al. 2010), or glutaminases (Ito et al. 2013), has been demonstrated.
1.1.3.4 *Aspergillus sojae* for pectinases production in fermentation processes

Production of pectinolytic enzymes with the *Aspergillus sojae* strain ATCC 20235 (*A. sojae*) has been broadly studied in the last years. For instance, in fermentation studies with this fungus, the effect of different parameters for optimization of its bioprocess has been evaluated through advanced statistical design tools (Demir et al. 2012; Gogus et al. 2006; Tari et al. 2008; Tari et al. 2007; Ustok et al. 2007). In most of these studies, the effect of solid substrate kind, inoculum, incubation time and acidity of the media were evaluated for the production of pectinolytic enzymes. In a recent study (Heerd et al. 2012), the pectinolytic enzyme production of three *A. sojae* strains by means of solid-state fermentation (SSF) was evaluated and enzymatic extracts from these bioprocesses were assayed for pectinases activities exo-PMG, endo-PMG, exo-PG and endo-PG. Among the *A. sojae* strains assessed for pectinase production, the ATCC 20235 strain had the highest pectinolytic activities with maximum yields of 33.4 U/g of exo-PMG, 28.3 U/g of exo-PG, 32.9 U/g of endo-PMG and 30.1 U/g of endo-PG, and was therefore considered as the best candidate for industrial pectinase production. Moreover, descending mutants of this fungus obtained through strain improvement strategies based on physical (ultraviolet irradiation) and chemical mutagens, have improved its pectinases titers by 2.4-fold in SmF and SSF (Heerd et al. 2014a). The potential of some of these pectinases overproducing mutants was further explored by measuring various pectinases activities as exo-PG, endo-PG, exo PMG and PL using two different carbon sources in SSF (Mata-Gomez et al. 2014). In the most recent study with *A. sojae* ATCC 20235, it was demonstrated that optimization of crude plant compounds, such as wheat bran and sugar beet pulp as substrate, increased 6.9 times the pectinases production of this fungus in comparison to *A. sojae* CBS 100928 in SSF (Heerd et al. 2014b). Specifically, an
enzyme yield of 909.5 ± 2.7 U/g was obtained by *A. sojae* ATCC 20235 after 8 days at 30°C applying 30% sugar beet pulp as inducer substrate in combination with wheat bran as medium wetted at 160% with 0.2 M HCl. The listed studies were of significance to increase protein production of *A. sojae* ATCC 20235 in SSF, and provide a good basis for follow-up studies.

There are not many reports available concerning strain improvement strategies for *A. sojae* ATCC 20235 and pectinases production. As previously mentioned, the earlier studies have been focused on media design and fermentation process optimization applying advanced statistical design tools. However, the strain improvement approach has been less explored. The few studies about improved *A. sojae* strains are based on repeated rounds of mutagenesis and screening for improved filamentous fungi using physical (ultraviolet irradiation) and chemical mutagens (Demir et al. 2012; Heerd et al. 2014a). These classical strain development approaches are robust and widely acceptable, but labor intensive. Furthermore, the induced changes via the methods are not easily traceable or movable to another host strain, and the organisms used for high production of a desired compound are often genetically uncharacterized. Alternative studies to these classical strain development approaches are scarce for this fungus (e.g. Ref1, Ref2). As explained in detail in Chapter 2 and Chapter 3, this thesis deals with the development of a methodology based on genetic molecular tools to systematically improve *A. sojae* for SSF applications.
1.1.3.5 Fungal strain development to enhance bioprocesses

The science and technology of manipulating and improving fungal or other microbial strains in order to enhance their metabolic capabilities for biotechnological applications are referred as strain improvement strategies. As for all commercial fermentation processes, improved microbial strains have gone through strain improvement campaigns to achieve overproduction of industrial products. However, the overproduction of primary or secondary metabolites is a complex process, which in addition requires mastery of the fermentation process for each new strain, as well as sound engineering know-how for media optimization and the fine-tuning of process conditions (Sharma et al. 2009; Ward 2012). Conventionally, strain improvement has been achieved through mutation, selection, or genetic recombination.

Recombinant DNA technology approaches had enabled targeted mutagenesis to create desired phenotypes in filamentous fungi. These molecular techniques such as genetic transformation and protein engineering have been developed as further refinement of fungal products and productions systems (Nevalainen 2001). Within their use various metabolic processes have been enhanced through knocking out genes or overproduction of specific molecules (Meyer et al. 2010; Nevalainen and Peterson 2014; Nevalainen 2001; Nevalainen et al. 2005; Sharma et al. 2009). Moreover, these methodologies overcome disadvantages associated to classical mutagenesis strategies based on induced mutation via physical and chemical mutagens, which are listed as lack of genetic characterization and mobility of induced changes to another host. Genetic engineering approaches for filamentous fungi require fundamental knowledge of the organism such as physiology and culturing. Moreover, the utilization of these methods is
based on the availability of transformation procedures, which in the case of filamentous fungi, are scarce.

**1.1.3.6 Transformation of filamentous fungi**

The attraction of filamentous fungi for protein expression studies is based on the gained capacity to genetically transform them. The availability of transformation procedures for filamentous fungi plays a key role to enable gene manipulation strategies for purposes ranging from the elucidation of fundamental biological phenomena to the improvement of commercial biological processes (Nevalainen and Peterson 2014). However, in filamentous fungi the number of efficient transformation methods is not as vast as for other microorganisms such as bacteria or yeast. Filamentous fungi are multicellular microorganisms characterized by a thick cell wall, and in most cases, they lack the ability to maintain a self-propagating plasmid. These characteristics constrain the development of efficient transformation techniques for filamentous fungi compared with *E. coli* and *S. cerevisiae*, two model organisms commonly used for heterologous gene expression (Lubertozzi & Keasling, 2009).

In the last two decades, the most common transformation method used in filamentous fungi has been the calcium ion-polyethylene glycol (PEG) procedure that requires first the conversion of the target organism to spheroplasts for the uptake of the DNA and subsequently the regeneration of cells walls for the resumption of hyphal growth. Other methods, including electroporation and particle bombardment (biolistic transformation), are less labor intensive and inherently faster, but many times they are not applicable to specific fungal strains. A usual outcome with common transformation
systems for filamentous fungi is low-efficiency transformation rates. Furthermore, some common disadvantages of these methods include expensive equipment and long optimization times to generate protoplast in good quality and quantity for biolistic and protoplast transformation, respectively (Ruiz-Diez 2002). In the last years, research on novel transformation techniques for filamentous fungi has been intensified, with an increase in the number of new filamentous fungi genetically transformed. In this regard, genetic transformation mediated by the bacterium Agrobacterium tumefaciens has emerged as a reliable method for various fungal species, among other common transformation procedures (Michielse et al. 2005; Nageatte et al. 2004).

### 1.1.4 Agrobacterium tumefaciens mediated transformation

The Agrobacterium tumefaciens-mediated transformation (ATMT) is a mutagenesis method routinely used for transformation of plants. The applicability of this method for transformation of filamentous fungi was first described by de Groot in (1998). Since then, ATMT had gained important terrain as a reliable tool for mutagenesis studies in filamentous fungi (Tkacz et al. 2004).

As pointed out by Frandsen (2011), ATMT has become a common technique to conduct molecular genetics experiments such as random mutagenesis, targeted genome modification and random integration and introduction of reporter genes (e.g., GFP, RFP and GUS). ATMT is based on the property of Agrobacterium tumefaciens to transfer part of its Ti vector, the transfer DNA (T-DNA), to plants cells during tumorigenesis (Figure 1.7). The T-DNA is delimited by 25-bp directional imperfect repeat sequences, known as the left and right border (LB and RB, respectively). At least 25 vir genes on the Ti
plasmid are necessary for tumor induction. Phenolic compounds, such as acetosyringone and other released compounds by the plant cells regulate the vir genes activation. The induced vir genes encode the T-DNA transfer machinery of A. tumefaciens, responsible for the transformation of the eukaryotic cells (Hooykaas et al. 1994). ATMT is routinely used for the genetic modification of a wide range of plant species. However, the applicability of this technique had gone nowadays beyond the plant kingdom providing a promising alternative to traditional transformation methods for filamentous fungi such as protoplasting and electroporation methods. These traditional methods besides being limited to some fungal species are commonly referred as time-consuming and low-efficiency techniques with no guarantee of success (Meyer et al. 2003; Meyer et al. 2010).

ATMT has been used in filamentous fungi since more than 15 years. The efficiency of A. tumefaciens to transfer its T-DNA has been demonstrated in a wide variety of fungal species. On this subject, Tkacz et al. (200), Michielse et al. (2005) and Fradsen, (2008 and 2011) and have broadly reviewed many studies. The Table 1.3 shows various fungal strains successfully transformed by ATMT. These include fungal species such as Ascomycetes, Basidiomycetes and Oomycetes. As observed in this table, main factors to be evaluated when setting up an ATMT for a given fungus, are the selection marker (e.g. antibiotic resistance gene) and the starting material to be transformed (e.g. conidia, mycelium or protoplast). It can be noticed that the optimal factors for successful ATMT vary among the diversity of fungi.
Figure 1.7. T-DNA transfer process into the plant cell by Agrobacterium tumefaciens. A. tumefaciens can transfer part of its Ti plasmid, the T-DNA to the plant cells. Integration of T-DNA into the plant nuclear genome is induced by a set of virulence (vir) genes (a-d), which are located in the Ti plasmid. Phenolic compounds produced by the plant induce the vir genes that encode the T-DNA transfer machinery of A. tumefaciens.
Table 1.3. Example of fungal species genetically transformed with *Agrobacterium tumefaciens*. Adapted from Michielse et al. (2005).

<table>
<thead>
<tr>
<th>Specie</th>
<th>Selection marker</th>
<th>Starting material</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basidiomycetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Agaricus bisporus</em></td>
<td><em>hph</em></td>
<td>Germinating conidia, mycelium, and fruiting body tissue</td>
<td>Vacuum infiltration used for fruiting body tissue</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td><em>NAT</em></td>
<td>Yeast-like cells</td>
<td></td>
</tr>
<tr>
<td><em>Hebeloma cylindrosporum</em></td>
<td><em>hph, Sh ble</em></td>
<td>Mycelium</td>
<td></td>
</tr>
<tr>
<td><em>Hypholoma sublateritium</em></td>
<td><em>hph</em></td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Omphalotus olearius</em></td>
<td><em>hph</em></td>
<td>Mycelium</td>
<td></td>
</tr>
<tr>
<td><em>Paxillus involutus</em></td>
<td><em>Sh ble</em></td>
<td>Mycelium</td>
<td></td>
</tr>
<tr>
<td><em>Suillus bovinus</em></td>
<td><em>hph, Sh ble</em></td>
<td>Mycelium</td>
<td></td>
</tr>
<tr>
<td><strong>Ascomycetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergilus awamori</em></td>
<td><em>hph, ble, Aa pyrG</em></td>
<td>Protoplasts, conidia</td>
<td>Targeted gene disruption of orotidine-5'-monophosphate dehydrogenase (pyrG) and glucosamine/fructose-6-phosphate aminotransferase (gfaA)</td>
</tr>
<tr>
<td><em>Aspergilus niger</em></td>
<td><em>hph, Aa pyrG</em></td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td><em>hph</em></td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Beauveria dermatiditis</em></td>
<td><em>hph, Hc ura5</em></td>
<td>Yeast-like cells, germinated conidia</td>
<td></td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td><em>hph</em></td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Calonectria morganii</em></td>
<td><em>hph</em></td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td><em>hph</em></td>
<td>Yeast cells</td>
<td></td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td><em>hph</em></td>
<td>Yeast cells</td>
<td></td>
</tr>
<tr>
<td><em>Ceratocystis resinifera</em></td>
<td><em>hph</em></td>
<td>Germinated conidia</td>
<td>Targeted gene disruption of a polyketide synthase (PKS1)</td>
</tr>
<tr>
<td><em>Coccidiodes immitis</em></td>
<td><em>hph</em></td>
<td>Protoplasts, germinated conidia</td>
<td></td>
</tr>
<tr>
<td><em>Coccidiodes posadasii</em></td>
<td><em>hph</em></td>
<td>Germinated conidia</td>
<td>Targeted gene disruption of a 1,3-beta-glucan synthase (FKS1)</td>
</tr>
<tr>
<td>Organism</td>
<td>Marker(s)</td>
<td>Material</td>
<td>Description</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------</td>
<td>-------------------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Colletotrichum gloeosporioides</td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td>Coniothyrium minitans</td>
<td>hph</td>
<td>Germinated conidia</td>
<td></td>
</tr>
<tr>
<td>Cryphonectria parasitica</td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>hph, nptII</td>
<td>Conidia</td>
<td>Targeted gene disruption of a kinase (SNF) in combination with the two-markerv gene technique</td>
</tr>
<tr>
<td><em>Fusarium venenatum</em></td>
<td>hph</td>
<td>Conidia, freeze-dried mycelium</td>
<td>Targeted gene disruption of polyketide synthase (PKS1)</td>
</tr>
<tr>
<td><em>Glarea lozoyensis</em></td>
<td>hph</td>
<td>Mycelium</td>
<td>Liquid co-cultivation</td>
</tr>
<tr>
<td>Helminthosporium turcicum</td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Histoplasma capsulatum</em></td>
<td>hph, <em>Hc ura5</em></td>
<td>Yeast-like cells, germinated conidia</td>
<td>Targeted gene disruption of N-(5’-phosphoribosyl)lanthranilate isomerase (TRP1) and a centromereassociated factor gene (SKP1)</td>
</tr>
<tr>
<td><em>Kluyveromyces lactis</em></td>
<td><em>Sc ura3</em></td>
<td>Yeast cells</td>
<td>Targeted gene disruption of ABC transporter (LmAABC4), histidine kinase gene (LmHK1) and peptide synthetase (sirP) in combination with the two-marker gene technique</td>
</tr>
<tr>
<td><em>Leptosphaeria maculans</em></td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Magnaporthe grisea</em></td>
<td>hph, nptII</td>
<td>Conidia</td>
<td>Targeted gene disruption of a class II hydrophobin (MHP1) in combination with the two-marker gene technique</td>
</tr>
<tr>
<td><em>Metarhizium anisopliae</em> var. acridum</td>
<td>beta-tubulin</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Monascus purpureus</em></td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Mycosphaerella fijensis</em></td>
<td>hph</td>
<td>Mycelium</td>
<td></td>
</tr>
<tr>
<td><em>Mycosphaerella graminicola</em></td>
<td>hph</td>
<td>Protoplasts, yeast-like cells</td>
<td>Targeted gene disruption of ABC transporter genes (MgAtr1, MgAtr2)</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma floccosum</em></td>
<td>hph</td>
<td>Yeast-like cells</td>
<td>RNA interference of a polyketide synthase gene (PKS)</td>
</tr>
<tr>
<td><em>Ophiostoma piliferum</em></td>
<td>hph</td>
<td>Yeast-like cells</td>
<td>Targeted gene disruption of subtilase albin1</td>
</tr>
<tr>
<td><em>Paecilomyces fumosoroseus</em></td>
<td>hph</td>
<td>Conidia</td>
<td></td>
</tr>
<tr>
<td><em>Paracoccidioides brasiliensis</em></td>
<td>hph</td>
<td>Yeast-like cells</td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td><em>trp1, Sc ura3, aph1</em></td>
<td>Yeast cells</td>
<td>Autonomous replication of the T-DNA</td>
</tr>
<tr>
<td><em>Trichoderma atroviride</em></td>
<td>hph</td>
<td>Conidia</td>
<td>Reduction background growth by overlay method, targeted gene disruption of MAP kinase (tmk1) and alpha subunit heterotrimeric G</td>
</tr>
</tbody>
</table>
The recent examples of successful ATMT in filamentous fungi include *Penicillium digitatum* to generate knockout mutants for the pectin lyase (PnI1 and naphthalene dioxygenase (Ndo1) genes (Lopez-Perez et al. 2014), and *Tuber borchii* to study gene integration and genetic tractability on this fungus (Brenna et al. 2014).
Regarding *Aspergillus* species, which are well-known for biotechnological applications, *A. carbonarius* (Crespo-Sempere et al. 2011) and *A. terreus* (Wang et al. 2014a) are among some of the more recently investigated species. However, to my knowledge, the applicability of this approach in *A. sojae* has not been explored so far. As earlier mentioned, this fungus has been developed as a platform for protein production; therefore, the setup of an ATMT procedure for *A. sojae* is relevant for mutagenesis and expression studies on this fungus. In this respect, besides the diversity of genetics experiments before mentioned, an available ATMT protocol may enable cloning and expression of heterologous genes that improve the metabolism of *A. sojae*.

### 1.1.5 The vgb/VHb technology and its application for strain improvement

Genetic engineering of the *Vitreoscilla* hemoglobin (VHb) gene (*vgb*) in strain improvement strategies are referred as “vgb/VHb technology” (Stark et al. 2011), and their applications have been summarized by Zhang et al. (2007), Wei et al. (2008) and Stark et al. (2011 and 2015). The application of this bacterial hemoglobin for strain improvement strategies had been broadly explored in various organisms to date. Heterologous expression of VHb has been proven to improve cell growth and protein synthesis under oxygen-limited conditions by enhancing the respiratory metabolism in several aerobic microbial hosts. The application studies of VHb deal with enhancement of bioproducts production such as antibiotics, enzymes, polymers and other miscellaneous biochemicals (e.g. fatty acids), and bioremediation from a wide range of hosts (Table 1.4). Year after year, novel biotechnological applications of this bacterial hemoglobin are reported on different hosts and demonstrate thus its broad application for strain improvement strategies.
1.1.5.1 The Vitreoscilla hemoglobin

Since VHb identification as hemoglobin by its amino acid sequence (Wakabayashi et al. 1986), this protein has been broadly studied and is the best-characterized member of the bacterial hemoglobin proteins. Studies about VHb have been well reviewed (Stark et al. 2015; Stark et al. 2011; Wei and Chen 2008) and focus on ligand binding properties and mechanisms, structure, biochemical functions, and the mechanisms by which its expression is controlled. Unlike classic hemoglobins, VHb is composed of only a single globin molecule (Stark et al. 2012) (Figure 1.8), and is known to play a number of functions. The main role of VHb is assumed to be the binding of oxygen at low concentrations and its direct delivery to the terminal respiratory oxidase(s) such as cytochrome o. It is also involved in the delivery of oxygen to oxygenases, detoxification of NO by converting it to nitrate, and sensing oxygen concentrations and

![Viewed from the front Viewed from the side](image)

Figure 1.8. Depiction of the Vitreoscilla hemoglobin (VHb) monomer in 3D. The haeme group is highlighted in sticks and balloons. Adapted from the Protein Data Bank query 3tm3.
passing this signal to transcription factors (Stark et al. 2011). It has a peroxidase-like activity and effectively eliminates autoxidation-derived \( \text{H}_2\text{O}_2 \), which is a cause of haeme degradation and iron release (Isarankura-Na-Ayudhya et al. 2010). In comparison to the currently known globins, the functional investigation of VHb has demonstrated its suitability for engineering the energy metabolism of diverse aerobic microorganisms.

As observed on Table 1.4, most of the research about the \( vgb/VHb \) technology has been done on bacterial and yeast strains. The recent applications of VHb include production of fatty acids by \( \text{Escherichia coli} \) (Wu and San 2014) and enhancement of betulinic acid production by \( \text{Saccharomyces cerevisiae} \) (Li and Zhang 2015). However, the number of VHb applications in fungal species is still fall short in comparison to bacterial and yeast strains. The reduced number of studies is possibly due to fewer molecular tools available to genetically engineer fungal strains. As advances in biochemistry, engineering, and genetic manipulative techniques of filamentous fungi are discovered, the potential of VHb can be explored more extensively in these hosts.

### 1.1.5.2 Application of the \( vgb/VHb \) technology in fungi and SSF processes

The study of the \( vgb/VHb \) technology has not been broadly assessed in filamentous fungi. Moreover, the application of this bacterial hemoglobin has particularly remained poorly explored for SSF processes. As earlier mentioned in Section 1.1.1, SSF of filamentous fungi are considered valuable biotechnological processes as they have a series of advantages over the more traditional SmF processes (e.g. higher productivities, lower operational costs, etc.). However, as also previously pointed out, a major drawback in solid-state cultures with aerobic microorganisms is the poor diffusion of oxygen in the biomass which in turn limits the production of proteins (te Biesebeke et al. 2006). The
low availability of oxygen affects considerably the viability of the fungal host during its 
culturing. In this regard, co-expression of globins such as VHb has shown to be a reliable 
strain improvement strategy in several aerobic hosts to alleviate the hypoxic conditions 
found in a typical solid-state cultures (Stark et al. 2011; te Biesebeke et al. 2006; Wei and 
Chen 2008). Thus, genetic engineering strategies to express proteins such as VHb can be 
powerful approaches to improve oxygen uptake in fungal hosts and in turn increase their 
cell growth and biological production.

### Table 1.4. Selected applications (since 2005) of \( \text{vgb/VHb} \) technology. Adapted from Stark et al. (2011 and 2015).

<table>
<thead>
<tr>
<th>To enhance production of antibiotics</th>
<th>Antibiotic</th>
<th>Specie</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natamycin</td>
<td>Streptomyces gilvosporeus</td>
<td>Wang et al. (2014)</td>
<td></td>
</tr>
<tr>
<td>Polypeptide antibiotic</td>
<td>Paenibacillus peoriae</td>
<td>An et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>Flavones, exopolysaccharides</td>
<td>Phellinus igniarius</td>
<td>Zhu et al. (2011)</td>
<td></td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>Acremonium chrysogenum</td>
<td>Liu et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>Rifamycin b</td>
<td>Amycolatopsis mediterranei</td>
<td>Priscila et al. (2008)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>To enhance production of polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer</td>
</tr>
<tr>
<td>Salecan (exopolysaccharide)</td>
</tr>
<tr>
<td>3-hydroxybutyrate and 3-hydroxyalkanoate</td>
</tr>
<tr>
<td>Gellan gum</td>
</tr>
<tr>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>Poly(hydroxyalkanoate)</td>
</tr>
<tr>
<td>Bacterial cellulose</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
</tbody>
</table>

**To enhance production of miscellaneous biochemcials**

<table>
<thead>
<tr>
<th>Biochemical</th>
<th>Specie(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betulinic acid</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Li and Zhang (2015)</td>
</tr>
<tr>
<td>Fatty acids (C11:0; C13:0; C15:0)</td>
<td><em>Escherichia coli</em></td>
<td>Wu and San (2014)</td>
</tr>
<tr>
<td>Fatty Acids and Astaxanthin</td>
<td><em>Aurantiochytrium sp</em></td>
<td>Suen et al. (2014)</td>
</tr>
<tr>
<td>L-Asparaginase</td>
<td><em>Escherichia coli, Enterobacter aerogenes, and Pseudomonas aeruginosa</em></td>
<td>Erenler and Geckil (2014)</td>
</tr>
<tr>
<td>Poly-γ-glutamic acid (γ-PGA)</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>Zhang et al. (2013)</td>
</tr>
<tr>
<td>Ethanol</td>
<td><em>Escherichia coli</em></td>
<td>Arnaldos et al. (2012)</td>
</tr>
<tr>
<td>Flavones and exopolysaccharides</td>
<td><em>Phellinus igniarius</em></td>
<td>Zhu et al. (2011)</td>
</tr>
<tr>
<td>Poly c-glutamic acid</td>
<td><em>Bacillus subtilis</em></td>
<td>Su et al. (2010)</td>
</tr>
<tr>
<td>L-DOPA and dopamine</td>
<td><em>Citrobacter freundii and Erwinia herbicola</em></td>
<td>Kurt et al. (2009)</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td><em>Bacillus subtilis</em></td>
<td>Chien and Lee (2007)</td>
</tr>
<tr>
<td>S-adenosylmethionine</td>
<td><em>Pichia pastoris</em></td>
<td>Chen et al. (2007)</td>
</tr>
</tbody>
</table>

**To enhance bioremediation**

<table>
<thead>
<tr>
<th>Substance(s) remediated or <em>compound produced</em></th>
<th>Specie</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rock phosphate in acidic Alfisols</td>
<td><em>Pseudomonas fluorescens</em></td>
<td>Yadav et al. (2014)</td>
</tr>
<tr>
<td>Nitrification</td>
<td><em>Mixed culture of nitrifiers</em></td>
<td>Arnaldos et al. (2013, 2014)</td>
</tr>
<tr>
<td>Benzene, toluene, chlorobenzene</td>
<td><em>Pseudomonas putida</em></td>
<td>Ouyang et al. (2007)</td>
</tr>
<tr>
<td><em>Biosurfactant</em></td>
<td><em>Gordonia amarae</em></td>
<td>Dogan et al. (2006)</td>
</tr>
<tr>
<td>2-chlorobenzoate</td>
<td><em>Burkholderia cepacia</em></td>
<td>Uurgun-Demirtas et al. (2006)</td>
</tr>
<tr>
<td>Benzene, toluene, xylene</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Kahraman and Geckil (2005)</td>
</tr>
</tbody>
</table>
1.2 The PGSYS project

The research presented in this dissertation forms part of the larger project entitled “PGSYS EXCHANGE (Bioprocess Platform for the Aspergillus sojae PGzyme system)“ which consists of a cooperation between several international partners, including academic research groups and private companies in Europe, Asia and America. The PGSYS project aim is to define and optimize bioprocesses for production and downstreaming of pectinases using *Aspergillus sojae* ATCC 20235 (A. sojae). Some main goals of the project are focused on (I) the comparison of pectinase production within solid-state and submerged fermentation systems (SSF and SmF, respectively), (II) microbial strain improvement to enhance pectinase production, (III) optimization of culture conditions for improved enzyme production employing advanced statistical designs, and (IV) the development of an efficient product recovery and purification process.

The achievements of the PGSYS project have been previously summarized in Section 1.1.3.4 with the work of Gögus et al. (2006), Tari et al. (2007), Tari et al. (2008), Ustok et al. (2007), Heerd et al. (2012), Demir et al. (2012), Mata-Gomez et al. (2014), Ortiz et al. (2014), and Heerd et al. (2014a and 2014b). For instance, the project has already succeeded a) to generate improved A. sojae strains, b) to establish SSF and SmF systems, c) to provide a general downstream processing route, and d) to characterize the crude enzyme mixture in relation to the potential final applications.

In the recent work, mainly focus in SSF systems, *A. sojae* was shown superior pectinases yields in comparison to other well-known pectinases producers such as
Aspergillus niger and A. oryzae (Heerd et al. 2012). Also, through classic mutagenesis screening strategies based on physical (ultraviolet irradiation) and chemical mutagens, pectinases titers of descending mutants of A. sojae were improved in SmF and SSF (Heerd et al. 2014a). However, until now, a methodology to rational improve A. sojae was not available for SSF applications. Genetic engineering strategies can be powerful approaches to improve the productivities of filamentous fungi, however, their implementation is a challenging task, especially in filamentous fungi. Thus, the availability of a modern strain improvement strategy for SSF applications of A. sojae is anticipated to be of great importance for the general aim of the PGSYS EXCHANGE project. Hence, the main goal of this dissertation was to develop and evaluate in detail a systematic strain improvement strategy for A. sojae in SSF based on genetic molecular tools.
1.3 Scope of this thesis

1.2.1 Background

The main goal of this dissertation was to develop and evaluate a strain improvement strategy for Aspergillus sojae ATCC 20235 (A. sojae) based on the vgb/VHb technology (Section 1.1.5) to overcome typical drawbacks associated to fungal solid-state fermentation (SSF) systems (Section 1.1.1). A. sojae has demonstrated its potential for pectinases production particularly in SSF processes (Section 1.1.3.4). Thus, the main focus was to evaluate solid-state cultures of an A. sojae strain genetically engineered with the Vitreoscilla hemoglobin (VHb) gene (vgb) in terms of cell growth and protein production. As the particular interest was focused in SSF, related knowledge previously developed in this research group was integrated and adapted in this study, including optimized media and fermentation settings for pectinases production with A. sojae at flask culture and reactor scale. An important milestone and fundamental prerequisite to carry out protein expression studies on A. sojae was the development and evaluation of a transformation system on this fungus. Agrobacterium tumefaciens-mediated transformation (ATMT) was selected as this method has shown its reliability and robustness in various filamentous fungi (Section 1.1.4). The applicability of neither an ATMT protocol nor the vgb/VHb technology has been explored in A. sojae until now, making this study of relevance in systematic strain improvement strategies of this fungus. Furthermore, very little has been published on the use of these methodologies to improve SSF processes with filamentous fungi.
1.2.2 Main questions

The main inquiries that arose during the development of a strain improvement strategy for *A. sojae* based on ATMT and the *vgh/VHb* technology were the following:

1) Would it be possible to genetically transform *A. sojae* using an ATMT procedure?

2) Upon the setup of an ATMT system for *A. sojae*, how would its competence be in terms of:
   a. transformation efficiency;
   b. mitotic stability;
   c. practicality?

3) Would it be possible to genetically modified *A. sojae* with VHb?

4) Upon genetic modification of *A. sojae* with VHb, how would its performance in SSF be in terms of:
   a. cell growth or biomass production;
   b. protein production;
   c. respiratory metabolism?

5) Would an *A. sojae* strain modified with VHb be a better candidate for a SSF process in comparison to its wild-type at reactor scale?
6) How will the enzymatic complexes of a modified *A. sojae* strain perform in technological applications, such as the clarification of fruit juices, in comparison to enzymatic complexes of its wild-type?

### 1.2.3 Structure

The findings obtained in this research study should provide a rational strain improvement strategy for *A. sojae* in SSF, and contribute to its development as a microbial biomanufacturing platform for pectinolytic enzymes. For its comprehension, this thesis has been divided in five Chapters.

In **Chapter 1**, a general background about filamentous fungi as cell factories for protein production was given. The main focus lies on the study of *A. sojae* and how the ATMT and *vgb/VHb* technologies can enhance its potential for pectinases production in SSF. Recent applications of ATMT and *vgb/VHb* technologies are reviewed as well as the latest SSF of *A. sojae*. Based on these technologies and the previous knowledge about *A. sojae*, a strain improvement strategy for this fungal strain is justified.

**Chapter 2** describes the setup of a transformation system for *A. sojae* based on the ATMT approach, a fundamental prerequisite to carry out protein expression studies on this filamentous fungus. The applicability and robustness of this transformation protocol is evaluated for *A. sojae*. 
Chapter 3 is about the cloning of the bacterial VHb in *A. sojae* using the developed ATMT procedure described in Chapter 2. The effect of VHb on *A. sojae* is evaluated in SSF in flask-cultures. The fermentation performance between the fungal VHb-expressing strain (*A. sojae vgb*+) and its wild-type counterpart (*A. sojae* wt) is compared in terms of fungal biomass and various pectinolytic enzymes productivities.

Chapter 4 deals with the performance of the genetically engineered *A. sojae vgb*+ strain and its parental *A. sojae* wt strain in SSF at reactor level. As implemented in Chapter 3, both fungal strains are assessed for various protein contents. Finally, the SSF process at flask cultures and reactor scale are compared to evaluate the overall impact of the VHb on protein production of *A. sojae*. In addition to these, the application of experimental enzymatic extracts of these fungi for industrial processes is evaluated, *i.e.* clarification of fruit juices.

Chapter 5 combines general remarks and conclusions. Additionally, future perspectives and the potential of this study are discussed.
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Chapter 1


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CHAPTER 2

Development of a transformation system for *Aspergillus sojae* based on the *Agrobacterium tumefaciens*-mediated approach
Abstract

Aspergillus sojae has been an important filamentous fungus in Biotechnology due to its use in diverse fermentative processes for the production of various food products (Section 1.1.3.3). Furthermore, this fungus is a common expression system for the production of enzymes and other metabolites (Section 1.1.3.4). The availability of molecular genetic tools to explore its biology is thus of big interest (Section 1.1.3.5 & 1.1.3.6). In this study, an Agrobacterium tumefaciens-mediated transformation (ATMT) system (Section 1.1.4) for the A. sojae ATCC 20235 strain (A. sojae) was developed and its applicability evaluated. The donor plasmid named pRM-eGFP was constructed for ATMT of A. sojae. This plasmid contains the ble and egfp genes in its transfer DNA element (T-DNA) to confer phleomycin resistance and express the enhanced green fluorescent protein (EGFP) in A. sojae, respectively. Agrobacterium tumefaciens (LBA4404) harboring the donor plasmid and A. sojae were co-cultured under diverse conditions to achieve ATMT. The maximum number of transformed fungi was obtained after three days of co-culturing at 28°C, and selection with 50 µg/ml phleomycin. Polymerase chain reaction (PCR), fluorescence microscopy and Western Blot analysis for EGFP expression confirmed successful genomic integration of the T-DNA element in A. sojae. The T-DNA was mitotically stable in approximately 40% of the fungal transformants after four generations of sub-culturing under phleomycin pressure. We successfully established a new ATMT protocol for A. sojae. This transformation system should enable further protein expression studies on this filamentous fungus.

2.1 Outline

*Aspergillus sojae* is a filamentous fungus and a well-known *koji* mold. Like *Aspergillus oryzae*, *A. sojae* is widely used for the production of oriental food and beverage products such as soy sauce, sake (rice wine) and miso (soybean paste) (Ushijima et al. 1990). Moreover, these fungi have the ability to secrete large amounts of hydrolytic enzymes (Section 1.1.3.4). Diverse homologous and heterologous proteins have been expressed in *A. sojae* (Heerikhuisen et al. 2008), and its potential for the production of commercially important enzymes such as pectinases (Heerd et al. 2012), mannanases (Ozturk et al. 2010), or glutaminases (Ito et al. 2013), has been demonstrated. Its GRAS status (generally recognized as safe) has been advantageous over other toxigenic (aflatoxin-producing) filamentous fungi for many bioprocess applications (Matsushima et al. 2001). Thus, the availability of molecular genetic tools to explore its biology is of big interest.

In the last years, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has become a common technique for selected filamentous fungi (Fransen et al. 2008; Frandsen 2011). ATMT is based on the capacity of *A. tumefaciens* to transfer part of its DNA (T-DNA), contained in the tumor-inducing (Ti) plasmid, to the host cell. Such T-DNA, delimited by imperfect 25-base pair repeats called, the right and left border sequences (RB and LB, respectively), is typically randomly inserted in the host genome as a single copy (de Groot et al. 1998). Until now, a wide variety of different fungal species have been transformed using this approach, with *Aspergillus terreus* (Wang et al. 2014a) and *Aspergillus carbonarius* (Crespo-Sempere et al. 2011) as some of the last examples. However, to our knowledge, the applicability of ATMT in an *A. sojae* strain has so far not been tested.
In this study, we set up an ATMT procedure for *A. sojae* ATCC 20235 (Figure 2.1), using the *ble* gene as an antibiotic selector marker for recombinant fungi. The effectiveness of this method was measured and further validated as a genetic molecular tool for *A. sojae*.

![Image](image.png)

**Figure 2.1.** Experimental set-up of *Agrobacterium tumefaciens*-mediated transformation of *A. sojae*. 

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Page | 50
2.2 Material and methods

2.2.1 Microorganisms

*Aspergillus sojae* ATCC 20235 (*A. sojae*) was obtained from Procochem Inc (Teddington, United Kingdom), an international distributor of the American Type of Culture Collection (ATCC) in Europe. This fungal strain was grown at 28°C on potato dextrose agar (PDA) plates until conidiation (5-8 days). Spores were harvested using 0.02% (v/v) Tween 80, and filtered through cotton to remove hyphae. The spore concentration was determined using a hemocytometer (Thoma, Germany). Top 10 *Escherichia coli* cells (Invitrogen, USA) were used as a host for all DNA manipulations (Appendix A3). DNA plasmids were isolated from Luria-Bertani (LB) overnight cultures supplemented with 100 µg/ml streptomycin or 50 µg/ml kanamycin as required, using the “NucleoSpin Plasmid” commercial kit (Macherey-Nagel, Germany). *Agrobacterium tumefaciens*, strain LBA4404 (ElectroMAX™, Invitrogen, USA) was used as T-DNA donor for fungal transformation of *A. sojae*.

2.2.2 Phleomycin minimum inhibitory concentration for *A. sojae*

The sensitivity of *A. sojae* to phleomycin was assayed. Phleomycin is a glycopeptide antibiotic of the bleomycin family, which binds and intercalates DNA thus destroying the integrity of the double helix. A total amount of $10^5$ *A. sojae*-spores was inoculated in minimal media agar plates (MM) (10 mM K$_2$HPO$_4$, 10 mM KH$_2$PO$_4$, 2.5 mM NaCl, 2 mM MgSO$_4$·7H$_2$O, 0.7 mM CaCl$_2$, 9 µM FeSO$_4$·7H$_2$O, 4 mM (NH$_4$)$_2$SO$_4$, 10 mM glucose and 2% (w/v) agar) with various antibiotic concentrations: 10, 25, 50, 100
µg/ml and no antibiotic. The plates were incubated at 29°C, and the appearance of fungal colonies was examined during 7 days.

### 2.2.3 Construction of T-DNA donor vector

The *A. tumefaciens* transforming vector named pRM-eGFP (Figure 2.2 and Appendix A1), was designed to confer phleomycin resistance and express EGFP reporter protein in *A. sojae* (*ble* and *egfp* genes, respectively) through an ATMT procedure. The expression of both genes is driven by the strong constitutive *Aspergillus nidulans* gpdA and trpC promoters (*P_{gpdA}* and *P_{trpC}*, respectively), extensively used for protein expression in *Aspergillus* species (Meyer et al. 2011). This vector was derived from the pRFHUE-eGFP vector (Crespo-Sempere et al. 2011) by replacement of the hygromycin B phosphotransferase gene (*hph*), with the phleomycin gene (*ble*). To construct the pRM-eGFP vector, a two-step subcloning strategy was followed. Firstly, multiple ClaI and BamHI sites in the pRFHUE-eGFP vector were deleted by amplifying a 2.5-kbp-length fragment from this vector with the primers ML1.3-F and ML1.4-R, which incorporates each a BssHII restriction site. This PCR product was digested and ligated by BssHII restriction site into the pRFHUE-eGFP vector, resulting in a 9068 bp plasmid with unique ClaI and BamHI sites delimitating the *hph* gene. In the second step, the *hph* gene was replaced by the *ble* gene using the ClaI and BamHI restriction sites. The *ble* gene was amplified from the pGAPZαA vector (Invitrogen) with the primers cla-F and bam-R, which incorporates ClaI and BamHI restriction sites (Table 2.1). Sequencing and restriction enzyme digestion analysis were carried out to verify correctly assembled plasmid. The resulting pRM-eGFP vector (Figure 2.2) was transformed into *A. tumefaciens* LBA4404 electrocompetent cells and selected on LB agar plates containing
100 µg/ml streptomycin and 50 µg/ml kanamycin. The oligonucleotide primer sequences and PCR conditions used are listed in Table 2.1.

Figure 2.2. The ATMT donor vector pRM-eGFP. The transfer DNA region (T-DNA) consists of the phleomycin-resistance conferring gene (ble) which is under control of the A. nidulans trpC promoter (PtrpC) and trpC terminator (TtrpC). The enhanced green fluorescent protein (EGFP) reporter gene is under control of the constitutive A. nidulans gpdA promoter (PgpdA). The blue arrows indicate the target sites for the oligonucleotide primers BLE-F, BLE-R, EGFP-F and EGFP-R. OriV = replication origin; KanR = kanamycin resistance gene; TrfA = trans-acting gene trfA.
2.2.4 Agrobacterium tumefaciens-mediated transformation

The ATMT was carried out following indications from previous reports (de Groot et al. 1998; Shi et al. 2012). The *A. tumefaciens* LBA4404 strain harboring the T-DNA binary vector pRM-eGFP was grown overnight at 28°C on a rotatory shaker (Innova 4000, New Brunswick Scientific, USA) at 200 rpm in 5 ml of LB broth supplemented with 50 µg/ml streptomycin and 50 µg/ml kanamycin. The overnight culture was centrifuged for 10 min and 3,200 x g at room temperature and the pellet was washed twice with one volume of fresh induction medium (IM) (MM plus 0.5% (v/v) glycerol, 200 µM acetosyringone (AS) and 40 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.3). Finally, the bacterial pellet was resuspended in 10 ml of IM to an OD$_{600}$ of 0.1. The bacterial cells were further grown under the same conditions to an OD$_{600}$ of 0.5–0.6. In parallel, a $10^5$ *A. sojae*-conidia/ml water suspension was prepared with fresh spores harvested from a PDA agar plate as described above. For the co-cultivation of *A. tumefaciens* and *A. sojae*, 100 µl of each the bacterial and fungal suspensions, were spread evenly on a 50 mm filter paper laid on top of 25 ml of an IM agar plate (IM plus 1.8% (w/v) agar) and incubated in the dark at 28°C. The co-culture growth from this plate was harvested in 5 ml of 0.02% (v/v) Tween 80 after the co-culture times to be evaluated (two, three or four days). The total cells’ suspension volume was equally distributed into five MM-selection-agar plates supplemented with 200 µg/ml cefotaxime and variable concentration of phleomycin (25, 50 and 100 µg/ml) to inhibit the growth of *A. tumefaciens* and select for the positive *A. sojae* transformants, respectively. The plates were incubated in the dark at 28°C until growth of fungal colonies was observed.
2.2.5 Extraction of genomic DNA

Genomic DNA (gDNA) from *A. sojae* was extracted as described by Melo et al. (2006) with some modifications (Appendix A6). A mixture of 100 mg of fungal growth (either mycelia or spores from PDA), one volume of lysis buffer (1 mM Na$_2$EDTA, 100 mM NaCl, 1% (w/v) SDS, 2% (v/v) Triton X-100 and 10 mM Tris, pH 8.0) and one volume of glass beads (212-300 µm; Sigma, Germany) was vortexed for 5 min for breaking of fungal cells. The released gDNA was then isolated with a phenol-chloroform mixture according to standard procedures (Sambrook and Russell 2001).

2.2.6 Gene expression analysis

PCR analyses

The T-DNA integration in the fungal genome of randomly selected transformants was assessed by PCR. The specific primers sets BLE-F/BLE-R and EGFP-F/EGFP-R (Table 2.1) were used to target the *ble* and the *egfp* gene located in the T-DNA region, respectively. Genomic DNA from untransformed *A. sojae* (wild-type; wt) was used as a control.

Fluorescence microscopy

The expression of EGFP in *A. sojae* transformants was analyzed by fluorescence microscopy. Randomly selected phleomycin-resistant fungal colonies were picked from the selection plates, inoculated on PDA plates supplemented with 100 µg/ml phleomycin, and grown at 28°C for 2-5 days. Fungal growth fractions were immersed in a drop of water on glass slides. The fluorescence signal was visualized using an Axiplan 2 imaging (Zeiss) microscope equipped with a filter set matching the excitation and emission
spectra for the EGFP (Ex/Em = 488/509). Images were acquired with the AxioVision (V. 4.8) software, setting an exposure time of 900 ms to subtract the background autofluorescent signal of the A. sojae wt.

2.2.7 Western Blot

The expression of EGFP in selected transformants was further investigated by Western blot according to standard procedures (Sambrook and Russell 2001). The A. sojae transformants and wild-type strains were cultured in potato dextrose liquid media for 2-3 days at 28°C on a rotatory shaker at 150 rpm. The mycelia were harvested by filtration, washed with distilled water, dried, and frozen in liquid nitrogen. The frozen mycelia were lyophilized, ground to a fine powder by using a mortar and pestle, and mixed with 2 volumes of distilled water and 3 volumes of Laemmli-sample-loading buffer (100 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, and 200 mM β-mercaptoethanol). The mixture was boiled at 100°C for 5 min, cooled quickly on ice and then centrifuged at 13,000 x g for 5 min. The soluble proteins in the supernatant were separated on a 12% (w/v) SDS-PAGE gel and blotted onto a nitrocellulose membrane (Amersham, GE Healthcare) for 2 h at 100 V, by using a wet transblot system (Mini-PROTEAN, Bio-Rad, Germany). Protein transfer was confirmed by staining the membrane with Ponceau S (Roth, Germany). The blots were blocked by incubation for at least 8 h at 4 to 8°C in tris-buffered saline (TBS) containing 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20. Primary and secondary antibodies were diluted with the same buffer. The primary antibody used was rat monoclonal anti-GFP diluted at 1:500 (BioLegend, USA). The blots were incubated with the primary antibody at room temperature for at least 4 h and then washed three times with TBS
containing 0.1% (v/v) Tween 20. All washing steps were carried out for 10 min. The secondary antibody used was goat anti-rat immunoglobulin G conjugated to horseradish peroxidase (BioLegend, USA) diluted at 1:1,000. The blots were incubated with the secondary antibody at room temperature for at least 2 h, washed three times with TBS containing 0.1% (v/v) Tween 20, and then washed once with TBS. The chemiluminescent signal was revealed using ECL substrate and X-ray films for ECL detection (Thermo Scientific, Germany).

### 2.2.8 Mitotic stability of transformants

The mitotic stability of the transfected T-DNA was determined in a sample of 20 selected fungal transformants. Fungi were cultured on PDA plates supplemented with 100 µg/ml phleomycin for 4 generations. The stable integration of T-DNA into the genome of the transformants was investigated by PCR analysis, targeting the *ble* and the *egfp* gene with the specific primers sets BLE-F/BLE-R and EGFP-F/EGFP-R, respectively (Table 2.1).
Table 2.1. Oligonucleotide primers and PCR conditions used in this study.

<table>
<thead>
<tr>
<th>Name / Sequence (5´-3´)</th>
<th>Function</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML1.3-F \ TCCGCGGgcegcgeATCCTCTAGAAAGAGATTAC</td>
<td>Mutagenesis on pRFHUE-eGFP vector</td>
<td>ID: 94°C for 5 min. 5-cycle: 94°C for 15 sec, 60°C for 15 sec and 68°C for 3 min. 30-cycle: 94°C for 15 sec and 68°C for 3 min. FE: 68°C for 5 min.</td>
</tr>
<tr>
<td>ML1.4-R \ ATCATCGATgcegcgeGTGATGATCAGCCCTGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cla-F</td>
<td>Amplification of ble gene from pGAPZaA vector</td>
<td>ID: 94°C for 5 min. 30-cycle: 94°C for 15 sec, 62°C for 15 sec and 68°C for 45 sec. FE: 68°C for 5 min.</td>
</tr>
<tr>
<td>GAGGAatcgatCCATGGCACAAGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bam-R</td>
<td>Target ble gene contained on T-DNA</td>
<td>ID: 94°C for 5 min. 30-cycle: 94°C for 15 sec, 55°C for 15 sec and 68°C for 45 sec. FE: 68°C for 5 min.</td>
</tr>
<tr>
<td>TCggatccGTGATCTTCTGCTCCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLE-F</td>
<td>Target egfp gene contained on T-DNA</td>
<td>ID: 94°C for 5 min. 30-cycle: 94°C for 15 sec, 60°C for 15 sec and 68°C for 45 sec. FE: 68°C for 5 min.</td>
</tr>
<tr>
<td>CGTTTTATTTCTTGTGACATGGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLE-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTGGGCTTGGCTGGAGCTAGTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP-F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACCTACGGCAAGCTGACCCTGAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGTACGCTGCATCGCGAGAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lowercase letters gcgcge, atcgat and ggatcc indicate restriction sites BssHII, ClaI and BamHI respectively. The underlined sequences ATG and TCA, correspond to the start and stop codon, respectively. ID: initial denaturation; FE: final elongation.
2.3 Results and discussion

2.3.1 Phleomycin antibiotic as selectable marker for A. sojae

To be able to perform the ATMT procedure with Aspergillus sojae ATCC 20235 (A. sojae), the suitability of antibiotics from the bleomycin family was tested as selectable markers for this fungus. Zeocin showed little inhibition for A. sojae at concentrations up to 300 µg/ml (data not shown). We thus tested phleomycin, an antibiotic recommended from the manufacturer (InvivoGen, USA) for cells with low sensitivity to zeocin. Our analysis showed that minimal media plates supplemented with 50 µg/ml phleomycin completely inhibited the growth of an A. sojae-inoculum of 10⁵ spores for up to 7 days (Figure 2.3). We thus used this antibiotic concentration for the selection of A. sojae transformants in the ATMT.

![Figure 2.3. Phleomycin inhibition. The A. sojae growth on minimal media with various phleomycin concentrations after 7 days incubation. Initial inoculum: 10⁵ spores.](image-url)
2.3.2 Optimization of conditions for ATMT of \textit{A. sojae}

\textit{A. tumefaciens} LBA4404 containing the vector pRM-eGFP was utilized to test the ATMT method. Common ATMT conditions for filamentous fungi (de Groot et al. 1998; Shi et al. 2012) as mentioned in the methodology were used to verify the viability of the assay. However, in our procedure, the bacterial-fungal co-culture growth was harvested in a 0.02\% (v/v) Tween 80 solution and the solution equally distributed in several selection plates, contrary to other common ATMT methodologies, where the co-culture growth lying on a membrane is transferred directly onto the selection plates. In preliminary experiments, a concentration of 100 µg/ml of phleomycin was used in the selection plates, aiming to enhance the selectivity of the assay. However, the number of putative fungal transformants was as low as 10 colonies per $10^5$ conidia after three days. Consequently, the concentration of phleomycin in the selection plates was reduced to half, and the number of fungal colonies increased five to eight fold. This result reflected a possible limiting condition where a high concentration of antibiotic affects the survival rate of fresh transformants. On the other side, further reductions in phleomycin concentration led to fungal over-growth, which made the subsequent isolation of single transformants difficult; therefore, selection plates with 50 µg/ml phleomycin final concentration were used for following experiments.

Different co-cultivation times of two, three and four days were assessed in the transformation procedure. An increase of putative fungal transformants was observed on the selection plates with increasing co-cultivation time, ranging from 40 to 100 transformants per $10^5$ conidia from two to four days, respectively. However, co-cultivation times of three days led to the best ratio of positive to false positive transformants. The higher frequency of negative transformants during the four-day co-
cultivation was likely due to higher background growth on the selection plates resulting from longer co-cultivation times without antibiotic pressure. We thus used three days of co-cultivation time before selecting the transformants on 50 µg/ml phleomycin in the selection media that yielded approximately 80 putative fungal transformants per $10^5$ conidia.

**2.3.3 Gene expression analysis**

**PCR analysis**

Putative fungal transformants were examined by PCR analysis to confirm successful chromosomal integration of the T-DNA. The genomic DNA from selected *A. sojae* transformants and the wild-type as negative control were used to target *ble* and *egfp* genes with the specific primers sets BLE-F/BLE-R and EGFP-F/EGFP-R, respectively (Table 2.1). PCR products of expected sizes of 905 bp for the *ble* gene and 601 bp for the *egfp* gene were obtained from the transformants but not from the wild-type DNA (Figure 2.4). Subsequent sequencing analysis of the PCR products confirmed the presence of both genes in the *A. sojae* transformants.

**Fluorescence microscopy**

Fluorescence microscopy studies were carried out to verify the expression of the EGFP reporter gene in selected *A. sojae* transformants. The fungal transformants showed regular fluorescent signal in structures such as hyphae and conidiophores but in isolated conidia the signal was scarce (Figure 2.5). Moreover, the intensity of the fluorescent emission appeared to be higher in growing hyphal tips and conidiophores. The wild-type strain showed some autofluorescent background signal, which was considerably less
compared to the transformants. Therefore, an exposure time of 900 ms was used in the microscopy analysis, where no fluorescent signal was detected in the wild-type but in the transformants (Figure 2.5). These results indicate that the transformants were able to express the EGFP gene, and validate the PCR results, where integration of the EGFP gene was demonstrated. This analysis facilitated the selection of positive transformants vs. wild-type, as there was no noticeable morphological difference between the colonies of these fungi on the agar plates.

Figure 2.4. PCR analysis of A. sojae transformants. Amplified PCR products from genomic DNA of selected A. sojae transformants (T1-T4), confirming the presence of the ble gene (panel A) and the egfp gene (panel B); A. sojae wild-type sample used as negative control (W); pRM-eGFP vector used as positive control (C); molecular size marker (M).
Figure 2.5. Fluorescence microscopy analysis of *A. sojae* wild-type and selected transformants with EGFP. Highlighted with arrows are hyphae (h), conidiophores (p) and conidia (c) of fungal transformants showing fluorescent signal. A and B are bright field and fluorescence images, respectively. Scale bar = 50 µm.
2.3.4 Western Blot analysis

Western blot analysis was performed to confirm the presence of EGFP in *A. sojae* transformants. Intracellular fungal extracts were electrophoresed under denaturing conditions (12% (w/v) SDS-PAGE) (Figure 2.6). Prior antibody hybridization, Ponceau S staining was performed to verify the transfer of intracellular proteins onto the nitrocellulose membrane. After Western blotting with anti-GFP (rat) primary and anti-rat IgG-horseradish peroxidase conjugate secondary antibodies, chemiluminescent signals in form of unique bands were detected in the fungal transformant and the positive control (Figure 2.6). These bands were shown at the proximate 26.9 kDa molecular mass expected for the EGFP. No visible band was detected in the wild-type sample.

![Western Blot](image)

**Figure 2.6. Western Blot.** Intracellular protein profiles (left panel) and Western Blot analysis (right panel) of *A. sojae* transformant (T), wild-type (W), and control GFP-overexpressing *E. coli* (C) strains. Molecular size marker (M).
results indicated that EGFP protein had been produced in the \textit{A. sojae} transformants intracellularly and corroborate the fluorescence microscopic analysis, where EGFP expression was detected. Furthermore, the suitability of \textit{PgpdA} for heterologous gene expression in \textit{A. sojae} was demonstrated.

\subsection*{2.3.5 Mitotic stability of transformants}

The mitotic stability of the T-DNA in \textit{A. sojae} transformants was examined by subculturing them under phleomycin pressure aiming to increase the stability of the recombinant DNA. Previous experiments with no antibiotic in the media showed little T-DNA retention in the fungal transformants. Therefore, a relatively high concentration of phleomycin was used in the media to favor the survival of the most stable transformants. Twenty \textit{A. sojae} transformants were subcultured for four generations on PDA plates supplemented with 100 \(\mu\)g/ml phleomycin. After four generations, all the transformants were screened by PCR for the presence of the \textit{ble} and \textit{egfp} genes. Both genes were PCR-amplified in 8 of the 20 transformants, corresponding to a 40\% mitotic stability of genomic T-DNA integration. Unique amplification of either the \textit{ble} or the \textit{egfp} gene was not observed in any of the samples, thus discarding any partial integration of the T-DNA cassette. The negative transformants were further examined with the fluorescence microscope to check for EGFP expression. As expected, none of these fungal samples showed a fluorescent signal. These results indicate that the T-DNA was aborted in these fungi, and suggest that a resistance mechanism different from the \textit{ble} gene, played a role in the tolerance of these fungi to phleomycin.
It is known that transformation efficiencies in filamentous fungi are generally low (Ruiz-Diez 2002). In fungi such as A. oryzae and A. nidulants, high abortive rates of transformed DNA were reported. For example, an abortive rate of the A. nidulants argB gene in A. oryzae was evidenced in approximately 90% of the initial transformants (Hahm and Batt 1988; John and Peberdy 1984). Filamentous fungi with multinucleate conidia have been considered a probable cause for recalcitrant transformation and thus conidia with one nucleus are commonly considered to be useful in mutagenesis approaches (Ji et al. 2013). To test for multiple nuclei, we stained spores of the A. sojae ATCC 20235 used in this study with DAPI (4',6-diamidino-2-phenylindole) and found that its conidia contains multiple nuclei ranging in numbers from 1 to 6 (data not shown). Possibly, the abortive transformations we observed in ATCC 20235, was caused by insufficient T-DNA integration in all nuclei; for instance a single T-DNA locus could lead to mitotically unstable transformants. However, the exact mechanism resulting in abortive events in this fungus is not well-known. More studies about the reproductive cycle of this fungus are needed to explain this phenomenon. Nevertheless, the ATMT method described in this study demonstrated to be useful to produce A. sojae transformants, and our analysis showed that these transformants were able to successfully express the heterologous EGFP protein using the constitutive promoter PgpdA.
2.4 Conclusions

The capacity of the gram-negative Agrobacterium tumefaciens to genetically transform the fungal A. sojae strain ATCC 20235 (A. sojae).

The ATMT has become a common technique for protein expression studies on plants and fungi, despite the action mechanism and molecular basis of this transformation procedure ATMT have not been fully elucidated. For instance, over the past 16 years, ATMT has been applied in a wide variety of different fungal species, including members of the ascomycetes, basidiomycetes, zygomycetes, oomycetes and glomeromycetes groups (Section 1.1.4). However, as mentioned in some studies, the application of this transformation approach in some fungal species has been recalcitrant possibly due to genetic artifacts which are still unknown. Therefore, it was imperative to evaluate in detail the applicability of this methodology in A. sojae. The various ATMT studies have focused on the experimental parameters that affect transformation frequency, including co-cultivation conditions (i.e., temperature, duration, ratio between donor and acceptor organism), the use of particular marker genes for selection and the use of particular promoters for driving the expression of selection markers. Also, a key parameter was the design and construction of a Ti plasmid (expression vector) to enable cloning of a desired gene sequence into the T-DNA that will be inserted into the host DNA. In this study, the effects of the previous strategic parameters were systematically evaluated for successful ATMT of A. sojae.

Overall, successful chromosomal integration of the T-DNA cassette was achieved in A. sojae through the established ATMT protocol. On one hand, the ble gene under control of the constitutive Aspergillus nidulants trpC promoter (PtrpC) and trpC terminator (TtrpC) allowed selection of phleomycin-resistance A. sojae strains on agar
plates supplemented with phleomycin. On the other hand, the \textit{egfp} gene under control of the constitutive \textit{A. nidulants gpdA} promoter (P\textit{gpdA}) validated recombinant protein expression in \textit{A. sojae}, as demonstrated by fluorescence microscopy and Western blot analysis.

The relatively high rate of unstable ATMT of \textit{A. sojae} indicated the need for further research about the physiology of this fungus, which could be used to improve transformation frequencies. Nevertheless, the developed ATMT procedure should facilitate further protein expression studies in this fungus and may be applicable to other phleomycin-sensitive filamentous fungi.

Having achieved the planned milestones in this study, the next step in this dissertation was the cloning of the bacterial \textit{Vitreoscilla} hemoglobin (VHb) gene (\textit{vgb}) in \textit{A. sojae} using the developed ATMT procedure with the aim to improve the metabolism of this fungal strain for solid-state fermentation (SSF) (see Chapter 3).
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References


CHAPTER 3

Application of the *Vitreoscilla* hemoglobin in solid-state fermentation of *Aspergillus sojae* for pectinases production
Abstract

The biotechnological value of Aspergillus sojae ATCC 20235 (A. sojae) for production of pectinases in solid-state fermentation (SSF) has been demonstrated recently (Section 1.1.3.4). However, a common drawback of fungal solid-state cultures is the poor diffusion of oxygen into the fungi that limits its growth and biological productivity (Section 1.1.1). The bacterial Vitreoscilla hemoglobin (VHb) has favored the metabolism and productivities of various bacterial and yeast strains besides alleviating hypoxic conditions of its native host, but the use of VHb in filamentous fungi still remains poorly explored (Section 1.1.5). Based on the known effects of VHb, this study assessed its applicability to improve A. sojae performance in SSF. The VHb gene (vgb) under control of the constitutive Aspergillus nidulans gpdA promoter was introduced into the genome of A. sojae by Agrobacterium-mediated transformation (see background of this methodology in Chapter 2). Successful fungal transformants were identified by fluorescence microscopy and polymerase chain reaction (PCR) analyses. In solid-state cultures, the content of protease, exo-polygalacturonase (exo-PG) and exo-polymethylgalacturonase (exo-PMG) of the transformed fungus (A. sojae-vgb+) improved were 26, 60 and 44% higher, respectively in comparison to its parental strain (A. sojae wt). Similarly, biomass content was also 1.3 times higher in the transformant strain. No significant difference was observed in endo-polygalacturonase (endo-PG) content between both fungal strains, suggesting dissimilar effects of VHb towards different enzymatic productions. Overall, our results show that biomass, protease and exo-pectinases content of A. sojae in SSF can be improved by transformation with VHb.

3.1 Outline

Protein production by means of solid-state fermentation (SSF) has gained a lot of interest in the last years because it has many advantages over the most widely used submerged fermentation (SmF). For instance, the possibility to use low-cost substrates is a common benefit in solid-state cultures. Also, higher productivities, simpler equipment and less space requirements are advantages associated to SSF (Aguilar et al. 2008; De la Cruz Quiroz et al. 2014; Viniegra-Gonzalez et al. 2003). However, a major drawback in solid-state cultures with aerobic microorganisms is the lower diffusion of oxygen in the biomass which in turn limits the production of proteins (Stark et al. 2011; Wei and Chen 2008). To overcome oxygen limitations in fermentation processes, the co-expression of globins as a strain improvement strategy has proven to be useful in several aerobic hosts, including the filamentous fungi *Aspergillus oryzae* (Stark et al. 2011; te Biesebeke et al. 2006).

In various examples with prokaryotic and eukaryotic microorganisms, heterologous expression of the bacterial *Vitreoscilla* hemoglobin (VHb) has improved cell growth and protein synthesis under oxygen-limiting conditions most likely by enhancing respiratory metabolism (Stark et al. 2011; Stark et al 2015; Wei and Chen 2008). VHb is the best-characterized member of the bacterial hemoglobin proteins and since the identification of its amino acid sequence (Wakabayashi et al. 1986), its application for strain improvement in various organisms has been widely explored. Mostly in bacterial and yeast bioprocesses, cell growth, bioremediation and enhanced protein production have been improved within this so called “vgb/VHb technology” (Hofmann et al. 2009; Kahraman et al. 2011; Kahraman and Erenler 2012; Wu and Fu 2012; Zhu et al. 2011). However, the potential of VHb in filamentous fungi has not been
investigated in detail, mainly due to the availability of fewer genetic molecular tools to engineer them in comparison to bacterial and yeast hosts (Fleissner and Dersch 2010; Mora-Lugo et al. 2014; Ward 2012).

The potential of the filamentous fungus *Aspergillus sojae* ATCC 20235 (*A. sojae*) for production of pectinases by fermentative processes has been demonstrated in the last years (Ustok et al. 2007; Tari et al. 2008; Demir et al. 2012; Heerd et al. 2014a). Pectinases or pectinolytic enzymes are a heterogeneous group of related enzymes that hydrolyze pectic substances or pectins, and are valuable biocatalysts for food and industrial applications (Adapa et al. 2014). *A. sojae* has yielded higher amounts of pectinases in comparison with the well-known pectinase producer *Aspergillus niger* and other *A. sojae* strains (CBS 100928 and IMI 191303) in SSF (Heerd et al. 2012). Moreover, through classic mutagenesis screening strategies based on physical (ultraviolet irradiation) and chemical mutagens, pectinases titers of descending mutants of *A. sojae* have been improved in SmF and SSF (Heerd et al. 2014b). The potential of some of these high-yield pectinases mutants has been further explored by measuring various pectinases activities including exo-/endo-polygalacturonase (exo-PG/endo-PG), exo-polymethylgalacturonase (exo-PMG), and pectin lyase using two different carbon sources in SSF (Mata-Gomez et al. 2014). Recently, an *Agrobacterium tumefaciens*-mediated transformation (ATMT) method was described for *A. sojae* and heterologous expression of the enhanced green fluorescent protein (EGFP) was demonstrated successfully (Mora-Lugo et al. 2014, Chapter 2). This study opens up new possibilities of protein expression studies in *A. sojae* and explore systematically strain improvement strategies for this fungus based on genetic molecular tools.
Based on the positive effects of VHb in previous microbial hosts (Chapter 1), this study describes a genetic engineering approach to improve A. sojae for SSF. This fungus was genetically engineered with VHb through an adapted transformation method mediated by Agrobacterium tumefaciens (see fundamentals of the protocol in Chapter 2). Subsequently, different pectinases, nonspecific protease and biomass content were measured and compared between the transformed fungus (A. sojae vgb+) and its parental strain (A. sojae wt) in solid-state cultures. The present study provides a new strain improvement strategy for A. sojae to further explore its potential as pectinases bio-factory.

3.2 Material and methods

3.2.1 Materials

All chemicals were purchased from AppliChem GmbH (Darmstadt, Germany), except citrus pectin, galacturonic acid, polygalacturonic acid sodium salt and D-(+)-glucosamine hydrochloride were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Microbial substrates such as wheat bran, sugar beet pulp pellets and molasses were obtained from local suppliers (Bremer Rolandmühle Erling GmbH & Co. KG, Bremen, Germany; Nordzucker AG, Uelzen, Germany; Golden Sweet, Meckenheim, Germany). Restriction enzymes, T4 DNA polymerase and T4 DNA ligase were purchased from New England Biolabs (Frankfurt am Main, Germany). Oligoprimers, synthetic genes and DNA sequences were ordered from Eurofins (Ebersberg, Germany).
3.2.2 Microorganisms and media

*Aspergillus sojae* strain ATCC 20235 (*A. sojae*) was grown at 28°C until conidiation (3-6 days) on molasses agar plates (45 g/l molasses, 45 g/l glycerol, 18 g/l peptone, 5 g/l NaCl, 0.5 g/l KCl, 15 mg/l FeSO₄·7H₂O, 60 mg/l KH₂PO₄, 50 mg/l MgSO₄, 12 mg/l CuSO₄·5H₂O, 15 mg/l MnSO₄·H₂O and 20 g/l agar). Spores were harvested using 0.02% (w/v) Tween 80, and filtered through cotton to remove hyphae (Appendix A4). The spore concentration was determined using a Neubauer chamber (Celeromics, Grenoble, France) (Appendix A5). Top 10 *Escherichia coli* cells (Invitrogen, CA, USA) were used as a host for all DNA manipulations (Appendix A3). DNA plasmids were isolated from Luria-Bertani overnight cultures supplemented with 100 µg/ml streptomycin and 50 µg/ml kanamycin, using the “NucleoSpin Plasmid” commercial kit (Macherey-Nagel, Düren, Germany). *Agrobacterium tumefaciens*, strain LBA4404 (ElectroMAX™, Invitrogen, CA, USA) was used as T-DNA donor for fungal transformation of *A. sojae*.

3.2.3 Construction of vgb-gene donor vector and fungal transformation

The ATMT donor vector named pRM-vgb (GenBank Accession No KT225581) was designed for cloning and expression of the *vgb* gene under control of the constitutive *A. nidulants* *gpdA* promoter (PgpdA) in *A. sojae* (Figure. 3.1 and Appendix A1). This vector was derived from the pRM-eGFP vector (Mora-Lugo et al. 2014, Chapter 2) by subcloning a 1.7-kb-synthetic-cassette (ordered from Eurofins, Ebersberg, Germany) between *SalI* and *PvuI* restriction sites. This cassette contains *vgb*, which was codon-usage optimized for *Aspergillus* species according to the CUTG database (GenBank) and fused to the 5’ end of the reporter egfp gene. Moreover, other additional restriction sites
for forthcoming cloning strategies were added in this operon including: two KpnI to enable deletion of egfp, if required, and two Eam1105I to allow TA cloning (by 3’T overhanging ends) for gene expression under control of the PgpdA (Alibu et al. 2005). The constructed pRM-vgb vector was electro-transformed into A. tumefaciens LBA4404 and recombinant bacteria were selected on LB agar plates containing 100 µg/ml streptomycin and 50 µg/ml kanamycin. Accuracy of the plasmid sequence was examined by restriction enzyme digestion and sequencing analysis. The recombinant A. tumefaciens strain containing the pRM-vgb vector was used to transform A. sojae by the ATMT procedure as described by Mora-Lugo et al. (2014) (Chapter 2).

3.2.4 Analysis of the fungal transformants

Putative A. sojae transformants were assessed by PCR and fluorescence microscopy analysis to verify chromosomal integration and gene expression of the transformed or transferred DNA (T-DNA), respectively as described by Mora-Lugo et al. (2014) (Chapter 2). For the PCR analysis, the genomic DNA from putative fungal transformants was isolated and used as DNA template. The specific primers sets BLE-F/BLE-R and VHb-F/VHb-R (Table 3.1) were used to target the ble and the vgb genes located in the T-DNA region, respectively. Amplification included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing for 40 s (at 55°C for ble and at 59°C for vgb), and elongation at 68°C for 60 s, and a final elongation step at 68°C for 5 min. Genomic DNA from the parental A. sojae wt strain and purified pRM-vgb plasmid were used as a negative and positive control, respectively. For the fluorescence microscopy analysis, the expression of EGFP reporter gene in A. sojae transformants was visualized using an Axioplan 2 imaging (Zeiss) microscope equipped
with a filter set matching the excitation and emission spectra for the EGFP (Ex/Em = 488/509). Images were acquired with the AxioVision (V. 4.8) software, setting an exposure time of 900 ms to subtract the background autofluorescence signal of the A. sojae wt.

### Table 3.1. Sequences of the primers used for PCR amplification.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLE-F</td>
<td>5’-CGTTTTATCTCTGACATGGAGC-3’</td>
<td>Mora-Lugo et al. (2014)</td>
</tr>
<tr>
<td>BLE-R</td>
<td>5’-TTGGGCTGGCTGGAGCTAGTGGAG-3’</td>
<td></td>
</tr>
<tr>
<td>VHb-F</td>
<td>5’-CAGTTCAGCTTCCACCTTCATCG-3’</td>
<td>This study</td>
</tr>
<tr>
<td>VHb-R</td>
<td>5’-TGTACAGCTGTCATGGCCAGG-3’</td>
<td></td>
</tr>
</tbody>
</table>

### 3.2.5 Solid-state fermentation at flask scale

SSF experiments were carried out independently, in triplicates, and for ten days with an untransformed (A. sojae wt) and a selected transformed fungal strain (A. sojae vgb+) according to the method provided by Heerd et al. (2014) with slight modifications. Erlenmeyer flasks (300 ml) containing 10 g of wheat bran and ground sugar beet pulp in the ratio 70:30, and wetted at 160% with 16 ml of 0.2 M HCl solution were sterilized at 121°C for 20 min. Each flask was inoculated with a total number of 2x10⁷ fungal spores and incubated at 30°C. A non-inoculated flask was used as a control sample blank.

A replicate for each fungal strain consisted of ten individual flasks and one of each was used once every day for sampling. Fermentation samples were collected by adding 80 ml distilled water per flask, homogenized partially with a spatula; and mixed in an incubator shaker (Innova 4230, New Brunswick Scientific) at 250 rpm and 25°C for 1 h. The
supernatants were clarified by centrifugation at 3,200×g and 4°C for 20 min and filtration through Whatman #1 (11 µm pore size), and set aside for protein content analyses. The precipitated pellets (wet fermented substrate) were lyophilized for 2 days with freeze dryer (Alpha 1-2/LD plus, Christ, Osterode am Harz, Germany), ground to a fine powder with mortar and pestle and set aside for glucosamine content (fungal biomass analyses).

3.2.6 Protein production

Different protein contents (exo-PG, exo PMG, endo-PG and nonspecific protease) were measured from the clarified extracts and expressed as enzymatic activity unit per gram of substrate (U/g). The data obtained were represented as mean ± SD. See Appendix A8 – A12 for detailed procedures.

3.6.6.1 Exo-polygalacturonase and exo-polymethylgalacturonase

Exo-PG and exo-PMG activities were measured according to previously described protocols (Blandino et al. 2002; Heerd et al. 2012; Silva et al. 2005) with slight modifications. The assays were performed in a microplate by mixing 10 µl of enzymatic sample and 90 µl of 5 µg/µl of substrate (polygalacturonic acid, PGA for exo-PG activity and citrus pectin for exo-PMG activity) in a 100 mM citrate-Na biphosphate buffer pH 5.0. The reduced galacturonic acid (GA) released by the reaction after 30 minutes of incubation at 30°C for exo-PG activity and 10 minutes of incubation at 45°C for exo-PMG activity, was quantified by the DNS method at 575 nm (Miller 1959) and compared to a GA standard curve. One unit of exo-enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of GA per minute at the standard assay conditions mentioned above.
3.6.6.2 Endo-polygalacturonase

Endo-PG activity was measured according to an adapted method from (Ortiz et al. 2014). The assay was performed in a microplate by mixing 8 µl of enzymatic sample and 8 µl of 5 µg/µl PGA substrate in a 100 mM citrate-Na biphosphate buffer pH 5.0. The hydrolyzed PGA unable to precipitate with ruthenium red dye (RR) after 20 minutes of reaction at 40°C, was measured at 535 nm and compared to a PGA standard curve (from 0 to 36 µg). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µg of PGA in smaller fragments unable to precipitate with RR per minute at the standard assay conditions mentioned above.

3.6.6.3 Protease

Nonspecific protease activity was measured according to the commercial Pierce protease assay kit (Thermo Scientific, Illinois, USA). The assay was performed in a microplate by mixing 50 µl of enzymatic sample and 100 µl of 2 µg/µl succinylated casein (substrate) in a 50 mM borate buffer pH 8.5. The released tyrosine (product) by the reaction after 20 minutes of incubation at 37°C, was measured at 450 nm and compared to a TPCK-trypsin standard curve. One unit of protease activity was defined as the amount of enzyme that converts as much substrate as 1 µg of TPCK-trypsin (standard protease) per minute at the standard assay conditions mentioned above.

3.6.6.4 Total soluble protein

Total soluble protein was measured in SSF-supernatant samples according to the modified Bradford method (Bradford 1976) from the commercial Coomassie Plus™ Protein Assay Kit (Thermo Scientific, Illinois, USA). The assay was performed using the
standard microplate protocol and bovine serum albumin (included in the kit) as a standard. Absorbance values were measured at 595 nm.

3.2.7 Biomass production

The glucosamine (GlcN) released by acid hydrolysis of chitin present in the cell wall of the filamentous fungi was measured as an indirect method to estimate biomass content in the fermented samples. GlcN content in samples of 0.1 g of dried fermented substrate (dfs) was assessed according to Zamani et al. (2008) with slight modifications, and compared to a standard curve of D-(+)-glucosamine hydrochloride. Fungal biomass was expressed in terms of mg of GlcN (mgGlcN) per g of dfs (from here on referred just as mg/g). The data obtained were represented as mean ± SD.
3.3 Results and discussion

3.3.1 Cloning of vgb-gene in A. sojae

The VHb gene (vgb) was transformed into Aspergillus sojae ATCC 20235 (A. sojae wt) following the protocol described by Mora-Lugo et al. (2014) (Chapter 2). A. tumefaciens LBA4404 containing the vector pRM-vgb (Figure. 3.1a) was utilized for chromosomal integration of the T-DNA element containing vgb as well as the ble gene for phleomycin resistance (Figure. 3.1b). A. sojae transformants were selected from minimal media agar plates containing 50 µg/ml phleomycin. After four days of incubation, eight fungal colonies per 10^5 conidia were obtained from the selection plates. These putative transformants were sub-cultured individually on PDA plates supplemented with 100 µg/ml phleomycin for four generations to evaluate the stability of the T-DNA that confers antibiotic resistance. Three out of eight transformants survived after the antibiotic treatment and were used for further analysis.

3.3.2 Verification of transformation using PCR

The three obtained phleomycin-resistant transformants were examined by PCR analysis to confirm successful chromosomal integration of the T-DNA cassette. Genomic DNA from these strains and their parental strain (A. sojae wt) as negative control were used to target the ble and vgb genes by PCR with the specific primers sets BLE-F/ R and VHb-F/ R (Table 3.1), respectively. PCR products of expected sizes of 905 bp for the ble gene and 1281 bp for the vgb gene were obtained from the transformants while in the wild-type sample these DNA regions were absent (Figure. 3.2a). Bidirectional
The vector pRM-vgb was used for ATMT of *A. sojae*, and contains the kanamycin resistance gene (*kanR*), trans-acting factor A gene (*trfA*), replication origin (*oriV*) and transfer DNA region (T-DNA). B, Enlargement of the T-DNA region that is delimited by the right and left border (RB and LB, respectively), and contains the *Vitreoscilla* hemoglobin gene (*vgb*) and the enhanced green fluorescent protein (*egfp*) reporter gene under control of the constitutive *Aspergillus nidulans* *gpdA* promoter (*P_{gpdA}*), the phleomycin resistance gene (*Sh ble*) under control of the *A. nidulans* *trpC* promoter (*P_{trpC}*), and *trpC* terminator (*T_{trpC}*). The red arrows indicate the target sites for the oligonucleotide primers BLE-F/R, VHb-F/R. Target sites for restriction enzymes are indicated in italics.

sequencing of the PCR products with the primers used for PCR confirmed the identity of
ble and vgb and thus successful integration of these genes into the genome of the A. sojae transformants.

3.3.3 Verification of egfp expression using fluorescence microscopy

Additionally to the presence of the egfp reporter gene by PCR analysis, expression of the gene in the fungal transformants was verified by fluorescence microscopy analysis. Fluorescence signals were observed in the fungal transformants containing the vgb gene and the egfp in the same operon. In contrast, we observed no fluorescence signal in the parental A. sojae wt (Figure. 3.2b). Fluorescence microscopy analysis facilitated further control and discrimination between transformed and untransformed fungus as there was no noticeable morphological difference between these fungal colonies.

After PCR and fluorescence microscopy analysis, the three positive fungal transformants were screened on agar plates supplemented with PGA to determine variation of pectinolytic activity between them as described by Martos et al. (2013). As there was neither difference in the zones of pectin hydrolysis on the agar plates nor phenotypical changes observed between the fungi, one of the positive transformants was randomly selected (A. sojae vgb+) to carry out all subsequent SSF experiments.
Figure 3.2. A, Verification of putative *A. sojae* transformants by PCR analysis. The amplified PCR products at the expected size (highlighted with arrows) confirm the presence of the *ble* gene and the *vgb* gene in the genomic DNA samples of the fungal transformants. Genomic DNA of *A. sojae* wt was used as negative (W) and purified pRM-vgb vector as positive control (C); molecular size marker (M). B, Verification of *egfp* expression in the selected *A. sojae* vgb+ transformant by fluorescence microscopy analysis. *A. sojae* wt was used as negative control.
### 3.3.4 SSF with the transformed and untransformed fungus

The growth of the transformant A. sojae vgb+ and parental A. sojae wt strain in the solid-state cultures was visually examined. Both fungal strains showed a similar grow pattern during each of the entire fermentation period of ten days (Figure. 3.3). The first mold growth was observable two days after inoculation and mycelia were patchily distributed on the substrate. Abundant mycelial growth was observed after four days of fermentation with total colonization of the solid substrate by day six. The first conidia production was observed after six to seven days of fermentation reaching its maximum content by the end of the fermentation at day ten, where conidia are indicated by the green color (Figure. 3.3).

![Figure 3.3.](image)

Figure 3.3. Typical growth of A. sojae wt and A. sojae vgb+ on solid substrate during the 10-day fermentation period (only selected days are shown). The column on the left shows a representative flask of the inoculation day (0) and the column on the right shows the non-inoculated control media after ten days of incubation under the same conditions(C).
3.3.5 Production of extracellular proteins

To evaluate whether VHb had a positive effect on the production of extracellular proteins of *A. sojae* in SSF, fermented samples of the transformant *A. sojae* vgb+ and parental *A. sojae* wt strain were harvested daily and assessed for various enzymatic activities. A clear difference in protein production was observed between both fungal strains (Figure. 3.4). Regarding to pectinases production, the maximum exo-PG and exo-PMG titers of 562.1 U/g and 75.4 U/g after seven days of fermentation with *A. sojae* vgb+ were nearly 1.60-fold and 1.45-fold higher, respectively in comparison to the wild-type strain (Figure. 3.4a-b). However, no relative increase of endo-PG content was

![Figure 3.4](image)

Figure 3.4. Protein production of *A. sojae* wt and *A. sojae* vgb+ in SSF at flask scale. Exo-PG (a), exo-PMG (b), endo-PG (c) and protease (d) content was determined from enzymatic extracts collected every 24 h during the ten-day incubation period. Solid lines indicate the enzymatic yield and dashed lines indicate specific activity. Each data point represents the average ± SD from fermentations carried out in triplicates.
measured in the recombinant fungus, compared to the wild-type strain. In this case, the maximum endo-PG titer of 132.1 U/g was observed for the wild-type strain after six days of fermentation (Figure. 3.4c). Regarding to protease production, the maximum quantity of 39.2 U/g was nearly 1.25-fold higher in the transformed fungus compared to the parental strain after six days of fermentation (Figure. 3.4d). Overall, the maximum enzymatic titers for both fungal strains were between the sixth and seventh day of fermentation. The pH of all enzymatic extracts increased overtime ranging from pH 3.9±0.3 at the start to pH 5.8±0.5 at the end of the fermentation, in both the transformant and wild-type strains.

3.3.6 Biomass production

The production of biomass of A. sojae wt and A. sojae vgb+ on the solid-state cultures was indirectly evaluated by measuring the glucosamine content of the cell wall of the fungi. In general, biomass titers were higher in the transformed fungus in comparison to the wild-type cultures, with significantly improved levels between the seventh and tenth day of fermentation (Figure. 3.5). The maximum glucosamine content of 6.9 mg/g measured in the recombinant fungus after eight days of fermentation was 1.33-fold higher compared to the maximum content of the parental strain after six days of fermentation. Such improvement of biomass content in A. sojae vgb+ was similar in order of magnitude to its improved levels of extracellular protein such as proteases and exo-pectinases.
Figure 3.5. Biomass content of *A. sojae* wt and *A. sojae* vgb+ in SSF. The values plotted represent miligrams of glucosamine per gram of dried fermented mass collected every 24 h during the ten-day incubation time, and are expressed as mg/g. Each data point represents the average ± SD from fermentations carried out in triplicates.

Figure 3.6 summarizes the various enzymatic and biomass maximum yields for both fungal strains, where standard errors never exceeded 10%.

*A. sojae* ATCC 20235 has demonstrated recently its potential for pectinases production in fermentation systems and particularly in SSF (Demir et al. 2012; Gogus et al. 2006; Heerd et al. 2014a; Heerd et al. 2014b; Heerd et al. 2012; Mata-Gomez et al. 2014; Tari et al. 2008; Tari et al. 2007; Ustok et al. 2007). However, it is generally assumed that there is a limitation in the oxygen supply to the cells that are in close contact with the substrate in solid-state cultures with filamentous fungi (Oostra et al. 2001; Rahardjo et al. 2005). Previous studies have demonstrated beneficial effects of VHb to alleviate hypoxic conditions in several bacteria and yeast hosts (various summarized in Wei and Chen 2008, Stark et al. 2011 and Stark et al. 2015). Thus, this
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A study was aimed to alleviate hypoxic conditions for *A. sojae* in SSF by genetically engineering this fungus with the VHb in order to improve its cell growth and protein production levels. The effect of this hemoglobin in *A. sojae* was not explored until now and this so called “vgb/VHb technology” has rarely been used for SSF applications.

The VHb gene under control of the constitutive *A. nidulans* gpdA promoter (PgpdA) was integrated into the genome of *A. sojae* by an adapted method mediated by *A. tumefaciens* (ATMT). This transformation method was selected as its applicability for this fungus was previously demonstrated (Mora-Lugo et al. 2014, Chapter 2). Likewise, chromosomal integration of the transformed T-DNA cassette and expression of the reporter EGFP was successfully confirmed, indicating the presence of the protein product of *vgb* in the fungal transformants (Figure. 3.2). However, about ten times fewer putative

![Figure 3.6](image_url)
transformants (eight fungal colonies per $10^5$ conidia) were obtained in comparison to the previous study. A 482-bp larger T-DNA fragment was cloned in this study, which may have resulted in a lower number of transformants. Similarly, it is known from other studies on fungi, bacteria and plant cells that increasing DNA fragment size can result in lower transformation efficiencies (Fleming et al. 1995; Gouka et al. 1999; Kung et al. 2013). Nevertheless, the genetic stability of the T-DNA cassette in the transformants was comparable to the mitotic stability rate of 40% previously reported (Mora-Lugo et al. 2014, Chapter 2). Our results demonstrate thus that even though transformation efficiency was relatively low, mitotically stable transformants can be obtained by the adapted ATMT method, and indicate the applicability of this approach to explore the \textit{vgb}/VHb technology on \textit{A. sojae} for fermentation experiments.

Our results also show that \textit{A. sojae} harboring the \textit{vgb} gene (\textit{A. sojae vgb+}) yields higher amounts of biomass, protease and exo-pectinases in comparison to its parental strain \textit{A. sojae} wt in SSF (Figure. 3.4- 3.6). The results are in good agreement with recent reports of fungi, where increased levels of biomass and metabolites yields were associated with heterologous expression of VHb. For instance, improved biomass, spore and protease production by the filamentous fungus \textit{Paecilomyces lilacinus} and increased yields of total flavones and exopolysaccharides by \textit{Phellinus igniarius} were obtained in SmF when engineering these fungi with VHb (Zhang et al. 2014; Zhu et al. 2011). Also, expression of the VHb in \textit{Aspergillus niger} resulted in advantageous effects on the physiology of this fungus under oxygen-limiting conditions (Hofmann et al. 2009). Similarly, the increased biomass and extracellular-enzymatic content of \textit{A. sojae vgb+} in our fermentation showed that overall VHb improved the strain’s adaptability to the fermentation conditions. The maximum productivities of the transformed and
untransformed strain between the fourth and ninth day of fermentation were in good agreement with recent SSF with the parental fungus (Heerd et al. 2014b; Heerd et al. 2012; Mata-Gomez et al. 2014) and demonstrated thus an unaltered time shifting on enzyme productivity by VHB. The highest mycelial density observed during this period of maximum productivities indicates also the importance of this morphological stage for the production of proteins by A. sojae (Figure. 3.3).

Contrary to the increase of biomass, protease and exo-pectinases content in SSF, endo-PG production did not improve with A. sojae vgb+ but was rather slightly lower in comparison to its parental A. sojae wt strain, at least during the stationary phase of fermentation between the fourth and eighth day (Figure. 3.4c). Similarly, it was shown that heterologous expression of VHB in Escherichia coli appears to affect expression of several of its native genes in a different manner, either positively or negatively (Roos et al. 2004). In agreement with the previous study, the transformed VHB in A. sojae vgb+ did not affect the production of different extracellular protein equally but rather favored certain enzymatic activities. The higher exo- to endo pectinase activities suggests that under the SSF conditions, exo-pectinase activities are more essential for A. sojae vgb+, possibly to increase its biomass content. The more energy invested in exo-PG and biomass content may be connected to a trade-off concerning endo-PG production. Extending the investigation to other enzymatic activities besides hydrolases on A. sojae vgb+ may uncover novel potential applications of this fungus.

Even though VHB has been extensively assessed, to date there is not a comprehensive understanding of how its expression affects biological production. In recent applications of VHB on fungi and yeast, where beneficial effects on growth and
enzyme levels have been demonstrated, it has been pointed out that the mechanism of VHb can be rather complex (Shen et al. 2012; Wang et al. 2014; Wu and Fu 2012; Zhang et al. 2014). The common conception is that under oxygen limiting conditions, VHb is induced in order to bind the remaining oxygen and deliver it to the terminal respiratory oxidase(s) to maintain aerobic respiration at a high level under these conditions (Stark et al. 2011; Webster 1987). VHb may also take part in various steps of the respiratory chain as terminal electron acceptor by improving ATP production or showing peroxidase activity, hence the beneficial effects of VHb expression are presumably the result of one or more of its activities (Isarankura-Na-Ayudhya et al. 2010; Liao et al. 2014; Stark et al. 2015). Knowing that A. sojae has an aerobic metabolism, it may be implied that VHb favored the flux of oxygen in the transformant A. sojae vgb+ during the SSF conditions, making this fungal strain able to consume more solid substrate and in turn increase biomass and several of its enzymatic contents. Te Biesebeke et al. (2006) described that improvements on cell growth and enzymatic yields in an A. oryzae strain expressing hemoglobin domains similar to VHb may be due to an improved hyphae capacity to penetrate solid substrates. However, preliminary microscopic observations (data not shown) evidenced no remarkable difference on hyphal penetration depth between A. sojae vgb+ and A. sojae wt on fermented substrate. This indicates that the major contribution of VHb on the transformed fungus may lie in its metabolism rather than in its phenotype. Even though the exact VHb mechanism on A. sojae still needs to be shown, the present study demonstrated clearly that VHb had a positive effect on A. sojae metabolism in solid-state cultures. Future SSF studies with the transformant A. sojae vgb+ will show whether VHb will also have the same effect on the strain’s metabolism at a reactor scale.
3.4 Conclusions

The application of the \textit{vgb}/VHb technology to improve productivities of \textit{A. sojae} strain ATCC 20235 (\textit{A. sojae}) in SSF.

In solid-state cultures of filamentous fungi, it has been indicated that poor diffusion of oxygen into the fermented mass limits cell growth and protein production (Section 1.1.1). Thus, the \textit{vgb}/VHb technology (Section 1.1.5) was considered a good strategy to alleviate limiting conditions on SSF of \textit{A. sojae}. The bacterial \textit{Vitreoscilla} hemoglobin (VHb) is a simple protein with the natural function of transferring oxygen, and plays a vital role in the survival of its native and strictly aerobic host under oxygen-limited conditions, as stated in previous studies. Despite the action mechanism of VHb, as well as its regulatory mechanism have not been fully elucidated, functional expression studies of VHb have led to various levels of improvement in industrial fermentation and metabolite production under oxygen-restricted conditions. Heterologous expression of VHb in a wide variety of prokaryotes and eukaryotes was correlated to respiration and energy metabolism enhancements of the hosts. In this study, the VHb gene (\textit{vgb}) was successfully transformed into the genome of \textit{A. sojae} using a previously developed ATMT procedure (Chapter 2).

\textit{A. sojae} was genetically improved for SSF application by chromosomal integration of \textit{vgb} under control of the constitutive \textit{PgdA}. The transformed fungus (\textit{A. sojae vgb+}) showed improved biomass, protease and exo-pectinases production, while its endo-PG content appeared slightly diminished in comparison to its parental strain (\textit{A. sojae wt}) in solid-state cultures. The fungal transformant generated within this study is a suitable candidate to be evaluated in SFF scale-up studies in reactors. Based on our results, this genetic engineering strategy may also enable further optimization of
A. sojae as a microbial biomanufacturing platform for pectinolytic enzymes, e.g. by iterative cycles of mutagenesis.

Having demonstrated beneficial effects of VHb in SSF of A. sojae, the next step in this dissertation (see Chapter 4) was the performance study of the recombinant strain A. sojae vgb+ in a larger fermentation process in reactor-scale. In addition, enzymatic extracts of the recombinant fungal strain were compared against enzymatic extracts of the wild-type fungal strain for beverage processing applications.
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Chapter 3

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CHAPTER 4

Protein production of a genetically engineered *Aspergillus sojae* strain in a solid-state fermentation reactor
Abstract

Previously, a genetically modified Aspergillus sojae ATCC 20235 harboring the bacterial Vitreoscilla hemoglobin (VHb) (A. sojae vgb+) yielded higher amounts of extracellular protein in comparison to its parental strain (A. sojae wt) at flask scale (see Chapter 3). However, it is known that protein production varies particularly in solid state fermentation (SSF) at different size scales due to frequent variation of cultures conditions. In this study, protein production of A. sojae vgb+ was assessed in a scaled-up (100 times) SSF using a rotatory-drum reactor with 1.0 kg of solid substrate and compared to A. sojae wt and the previous flask-scale study. Furthermore, to test the industrial applicability of the produced proteins, enzymatic extracts of A. sojae vgb+ and A. sojae wt were assessed for clarification of fruit juices. Solid-state cultures at reactor scale of A. sojae vgb+ yielded up to 38% and 32% more enzymatic units (U) of exo-pectinases and protease per gram of fermented mass (g) in comparison to A. sojae wt; whereas endo-polygalacturonases production between both fungal strains was similarly high. The enzymatic extracts of the recombinant fungal strain clarified apple juice and blood orange juice with up to 37% and 50% more efficiency in comparison to the corresponding wild-type. Overall, the results of this study demonstrated that SSF of A. sojae vgb+ enhances protein production and that the protein complex of this recombinant strain could be of particular value for beverage processing applications.

4.1 Outline

Hydrolytic enzymes such as proteases and pectinases are incredible valuable in biotechnology due their various applications in industrial processes (Uhlig and Linsmaier-Bednar 1998). Pectinases or pectinolytic enzymes act on pectin, a polysaccharide highly distributed in nature as it forms part of the cell walls of plants. Consequently, industrial opportunities for these enzymes are counted as numerous and varied (Adapa et al. 2014). For instance, in the manufacturing of commercial beverages such as wine, juice or coffee, these enzymes are regularly utilized as they help to speed up fruit juice extraction and clarification (Sandri et al. 2011; Sandri et al. 2014; Bonnin et al. 2014). Commercial preparations of pectinolytic enzymes derive generally from bioprocesses with filamentous fungi. *Aspergillus, Trichoderma* and *Penicillium* strains are common microbial strains used in submerged (SmF) and solid-state fermentation (SSF) for the production of pectinolytic or other hydrolytic enzymes (Jayani et al. 2005; Lara-Marquez et al. 2011). Hence, investigations on these bioprocesses that lead into increases of protein productivities are of biotechnological interest.

*Aspergillus sojae* ATCC 20235 is a filamentous fungus with potential for pectinases production. The various studies have shown the production capacities of this fungus in SmF and SSF processes. (Demir et al. 2012; Heerd et al. 2012; Mata-Gomez et al. 2014; Tari et al. 2007; Ustok et al. 2007). Moreover, classical strain improvement approaches have been assessed in *A. sojae*, which are characterized by the introduction of random mutations through physical and chemical mutagens and following screening of desired phenotypes (Heerd et al. 2014). However, it was only recently described a methodology to explore systematically strain improvement strategies for fermentations with this fungus (Mora-Lugo et al. 2014, Chapter 2). Within the use of this methodology,
it was created an engineered *A. sojae* strain harboring the bacterial *Vitreoscilla* hemoglobin gene (*vgb*) in its genome, and the potential of this recombinant strain for SSF was demonstrated (Mora-Lugo et al. 2015, Chapter 3). Pectinases, protease and biomass yields of such modified fungal strain (named *A. sojae* vgb+) were improved in a SSF process at a flask-scale in comparison to its parental strain (*A. sojae* wt), and therefore positioned this engineered fungus as a suitable candidate to be evaluated at a larger SSF process.

SSF has emerged as an attractive alternative to SmF in the field of protein production with filamentous fungi (Aguilar et al. 2008; De la Cruz Quiroz et al. 2014; Pandey 2003). In SSF processes the absence or low content of water resembles the natural habitat of filamentous fungi and therefore higher enzymatic titers are generally achieved with these bioprocesses. Moreover, it is a recognized advantage associated to SSF processes to use numerous agro-industrial residues as substrate such as wheat bran, corn stover, rice stover and sugar beet pulp, among others, as this alleviates in certain degree the problem of solid waste disposal (Gowthaman et al. 2001; Toscano et al. 2013). The potential for protein production of numerous fungal SSF processes has been described in various lab-bench studies elsewhere. However, the number of reports is significantly lower when it comes to the study of larger SSF processes. As discussed by Mitchell et al. (2006) and Mitchell et al. (1999), up-scaling SSF process is not a straightforward approach as it is often difficult to mimic fermentation conditions such as aeration, mixing and distribution between the solid substrate and the microbial strain from a bench-scale fermentation. Moreover, increases of heat and evaporation in larger fermented beds affect negatively fungal growth and therefore protein production (Mitchell et al. 2006; Mitchell et al. 1999). Thus, verification of scaled-up SSF processes
in reactors specially designed for solid cultures is of significance to endorse the value of these biotechnological processes.

Based on the previous work with the genetically engineered *A. sojae vgb*+ strain in SSF, this study assessed the performance of this fungus at a scaled-up fermentation using a built-up reactor for solid cultures. The protein titers of this recombinant strain were compared against its corresponding wild-type counterpart and scaled-down bioprocess. Moreover, enzymatic extracts of both fungal strains were evaluated for clarification of fruit juices to determine the potential of these samples for industrial applications. The results of this study expand our knowledge about this biotechnological process and assess further the suitability of *A. sojae vgb*+ as protein biofactory platform.

### 4.2 Material and methods

#### 4.2.1 Materials

The chemicals used were of analytical grade and mostly purchased from AppliChem GmbH (Darmstadt, Germany). The Pierce protease assay kit for nonspecific protease activity measurements was acquired from Thermo Scientific (Illinois, USA). The chemicals citrus pectin, galacturonic acid, polygalacturonic acid sodium salt and D-(+)-glucosamine hydrochloride for pectinases activity measurements, as well as the commercial pectinases preparation Pectinex 3XL were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The cloudy fruit juices “Wies Gart” apple juice and “Pure Fruit” blood orange juice were obtained from Aldi mark (Bremen, Germany) for clarification experiments.
4.2.2 Microorganisms and media

The parental strain *Aspergillus sojae* ATCC 20235 (*A. sojae* wt) and its descendant strain harboring the bacterial *Vitreoscilla* hemoglobin *A. sojae vgb*+ (Mora-Lugo et al. 2015, Chapter 3) were grown at 28°C until conidiation for 3-6 days on molasses agar plates (45 g/l molasses, 45 g/l glycerol, 18 g/l peptone, 5 g/l NaCl, 0.5 g/l KCl, 15 mg/l FeSO$_4$·7H$_2$O, 60 mg/l KH$_2$PO$_4$, 50 mg/l MgSO$_4$, 12 mg/l CuSO$_4$·5H$_2$O, 15 mg/l MnSO$_4$·H$_2$O and 20 g/l agar). Fungal spores were harvested in 0.02% (w/v) Tween-80, filtered through cotton to remove hyphae, and concentration adjusted with a hemocytometer (Thoma, Germany) (Appendix A4 and A5). The substrates wheat bran, sugar beet pulp pellets and molasses for media preparation were obtained from local suppliers (Bremer Rolandmühle Erling GmbH & Co. KG, Bremen, Germany; Nordzucker AG, Uelzen, Germany; Golden Sweet, Meckenheim, Germany).

4.2.3 Solid-state fermentation

SSF experiments were carried out in a 15-l rotating drum type reactor, Terrafors-IS (Infors HT, Switzerland) as described by Heerd (2013) with slight modifications. The solid substrate was prepared by adding 960 ml of tap water to 300 g of sugar beet pulp pellets (not-grinded!) contained in a 5-l-autoclavable baker and was let it stand until swelling of pellets for ≥5 h. Subsequently, 700 g of wheat bran and 640 ml of 0.5 M HCl were added to reach a final concentration of 0.2 M HCl and 160% moisture level (equaly to 61.53% MC$_{wb}$). The moistened mixture was homogenized with a spatula and autoclaved at 121°C for 20 min. Under aseptic conditions, the autoclaved substrate was poured into the reactor vessel previously in-situ autoclaved. The media was inoculated with 50 ml of a 4x10$^7$ fresh spore solution (2x10$^6$ spore/gds) and incubated for 7 days at 30°C. To favor the mixing of spores, an intermittent mixed process of 1 rpm for 10 min.
clockwise followed by 10 min anticlockwise was applied twice in the first day of the cultivation period. An air flow rate of 2 l/min (0.13 vvm; 0.77 l/min/kg) was applied during the first day of cultivation, following by a 5 l/min (0.33 vvm; 1.92 l/min/kg) rate for the rest of the cultivation. Levels of oxygen and carbon dioxide in the exit gas stream were detected by an exit gas analyzer coupled to the reactor. Data logging of on-line parameters was done with Iris V5 control software. Fermentations were carried out independently three times with A. sojae wt and A. sojae vgb+.

4.2.4 Fermentation sampling

Fermented samples of ~50 g were collected at every 24 h interval of cultivation and split into two aliquots. An aliquot of ~20 g was used to determine moisture content of the samples as indicated ahead, while another aliquot was used for enzyme leaching as follow. An amount of 20 g of fermented mass and 80 ml of distilled water were homogenized partially with a spatula in a 300-ml Erlenmeyer flask, mixed in an incubator shaker (Innova 4230, New Brunswick Scientific, USA) at 250 rpm for 1 h and centrifuged at 3,200×g and 4°C for 20 min. The supernatants (enzymatic extracts) were further clarified by centrifugation at 13,000×g and 4°C for 10 min, filtrated through Whatman #1 and assessed for different protein contents.

4.2.5 Moisture content

The quantity of water or moisture content on wet basis (MC_{wb}) of fermented samples was calculated with their initial moisturized weight (weight_{wet}) and final dried weight (weight_{dry}; drying treatment of 70°C for 3 days), according to the following equation:
\[
\text{MC}_{\text{wb}} (\%) = \frac{\text{weight}_{\text{wet}}}{\text{weight}_{\text{wet}} - \text{weight}_{\text{dry}}} \times 100
\]

Afterwards, the weight\text{dry} value of fermented samples with known \text{MC}_{\text{wb}} were calculated as follows:

\[
\text{weight}_{\text{dry}} (g) = \text{weight}_{\text{wet}} - \frac{\text{MC}_{\text{wb}} \times \text{weight}_{\text{wet}}}{100%}
\]

### 4.2.6 Enzyme assays

Unspecific protease and different pectinases (exo-PG, exo PMG and endo-PG) activity contents of fermented samples were measured as described in Mora-Lugo et al. (2015) (Chapter 3 and Appendix A8 – A12). The protein yields were expressed in terms of enzymatic activity unit (U) per weight\text{dry} of fermented samples in grams (U/g). The data obtained were represented as mean ± SD.

### 4.2.7 Application of \textit{A. sojae} enzymatic extracts

Enzymatic extracts of \textit{A. sojae} vgb+ and \textit{A. sojae} wt were enzymatically characterized and assessed for clarification of fruit juices. As a reference control, the commercial preparation of pectinases “Pectinex 3XL” used in various food processing and plant biotechnology applications was included in these analyses. Enzymatic extracts of both \textit{A. sojae} strains were obtained from samples harvested after six days of fermentation for enzymatic profile determination. The experimental \textit{A. sojae} strains enzymatic extracts and commercial Pectinex 3XL protein preparation were diluted for experimental determinations in 100 mM potassium acetate pH 5.0 to a final concentration of 0.1 and 1.0 mg/ml of total protein. For clarification experiments, the enzymatic
extracts were used to clarify apple juice and blood orange juice as described by Sandri et al. (2014) with slight modifications (Appendix A7). Shortly, glass tubes with 5 ml of cloudy juice/tube were prepared in triplicate for each enzymatic sample. Volumes of 1 ml of enzymatic extract were added to the corresponding tubes containing juice and incubated at 50°C for a reaction time of 30 and 150 min. Distilled water instead as enzymatic extract was used as a blank control. The samples were boiled at 100°C for 5 min to interrupt enzymatic reactions, cooled in an ice bath and centrifuged at 1,000 x g and 4°C for 10 min. Subsequently, supernatants were filtered through Whatman #1 (11 µm pore size) and absorbance (A) measured at 420 and 550 nm. Clarification was expressed in terms of % of transmittance (%T) as a measure of turbidity (as indicated in Balch 1931) and calculated as %T = 10^(2 - ΣA). The %T of the blank control was subtracted from other samples. The data obtained were represented as mean ± SD.
4.3 Results and discussion

Solid-state cultures of the *Aspergillus* strains *A. sojae* vgb+ and *A. sojae* wt were carried out independently in a rotating drum reactor to evaluate their performance in terms of fungal cell growth, protein production and respiratory metabolism. Subsequently, the extracellular protein complex of both fungal strains was assessed for clarification of fruit juices to evaluate their potential for industrial applications.

4.3.1 Fungal growth in the SSF reactor

Similar growth patterns of *A. sojae* vgb+ and *A. sojae* wt were observed on the solid media throughout the seven-day period fermentations (Figure 4.1). The first mold growth patchily distributed on the substrate was observable one day after the inoculation. Hyphal density increased particularly after three days of fermentation with also the first conidia production. Total colonization of solid substrate was reached after five days of fermentation with mycelial growth still dominating. Conidia indicated by the green color covered totally the surface of the solid substrate after six day of fermentation, reaching its maximum content after seven days of fermentation. The morphologies of these fungal strains were similar to other filamentous fungi such as *Penicillium* in solid substrates (Gutarra et al. 2009).

In comparison to fermentation in flasks (Mora-Lugo et al. 2015, Chapter 3), conidia production of *A. sojae* vgb+ and *A. sojae* wt were observed at an earlier stage in the reactor fermentations. Specifically, around half of the time was needed for both fungal strains to produce conidia in the larger scale as compared to the smaller cultures. As reported for the fungus *Alternaria solani* (Dynesen and Nielsen 2003), conidia production is induced when unfavorable conditions for vegetative growth such as
mycelial wounding and dehydration are present. Also, forced aeration, increased heat and evaporation in larger fermented beds are known factors that influence the fungal growth profile (Mitchell et al. 2006; Mitchell et al. 1999). In this study, mycelial damage by mechanical forces of agitation was excluded as a likely cause of early sporulation, because no agitation cycle was set during the fungal growth phase. Instead, the moisture content of fermented samples was determined to evaluate dehydration as a cause of early sporulation. Also this parameter was determined to subsequently calculate protein yields in weight_{dry} basis. A gradual drop of MC_{wb} was observed throughout the cultivation period of both fungal cultures. Specifically, water content decreased 28.6±2.9% and 30.7±4.1% in A. sojae vgb+ and A. sojae wt cultures, respectively after seven days of fermentation (Table 4.1). The results indicate that the premature sporulation in both A. sojae strains cultures was likely triggered by these dehydration conditions, which were harsher at reactor scale than in flask scale.

Microbial hosts modified with VHb have shown enhanced cell growth in fermentation systems which are correlated with improved protein contents (Stark et al. 2011; Stark et al. 2015; Wei and Chen 2008). In the previous study of A. sojae vgb+ and A. sojae wt in SSF at flask scale (Mora-Lugo et al. 2015, Chapter 3), higher contents of biomass were measured in the recombinant cultures even though a clear difference in the growth pattern between these fungal strains was not observed in flask cultures. It was speculated that an overgrowth of recombinant fungi would be more evident at larger fermentation settings on the basis of the previous analysis. However, in this study, it was shown that A. sojae vgb+ and A. sojae wt have a similar morphological growth in solid-state cultures at reactor scale (Figure 4.1), and hence different protein titers cannot be
predicted between these two fungal strains based on the morphology of the fermented beds.

Figure 4.1. A, Typical fungal growth on the solid substrate mixtures during the seven-day fermentation period. The figure example shows the growth of *A. sojae* vgb+, which was similar to the growth of *A. sojae* wt (not shown). B, Drum type reactor used in this study for solid-state cultures. An user-friendly software allows in situ sterilization and record of the O₂ and CO₂ exit during the fermentation process.
4.3.2 Protein production

Pectinases contents of A. sojae vgb+ and A. sojae wt were measured from fermented samples of the reactor cultures to evaluate whether the characteristic productivities of these fungal strains, previously shown in flask cultures (Mora-Lugo et al. 2015, Chapter 3), were reproducible in this larger setting. The results confirmed previous findings of highest protein production by the genetically engineered A. sojae vgb+ strain in SSF as follow.

4.3.2.1 Pectinases production

The exo-pectinases contents of the A. sojae vgb+ strain were improved in comparison to the A. sojae wt strain in the fermentations at reactor scale (Figure 4.2a and 4.2b). Particularly, there was significant variation in exo-PG and exo-PMG production after day four of the fermentations period. For instance, the maximum improved content of exo-PG of 726.2 U/g in the A. sojae vgb+ solid-cultures, corresponding to a specific activity of 204.6 U/mg and a productivity of 121 U/g/d, was reached after six days of fermentation and was nearly 1.4-fold higher in comparison to the corresponding A. sojae wt cultures. Regarding exo-PMG, the maximum improved content of 92.7 U/g in the recombinant fungal solid-cultures, corresponding to a specific activity of 27.4 U/mg and a productivity of 15.5 U/g/d, was reached after six days of fermentation and was nearly 1.3-fold higher in comparison to the corresponding wild-type cultures.

In contrast to exo-pectinases levels, endo-PG productions of both fungal strains were similar throughout the fermentations (Figure 4.2c). The maximum titer of 165.1 U/g was observed for the wild-type strain after six days of fermentation, which had no significant variation in comparison to its recombinant counterpart. This result agrees with
the trend observed in the flask cultures in which the endo-PG titers of the recombinant strain were slightly lower than the corresponding titers of the wild-type (Mora-Lugo et al. 2015, Chapter 3). Nevertheless, endo-PG titers were improved up to 36% at reactor scale in comparison to the flask cultures yields (Table 4.1).

4.3.2.2 Protease production

The protease content of A. sojae vgb+ was improved in comparison to A. sojae wt throughout the fermentations at reactor scale (Figure 4.2d). The maximum improved content of protease of 53.8 U/g in the A. sojae vgb+ solid-cultures, corresponding to a specific activity of 15.8 U/mg and a productivity of 10.8 U/g/d, was reached after five days of fermentation and was approximate 1.3-fold higher in comparison to the corresponding A. sojae wt cultures. The protease contents of both fungal strains were improved up to 37% at the reactor scale in comparison to the flask cultures yields (Table 4.1).

4.3.3 Respiration rates of fungal strains in SSF reactor

Enhanced respiratory metabolism has been inferred in several microbial hosts genetically engineered with VHb, as demonstrated by cell growth and protein production studies (Stark et al. 2015; Wei and Chen 2008). To evaluate the relationship between protein production and respiratory metabolism of A. sojae vgb+ and A. sojae wt, levels of oxygen and carbon dioxide were monitored during their fermentations in the reactor (Figure 4.3). The results indicated different trends in oxygen consumption and carbon dioxide production between both fungal strains. On one hand, a greater oxygen consume of A. sojae vgb+ cultures was evident after two days of fermentation and until the end of the fermentation. On the other hand and similar in trend to the oxygen consumption
profile, a greater carbon dioxide production of A. sojae vgb+ cultures was evident after two days of fermentation and until the end of the fermentation. The results demonstrated a favored respiratory capacity of the A. sojae vgb+ strain, and agreed well with the superior protein capacities of this recombinant strain in comparison to its wild-type. In a previous study, a similar phenomena was observed for an Aspergillus oryzae strain, expressing hemoglobin domains similar to VHB, in which increased oxygen consumption
rates during the fermentation process were associated with improved cell growth and enzyme production (te Biesebeke et al. 2006). Thus in agreement with the previous study, the analysis indicate that the improved protein production of *A. sojae vgb*+ is likely due to its enhanced respiration metabolism, as demonstrated by higher oxygen consumption and carbon dioxide production rates.

**Figure 4.3.** Oxygen consumption (exit O2 %) and carbon dioxide production (exit CO2 %) of *A. sojae vgb*+ and *A. sojae* wt in SSF. Levels of oxygen (solid lines) and carbon dioxide (dashed lines) were detected by an exit gas analyzer attached to the drum vessel.
### Table 4.1. Summary of SSF with *A. sojae* vgb+ and *A. sojae* wt at flask and reactor scale.

<table>
<thead>
<tr>
<th></th>
<th>Flask¹</th>
<th>Reactor²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate mix</strong></td>
<td>7:3 ratio of wheat bran &amp; sugar beet pulp with 160% moisture content and 0.2 M HCl final conc.³</td>
<td></td>
</tr>
<tr>
<td><strong>MC&lt;sub&gt;wb&lt;/sub&gt; (%)⁴ initial</strong></td>
<td>61.5±2.8 (~160% on dry basis)</td>
<td></td>
</tr>
<tr>
<td><strong>Inoculum (spore/g)</strong></td>
<td>2x10⁶</td>
<td></td>
</tr>
<tr>
<td><strong>Substrate amount (g)</strong></td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Scaling ratio</strong></td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td><strong>Forced aeration</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Fungal strain</strong></td>
<td><em>A. sojae</em> vgb+</td>
<td><em>A. sojae</em> wt</td>
</tr>
<tr>
<td><strong>MC&lt;sub&gt;wb&lt;/sub&gt; (%)⁴ final</strong></td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>**Exo-PG (U/g)**⁵</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>562.1±34.3</td>
<td>354.1±43.1</td>
</tr>
<tr>
<td>**Exo-PMG (U/g)**⁵</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>75.4±4.1</td>
<td>52.4±3.6</td>
</tr>
<tr>
<td>**Endo-PG (U/g)**⁵</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>116.1±11.4</td>
<td>132.1±10.5</td>
</tr>
<tr>
<td>**Protease (U/g)**⁵</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>39.2±3.4</td>
<td>31.1±2.8</td>
</tr>
<tr>
<td><strong>Clarification of apple juice (%)⁶</strong></td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>Clarification of blood orange juice (%)⁶</strong></td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

1. Data obtained from Mora-Lugo et al. (2015) (Chapter 3)
2. Data generated in this study.
4. Content of water on wet basis determined experimentally either at the start (initial) or end (final) of the fermentation process.
5. The maximum enzymatic yields are shown. Upper number indicate harvested day.
6. Maximum clarification levels achieved under experimental conditions stated in Figure 4.5
n.a. = not stated in the study.
4.3.4 Application of enzymatic extracts of *A. sojae* strains

Pectinases preparations have been used in fruit juice production for more than 60 years (Bonnin et al. 2014). Today, they are a prerequisite for obtaining clear and stable juices, satisfying juice yields, and high-quality concentrates, along with improving the economy of the process (Grassin and Coutel 2009). In this study, experimental enzymatic extracts of *A. sojae* vgb+ and *A. sojae* wt were enzymatically characterized and assessed for clarification of fruit juices, an industrial process generally involving pectinases activities. As a reference control, the commercial preparation Pectinex 3XL, used in food processing and plant biotechnology applications, was included in these analyses.

4.3.4.1 Characterization of enzymatic extracts

For enzymatic profile determination, the enzymatic extracts of *A. sojae* vgb+ and *A. sojae* wt were obtained from fermented samples harvested after six days of fermentation, as after this time period maximum contents of pectinases and protease were measured. Pectinases and protease are known to play a major role in the clarification of fruit juices (Pinelo et al. 2010; Lara-Marquez, et al. 2011; Bonnin et al. 2014). In order to achieve comparative enzymatic profiles, the selected enzymatic extracts of the *A. sojae* strains, as well as the commercial preparation Pectinex 3XL, were prepared to a final concentration of 0.1 and 1.0 mg/ml of total protein.

Protease (nonspecific) and different pectinases (exo-PG, exo PMG and endo-PG) activity contents were assessed (Figure 4.4). Significant differences of enzymatic contents were observed between the experimental *A. sojae* protein extracts and the commercial preparation. On one hand, the exo-PG, exo-PMG and protease enzymatic contents of the *A. sojae* vgb+ extracts were slightly higher in comparison to the
corresponding contents of the *A. sojae* wt extracts, whereas the endo-PG content of both fungal samples were comparable. For instance, contents of exo-PG of 20.4 and 16.7 U/ml, exo-PMG of 2.6 and 2.3 U/ml, endo-PG of 4.6 and 5.2 U/ml, and protease of 1.4 and 1.2 U/ml were measured in the 0.1 mg/ml samples of *A. sojae* vgb+ and *A. sojae* wt, respectively. These enzymatic contents of *A. sojae* vgb+ showed an increase in the 1.0 mg/ml preparations compared to *A. sojae* wt, with contents of exo-PG of 204.6 and 166.5 U/ml, exo-PMG of 26.1 and 23.3 U/ml, endo-PG of 44.5 and 52.4 U/ml, and protease of 14.4 and 12.2 U/ml, respectively. On the other hand, the commercial preparation Pectinex 3XL had higher enzymatic contents than the experimental enzymatic extracts of both *A. sojae* strains (Figure 4.4). This result was expected, as commercial preparations are

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**Figure 4.4.** Comparison of enzymatic profile of protein extracts used for clarification of apple juice and blood orange juice. All protein preparations were prepared to a final concentration of 0.1 mg/ml (A) and 1.0 mg/ml (B) of total protein.
commonly made by semi-pure protein contents, which increase greatly their enzymatic activities (Del Cañizo et al. 1994). Moreover, commercial enzyme preparations for fruit juice production and wine making generally contain other cell wall-digesting enzymes in addition to pectinases, to obtain greater juice yield and clarity, as stated in Heerd (2013). In this study, the commercial preparation Pectinex 3XL was shown to have a more concentrated enzymatic profile than the experimental A. sojae enzymatic extracts, and hence, it was considered to be a good reference for measuring juice clarification efficiency.

4.3.4.2 Clarification of fruit juices

Pectinases are mainly used in industrial applications for extraction and clarification of juice from citric fruits rich in pectin content (Bonnin et al. 2014; Kashyap et al. 2001). The previous characterized enzymatic extracts of both A. sojae vgb+ and A. sojae wt and the commercial protein preparation “Pectinex 3XL” (reference) were assessed for clarification of apple juice and blood orange juice following the instructions by Sandri et al. (2014).

Evident variations in clarifications levels were observed between all the protein preparations (Figure 4.5). Under all conditions tested, the commercial enzyme preparation Pectinex 3XL was the most effective for clarification of both types of fruit juices. This result was expected as higher pectinases and protease levels were measured in the commercial protein preparation (Figure 4.4). It was also shown that increasing protein concentration and reaction time favored clarification in both juices samples, but with little effects. The maximum clarification levels for all enzymatic preparations were observed with 1.0 mg/ml of total protein and 150 min of reaction time. These
clarification levels were not much higher as might have been expected in comparison to the more diluted samples with shorter times of incubation. Thus, this data indicates that a maximum clarification level of each kind of fruit juice was reached with each enzymatic preparation, independent of protein concentration and reaction time.

The cloudy apple juice samples were up to 37% more efficiently clarified by the *A. sojae* vgb+ enzymatic extracts in comparison to the *A. sojae* wt enzymatic extracts at the conditions with the maximum clarification levels in both types of samples (condition 4) (Figure 4.5). However, the clarification levels at these conditions were comparable with the ones in condition 3, where reaction time was extended from 30 to 150 minutes. This indicates that most of the enzymatic action of both *A. sojae* protein complexes took place within the first 30 minutes of incubation. Substantial improvements in apple juice clarification were more evident when the concentration of protein was increased from 0.1 mg/ml to 1.0 mg/ml. However, this improvement was not linearly proportional to the increased concentration of protein, which suggests that almost maximum enzymatic action was reached with the less concentrated protein complexes.

Regarding to the cloudy blood orange juice samples, higher clarification levels were achieved with the enzymatic extracts of both *A. sojae* strains in comparison to the apple juice samples (Figure 4.6). The cloudy blood orange juice samples were up to 50% more efficiently clarified by the *A. sojae* vgb+ enzymatic extracts in comparison to the *A. sojae* wt enzymatic extracts at the conditions with the maximum clarification levels in both type of samples (condition 4) (Figure 4.6). However, improvements in clarification levels were inferior when increasing concentration of protein and reaction time in blood orange juice samples in comparison to the apple juice samples (Figure 4.5).
Despite having comparable enzymatic contents (Figure 4.4), the protein preparations of *A. sojae vgb+* were more effective than the protein preparations of *A. sojae* wt for clarification of fruit juices, indicating that the effect of other enzymatic activities may have played a relevant role in the recombinant fungal strain protein preparations. Mata-Gomez et al (2014) demonstrated that besides exo-PG and endo-PG, other hydrolytic activities were produced by *A. sojae* when wheat bran and sugar beet
Figure 4.6 Clarification of blood orange juice by different pectinase extracts (W, V and C) under various conditions. Each data point represents the mean ± SD from triplicates trials. B = sample blank (unclarified juice); W = enzymatic extract of A. sojae wt; V = enzymatic extract of A. sojae vgb+; C = commercial protein Pectinex 3XL (reference)

pulp were used as a carbon source. A detailed study on the hydrolytic activities of the protein complex of A. sojae vgb+ could provide valuable insights for further biotechnological applications of the enzymatic extracts. Overall, the enzymatic preparations examined in this study can be ordered according to their clarification capacity in both apple juice and orange juice samples, as follows:

Pectinex 3XL (commercial) > A. sojae vgb+ > A. sojae wt
Previous investigations have demonstrated application of fungal enzymatic extracts for clarification of juice and have indicated the role of various factors affecting clarification of juice including temperature, reaction time, pH, enzymatic activities profile and purity of the proteins. Heerd (2013) previously demonstrated the efficiency of enzymatic extracts of *A. sojae* wt to improve extraction and clarification of cloudy pure apple juice and compared it against commercial pectinolytic enzyme preparations. The simultaneous action of several enzymes rather than a single enzyme was attributed to be the main factor for better clarification levels with the commercial preparations in that study. Likewise, in this study, it is tempting to assume that the higher clarification levels of the commercial enzyme preparation Pectinex 3XL were due to its richer enzymatic profile. For instance, the levels of clarification by diluted samples of the commercial preparation Pectinex 3XL in both fruit juices were not reproducible with concentrated samples of experimental extracts of *A. sojae*. In another study, a positive effect on clarification of apple juice has been demonstrated using pectinolytic enzyme preparation from *Aspergillus niger* combined with gelatin (Singh and Gupta 2004). Gelatin is used as fining agent during apple juice processing, which favors clarification by forming gelatin-tannin complexes. It can be assumed that the presence of gelatin may have similar positive effects in enzymatic extracts of *A. sojae* for clarification of apple juice. Pinelo et al. (2010) have used enzyme preparations derived from *Aspergillus* spp. to improve clarification of cherry juice, and pointed out that pectinas and proteases play a decisive role in the reduction of juice turbidity via pectin depolymerization and prevention of protein-polyphenol complexes formation, respectively. Dey and Banerjee (2014) have used semi-pure polygalacturonase of *Aspergillus awamori* for clarification of apple juice and indicated that clarification is favored by increasing enzyme concentration to reduce
electrostatic repulsion between cloud particles which cause them to aggregate into larger particles and eventually settle out.

Overall, the results of this study show that enzymatic extracts of A. sojae vgb+ are more efficient than the enzymatic extracts of A. sojae wt for clarification of apple juice and blood orange juice. However, there are various parameters that can be further optimized to enhance clarification levels, i.e. enzyme concentration, temperature, reaction time, use of additional enhancers. Purification of relevant enzymes such as cellulases, amylases and xylanases from the A. sojae protein complexes or from other microbial sources, and re-formulation or enrichment of these samples in iterative cycles may significantly enhance their potential for industrial applications. Also, further investigations with the enzymatic extracts of A. sojae vgb+ in other pectin-rich fruit juices may lead to novel applications of these biological samples.

4.4 Conclusions

The production of exo-pectinases and protease of the genetically modified Aspergillus sojae ATCC 20235, harboring the bacterial Vitreoscilla hemoglobin (VHb) (A. sojae vgb+), was up to 38% and 33% higher in comparison to its parental strain (A. sojae wt) in SSF at reactor scale. Nonetheless, the endo-polygalacturonases production was comparable between both fungal strains. Also, higher levels of protein production were achieved in the solid-state cultures at reactor scale, compared to a previous study using flask cultures with both A. sojae strains, indicating the reliability of
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the scaled-up SSF. Contrary to qualitative examination of fermented bed morphologies, monitoring of oxygen consumption and carbon dioxide production in fungal cultures were good indicators to predict and differentiate cell growth and protein production between *A. sojae* vgb+ and *A. sojae* wt in SSF. In addition to these results, the enzymatic extracts of the recombinant fungus clarified apple juice and blood orange juice with up to 37% and 50% more efficiency, respectively in comparison to their equivalent wild-type enzymatic extracts. Overall, the protein complex of *A. sojae* vgb+ showed a high potential for future application in the extraction and clarification of juice from pectin-rich fruits.

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CHAPTER 5

An improved solid-state fermentation with *Aspergillus sojae* for production of technical enzymes
5.1 The approach

State of the art

This thesis provides a novel genetic engineering approach to improve protein production of *Aspergillus sojae* ATCC 20235 (*A. sojae*) in solid-state fermentation (SSF). The proposed strain improvement strategy consisted of two key technologies (reviewed in Chapter 1): the *Agrobacterium tumefaciens*-mediated transformation (ATMT) approach, a procedure previously used for genetic transformation of various fungal species (Section 1.1.4), and the *vgb/VHb* technology, a strategy based on the heterologous expression of the *Vitreoscilla* hemoglobin protein (VHb) to enhance the host respiratory metabolism (Section 1.1.5). In this research study, a series of milestones were sequentially accomplished in order to establish the intended strain improvement strategy. Firstly, it was show that the genetic transformation of *A. sojae* can be achieved by the ATMT approach, as demonstrated by protein expression studies (Chapter 2). Subsequently, the successful transformation of the VHb gene (*vgb*) in *A. sojae* was demonstrated using the developed ATMT protocol (Chapter 3). Furthermore, it was shown that an *A. sojae* strain harboring the *vgb* gene (*A. sojae* vgb+) yields higher titers of protein and biomass in solid-state cultures at flask scale in comparison to its parental strain (*A. sojae* wt). In a follow-up study, the superior capabilities of *A. sojae* vgb+ over *A. sojae* wt for protein production were demonstrated in scaled-up fermentations (100 times) using a SSF reactor (Chapter 4). Finally, enzymatic extracts of *A. sojae* vgb+ showed better clarification of fruit juices *i.e.* apple juice and blood orange juice, than the corresponding enzymatic extracts of *A. sojae* wt. These results suggest that further characterization of these enzymatic extracts and application for beverage processing applications are valuable.
The context

The value of filamentous fungi as cell factories in the fermentation industry is based on their natural ability to secrete large amounts of proteins (mainly hydrolytic enzymes) into the growth medium, which is a favorable characteristic for downstream processing applications (Nevalainen and Peterson 2014). Moreover, growing fungi is cost-worthy as fungi can be cultivated on inexpensive raw materials (e.g. wheat bran, sugar beet pulp, orange peel) and in turn produce valuable molecules such as primary and secondary metabolites, specialized chemicals, pharmaceuticals and enzymes (Meyer 2008; Ward 2012). Motivated by its biotechnological potential, this thesis was focused on the study of the filamentous fungus *Aspergillus sojae* ATCC 20235 (along this Chapter referred just as *A. sojae*). Specifically, the main goals were the development and evaluation of a genetic engineering strategy to improve protein production of *A. sojae* in solid-state fermentation (SSF). Previous studies with *A. sojae* had demonstrated the potential of this fungal strain for production of pectinolytic enzymes in fermentation process, and particularly in SSF (Ustok, Tari et al. 2007; Tari, Dogan et al. 2008; Demir, Gogus et al. 2012; Heerd 2012 and 2014; Mata-Gomez et al. 2014). Pectinases are biocatalysts that are widespread and are applied in various industrial processes (e.g. extraction and clarification of fruit juices, wine making, processing of coffee beans, etc.). Hence, enzymatic complexes of some fungi such as *Aspergillus niger* and *A. oryzae* strains have been subject of a vast number of studies due to their advantages as models for pectinolytic enzymes production (Lara-Marquez et al. 2011). For instance, a recent study showed that *A. sojae* yields higher amounts of pectinases in comparison to other common fungal pectinase-producers such as *A. niger* and *A. oryzae* in SSF (Heerd et al. 2012). The present study also suggested that strain improvement strategies are necessary to further explore the potential of this fungal strain. Despite various studies with *A. sojae,*
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Attempts to enhance its capacities for pectinases production by strain improvement strategies were scarce in fermentation systems until now. There was only a study that focussed on improving A. sojae capabilities in SSF using classic mutagenesis techniques (Heerd et al. 2014). However, classic mutagenesis and screening procedures have some limitations, as the induced mutations in a host are not easily traceable or movable to another host strain. Recombinant DNA technologies for filamentous fungi emerged as an important milestone and attractive alternative to classical mutagenesis procedures. The use of these techniques has facilitated developments of traditional fungal fermentations and genetic engineering to understand the molecular basis of product formation (Madden 2000). Classical mutagenesis is a widespread approach and its application has been reported in various strain improvement campaigns of industrial fungi. However, improvement strategies based on genetic molecular approaches have been less applied in filamentous fungi. This was possibly due to the fewer reliable molecular tools available to genetically engineer them in comparison to bacterial or yeast strains. Genetic engineering strategies can be powerful approaches to improve the productivities of filamentous fungi (Meyer 2011), but their implementation is a challenging task, especially in filamentous fungi (Section 1.1.3.6). Therefore, the focus of this thesis was the development of a strain improvement strategy for A. sojae based on genetic molecular tools to enhance its pectinases production in SSF.

Choosing a transformation system for A. sojae

For the development of a strain improvement strategy for A. sojae based on genetic engineering several factors were taken into account. The first and very important requirement for genetic molecular studies in A. sojae was the availability of a transformation system, which allows functional analysis of genes of interest and
systematic genetic modification to improve its biological capacities. As pointed out by Frandsen (2008 and 2011), the ability to introduce foreign DNA into a fungus can be nevertheless quite a very challenging task and is still a pillar of modern fungal genetics nowadays. Thus, the starting point of this research study was focus on the development and evaluation of a transformation system for \textit{A. sojae}. Various transformation methods were available for genetic modification of filamentous fungi. However, they all vary in transformation efficiency. In this study, the \textit{Agrobacterium tumefaciens}-mediated transformation approach was selected as this methodology has been successfully applied in various filamentous fungi, and importantly, it has been demonstrated in \textit{Aspergillus} strains (Section 1.1.4). ATMT is based on the property of \textit{Agrobacterium tumefaciens} to transfer part of its Ti vector, the transfer DNA (T-DNA), to the host cells during tumorigenesis (Section 1.1.4). However, this methodology, which is routinely used in various plant species, can also be inapplicable in fungal species, which was shown in previous studies. Moreover, various parameters have to be taken into account at the time of establishing an ATMT protocol to maximize the chances of successful transformation. It was necessary to assess the various key parameters of this technique in detail, which had led to a successful ATMT of various filamentous fungi, to achieve effective transformation in \textit{A. sojae} as well.

\section*{5.2 ATMT of \textit{A. sojae}}

\textbf{Assessing the ATMT approach for \textit{A. sojae}}

The first and very important parameter at the time of setting up an ATMT protocol for \textit{A. sojae} was the selection of a dominant marker. A suitable marker for \textit{A. sojae} could lead to differentiation of recombinant and parental forms of this fungus.
Auxotrophic markers such as the orotidine-5′-monophosphate decarboxylase gene (pyrG) have been used in filamentous fungi, but they are originated from chemical or physical mutagenesis that may yield undesirable mutations along with the mutation of interest (Ling et al. 2013). For various strains of industrial importance, selection based on dominant drug resistance provides a more generally applicable approach because it requires only that the target organism is sensitive to an agent for which a resistance is available (Tkacz et al. 2004). Thus, the resistance the antibiotic marker option was preferred to carry out DNA recombination studies in A. sojae as these types of compounds have been extensively assessed in filamentous fungi in general (Michielse et al. 2005). The hygromycin B is among the most common antibiotics for selection of fungal species (see Table 1.3). However, this antibiotic did not have any inhibitory effect on A. sojae according to some preliminary tests, and was thus discarded as a selection marker for this fungal specie. Consequently, the phleomycin antibiotic effect on A. sojae was assessed, which is generally recommended for fungal species with little inhibition by hygromycin. Phleomycin is a glycopeptide antibiotic of the bleomycin family, which binds and intercalates DNA thus destroying the integrity of the double helix. It was found that minimal media plates supplemented with 50 μg/ml phleomycin completely inhibited the growth of an A. sojae-inoculum of 10⁵ spores for up to 7 days, and therefore, this antibiotic was considered a good selection marker for this fungal specie, which could allow eventually discrimination between recombinant and parental species (Chapter 2).

A key variable during ATMT of A. sojae was also the selection of strong promoters that could lead to good levels of gene expression. Various promoters from plants, bacterial and even mammalian sources have been used in expression studies in filamentous fungi; however, fungal promoters are among the most common promoters
used in genetic molecular strategies, as demonstrated in various protein expression studies. Specially, the promoters from Neurospora crasse (cps1) and Aspergillus nidulans (trpC and gpdA) have been among the most used in ascomycetes. Thus, the promoters from A. nidulants were selected to drive gene expression in A. sojae, as they also belong to an Aspergillus specie. Moreover, these promoters were tested in other Aspergillus species such as A. terreus and A. carbonarious (Crespo-Sempere et al. 2011; Wang et al. 2014), and gave a good reference basis. In addition to the choice of a promoter that drives gene expression was the choice of a reliable donor plasmid for the promoters and the genes meant to be expressed in A. sojae. These molecular elements were enclosed in the T-DNA of the donor or Ti plasmid, which is the fragment of DNA that integrates into the genome of A. sojae. The plasmid pRF-HUE, a binary vector designed for cloning strategies, was chosen as a donor T-DNA plasmid, as this plasmid demonstrated its efficiency in ATMT of A. carbonarious (Crespo-Sempere et al. 2011). During the setup of the ATMT protocol, co-culture conditions between the bacterial Agrobacterium tumefaciens strain and fungal A. sojae strain were found to have a major effect on the transformation frequency of A. sojae. Different co-culture conditions were assessed to favor Agrobacterium growth, as this bacterium was the infect agent or DNA transmitter. The results of this study show that the combination of selected cultivation parameters can lead to successful ATMT of A. sojae, as demonstrated by cell culturing and gene expression analysis (Chapter 2). It was also demonstrated that the selected promoters can drive good expression in A. sojae, as demonstrated plate culturing and microscopic analyses. For example, the trpC promoter enabled expression of the bleomycin gene (sh ble) in A. sojae leading to phleomycin resistance of this fungus when growing on plates supplemented with this antibiotic. In addition, the gpdA promoter
enabled expression of the enhanced green fluorescent protein (EGFP) gene (egfp) that led to detection of fluorescence signal in the phleomycin-resistance A. sojae transformants.

To the questions 1) and 2), stated in the Section scope of this thesis, the following statement can be presented:

It was possible to genetically transform A. sojae using an ATMT procedure, as demonstrated the results of Chapter 2. A maximum transformation efficiency of 32 mitotically stable transformants per $10^5$ conidia was achieved using this method. Upon setup of the ATMT protocol, it was possible to perform this procedure routinely within a time frame of one-two weeks. However, obtaining of mitotically stable transformants required a good deal of screening work by polymerase chain reaction, protein expression studies, fluorescence microscopy and Western blot analysis. Further research on the physiology of this fungus could provide novel insights about its reproductive cycle, and the use of this knowledge could eventually be used to improve transformation frequencies.

5.3 The vgb/VHb technology in SSF of A. sojae

Assessing the vgb/VHb technology in A. sojae

In the last 25 years, the application of the vgb/VHb technology has been used in a wide variety of prokaryotes and eukaryotes used for fermentation processes to enhance respiratory metabolism and cell growth, as well as to improve the production of different biological molecules of technological importance such as primary and secondary metabolites, antibiotics, enzymes, etc. (Section 1.1.5). Moreover, this technology,
Enhanced bioprocess with A. sojae

consisting of heterologous expression of VHB, has been considered useful for bioprocesses where oxygen supply is limited. During filamentous fungal growth on solid substrates, it is generally assumed that there is a limitation in the oxygen supply to the cells that are in close contact with the substrate (Oostra et al. 2001; Rahardjo et al. 2005). For instance, the aerial hyphae that occur only in solid state are mainly responsible for oxygen uptake (te Biesebeke et al. 2006). Thus, in this research study, it was aimed to improve the efficiency of a SSF process with A. sojae by genetically engineering the metabolism of this fungus with VHB. This strategy was envisioned to alleviate hypoxic conditions during solid-state culturing of A. sojae, as they affect negatively cell growth and protein production of this fungus.

The bacterial *Vitreoscilla* hemoglobin gene (*vgb*) was successfully integrated into the genome of A. sojae using the developed ATMT protocol (Chapter 2). The occurrence of this gene in fungal transformants was validated in a similar manner as previously did with the *ble* and *egfp* genes during the setting up of such transformation system (Chapter 3). For instance, the *vgb* gene was amplified from isolated genomic DNA of A. sojae transformants by PCR (Figure 3.2), indicating successful chromosomal integration. And, fluorescence signal was detected in the phleomycin-resistant transformants containing the genes *vgb* and *egfp* in the same operon, indicating protein expression. In comparison to previous studies, this current study was one of the few examples where the *vgb* gene was transformed by an ATMT approach as previous engineering campaigns of *vgb/VHb* have been relied on electroporation-based methods. Moreover, to the extent of our knowledge, this was the first successful attempt to incorporate the *vgb/VHb* into this fungus yet.
The mitotic stability of the T-DNA, which is the DNA fragment containing the \( vgb \) gene, was about 40% in the \( A. \ sojae \) transformants (Chapter 3), and was comparable to the one observed when transforming a similar DNA cassette but without the \( vgb \) gene (Chapter 2); this indicated that this fungal specie have a characteristic level of acceptance towards DNA recombination. In previous studies, high abortive rates of transformed DNA were reported for close relatives of \( A. \ sojae \) such as \( A. \ oryzae \). Specifically, in DNA recombinant studies with \( A. \ oryzae \), an abortive rate of the \( A. \ nidulants \ argB \) gene was evidenced in approximately 90 % of the initial transformants of this fungus (Hahm and Batt 1988; John and Peberdy 1984). Since a long time, multinucleate conidia have been considered a probable cause for unstable transformation of filamentous fungi, as stated in Ji et al. (2013). In this study, the nuclei of the \( A. \ sojae \) conidia were stained with DAPI (4',6-diamidino-2-phenylindole) and microscopic analysis showed that the conidia can contain multiple nuclei ranging in numbers from 1 to 6 (data not shown). Thus, this fact was considered as the main cause of unstable transformation in the initial \( A. \ sojae \) transformants that failed to retain phleomycin resistance after sub-culturing them on PDA plates supplemented with 100 µg/ml phleomycin for four generations, as described in Section 2.3.5. More studies about the reproductive cycle of \( A. \ sojae \) will be needed to explain the exact mechanism resulting in abortive mutagenesis events on this fungus and facilitate future genetic manipulations. Nevertheless, in this study, 40% of remaining stable \( A. \ sojae \) transformants were found reliable for fermentation experiments as they maintained the T-DNA that confers antibiotic resistance after sub-culturing the transformants for many generations (Chapter 3).

To evaluate whether VHb had a positive effect on the production of extracellular proteins of \( A. \ sojae \) in SSF, different enzymatic contents were measured in the fermented
samples of the transformed *A. sojae* strain harboring *vgb* (*A. sojae vgb+*) and its parental strain (*A. sojae wt*). The modified *A. sojae vgb+,* which was grown in solid-state cultures at flask scale yielded up to 60% more enzymatic units (U) of exo-pectinases and protease, and up to 33% more biomass per gram of fermented mass (g) in comparison to its parental strain (*A. sojae wt*) (Figure 3.6); this demonstrated that the transformed T-DNA had actually a positive effect in the metabolism of the recombinant fungus favoring its growth under the bioprocess conditions assessed. As the interest was particularly in SSF, an optimized substrate mixture to maximum pectinases production was the preferred media to carry out protein production studies with these fungal strains. The results show that VHb had clearly an effect on *A. sojae vgb+* growth, significantly different to the one observed in its parental *A. sojae wt*. However, the effect of VHb was contrasting towards different enzymatic productions. On one hand, VHb favored production of exo-pectinases and protease, as well as cell growth. On the other hand, the endo-pectinases levels of *A. sojae vgb+* remained unchanged throughout the fermentations, with comparable levels to its wild-type. Also, an unaltered morphology of the *A. sojae vgb+* strain remained, as similar solid-state cultures morphologies were observed between the two fungal strains. The results of this study suggest that in general VHb favored growth of *A. sojae vgb+* over *A. sojae wt* on the solid substrate mixture with particular enrichments for exo-pectinases and proteases. It is tempting to assume that besides the enzymatic activities measured, productions of other biomolecules were improved in *A. sojae vgb+. In this regard, it is imperative to extend the study of the protein complex of *A. sojae vgb+*.

The improved cell growth and protein production levels of *A. sojae* by the *vgb/VHb* technology were in good agreement with recent reports on other fungi. For instance, Zhang et al. (2014) increased the production of biomass, spore and protease in
the filamentous fungus *Paecilomyces lilacinus* by expression of VHb, while Zhu et al. (2011) reported a VHb-correlated increase of total flavones and exo-polysaccharides yields in *Phellinus igniarius*. The expression of *vgb/VHb* was also correlated with an increase of 3.2-fold production of the cancer and HIV drug betulinic acid in *Saccharomyces cerevisiae* (Li and Zhang 2014). As compared with the previous studies, genetic engineering of *A. sojae* with VHb resulted in advantageous effects on its biological productivities in solid-state cultures at flask scale, and therefore, the performance of the recombinant strain *A. sojae* *vgb*+ was examined further in a larger fermentation process at reactor scale.

**The protein yields of *A. sojae* *vgb*+ in SSF at reactor scale.**

The fungus *A. sojae* *vgb*+ yielded more protein and biomass in SSF at flask-scale in comparison to its parental *A. sojae* wt; therefore, it was compulsory to measure protein production with these fungal strains at a larger fermentation setting to validate the enhanced performance of the recombinant fungal strain. For instance, in SSF processes, variations in cell growth are expected to occur at the time scale-up due to technological difficulties implied to mimic culture conditions between different cultures sizes. Also, reduction in protein production arises commonly in larger fermented beds due to increases of heat and evaporation factors that affect negatively fungal growth (Mitchell et al. 2006; Mitchell et al. 1999). Even though the modern SSF reactors allow manipulation of external operating variables such as temperature and aeration to the vessel, internal variables such as temperature and oxygen supply in the insides of a fermented fungal bed are difficult to control and measure. Therefore, it was important to measure the performance of the *A. sojae* *vgb*+ strain in a scaled-up SSF process to evaluate whether fungal growth and protein levels can be emulated at different cultures sizes (flask-scale
and laboratory-scale reactor), and this modified fungus can have fermentation advantages over its wild-type strain at a larger process scale.

Solid-state cultures of *A. sojae vgb*+ and *A. sojae* wt were carried out independently in a rotating drum reactor using an optimized medium for production of pectinases consisting of wheat bran and sugar beet pulp. The fungal growth was examined throughout the seven-day period fermentations. The performance of both fungal strains was evaluated in terms of protein production and respiratory metabolism. During culturing, similar growth patterns of both strains on the solid substrate mixture were observed throughout the fermentations period (Figure 4.1), which indicated that at this point it was not possible to deduce a favored metabolism in *A. sojae vgb*+. Nevertheless, the availability of gases sensors in the reactor allowed monitoring of the respiratory metabolism of the fungal strains. For instance, the results indicated different trends in oxygen consumption and carbon dioxide production between both fungal strains. Greater oxygen consumption and carbon dioxide production rates were observed in the *A. sojae vgb*+ cultures, demonstrating functional expression of VHb and an enhanced metabolism of this fungal strain.

Following the same trend observed in the flask cultures, *A. sojae vgb*+ yielded more amount of protein in solid-state cultures at reactor scale in comparison to *A. sojae* wt. For instance, *A. sojae vgb*+ produced up to 38% and 32% more pectinases and protease, respectively compared to the *A. sojae* wt. Also, out of three pectinase activities measured, exo-polygalacturonase yielded the maximum content of 726.2 U/g after six days of fermentation in the *A. sojae vgb*+ cultures at reactor scale, which corresponds to a productivity of 121 U/g/d and specific activity of 204.6 U/mg. Furthermore, the protein
Yields of the recombinant and parental strain in the reactor cultures were improved up to 30\% and 49\%, respectively in comparison to the flask cultures, indicating the reliability of the scaled-up fermentation process.

**Application studies of experimental enzymatic extracts.**

It was aimed to measure the efficiency of experimental enzymatic extracts of *A. sojae* vgb+ and *A. sojae* wt for clarification of fruit juices to evaluate whether the enzymatic extracts of a recombinant fungal strain could have any technological advantage over the parental enzymatic extracts for drink processing applications.

Selected enzymatic extracts of *A. sojae* vgb+ and *A. sojae* wt, which were produced in the reactor cultures, were used to clarify fruit juices. The protein complexes of both fungal strains contain high level of pectinases, so apple juice and blood orange juice were selected for clarification studies as these fruits are rich on pectin. The enzymatic extracts of both fungal strains were standardized to equal concentrations of total protein and assessed for clarification of apple juice and blood orange juice. Under standard conditions, the enzymatic extracts of *A. sojae* vgb+ clarify up to 37\% and 50\% more efficiently the apple juice and blood orange juice samples, respectively in comparison to the enzymatic extracts of *A. sojae* wt. Despite having comparable enzymatic contents of exo-pectinases and protease (Section 4.3.4), the protein preparation of *A. sojae* vgb+ were more effective than the protein preparations of *A. sojae* wt for clarification of fruit juices, indicating that the effect of other enzymatic activities may have played a relevant role in the recombinant fungal strain protein preparations. Moreover, slightly differences in clarification levels were observed in both protein complexes at different reaction conditions *i.e.* concentration of total protein and reaction
time. The results of this study encourage further investigation of reaction conditions to increase the levels of clarification of the *A. sojae* vgb+ protein complex.

To the questions 3), 4), 5), and 6), stated in the Section scope of this thesis, the following statements can be presented:

It was possible to genetically engineer *A. sojae* with VHb (*A. sojae* vgb+) using the developed ATMT procedure, as demonstrated the results of Chapter 3. The fungal strain *A. sojae* vgb+, which was grown in solid-state cultures at flask scale yielded up to 60% more enzymatic units (U) of exo-pectinases and protease, and up to 33% more biomass per gram of fermented mass (g) in comparison to its parental strain (*A. sojae* wt).

It was possible to retain the cell growth and production levels of an *A. sojae* strain genetically engineered with VHb in a solid-state culture at reactor scale. In this larger scale, the fungal strain *A. sojae* vgb+ produced up to 38% and 32% more pectinases and protease, respectively in comparison to its parental strain *A. sojae* wt. Furthermore, the enzymatic extracts of the recombinant fungal strain clarified apple and blood orange fruit juices with up to 37% and 50% more efficiency, respectively in comparison to the corresponding wild-type extracts.
5.4 Conclusions and future remarks

Conclusions

In Chapter 1 of this thesis, I reviewed filamentous fungi as great cell factories for the production of industrially-valuable biomolecules, e.g. antibiotics, pharmaceuticals and enzymes through fermentation systems. This chapter focuses on solid state fermentation (SSF) of the filamentous fungus Aspergillus sojae ATCC 20235 (A. sojae) to produce pectinases, which are valuable industrial enzymes in food applications. I highlighted the importance of a modern strain improvement strategy for SSF applications of this fungus. The aim of this thesis was the development and evaluation of a systematic strain improvement strategy for A. sojae in SSF applications, based on Agrobacterium tumefaciens-mediated transformation (ATMT) and the vgb/VHb technology. In Chapter 2 I showed that the ATMT approach can be used for protein expression studies in A. sojae. I confirmed this using polymerase chain reaction, fluorescence microscopy and Western blot analysis. The successful genetic transformation of A. sojae was confirmed by obtaining recombinant strains that are able to grow in phleomycin-supplemented plates and that are able to express the enhanced green fluorescent protein (EGFP). Subsequently, having set a transformation procedure for A. sojae, the results of Chapter 3 show the applicability of the ATMT for cloning the VHb gene (vgb) into the genome of A. sojae. An A. sojae strain harboring the vgb gene (A. sojae vgb+) yielded up to 60% higher amounts of exo-pectinases, protease and biomass in comparison to its parental strain (A. sojae wt) in SSF at flask culture scale. After I revealed positive effects of the VHb to enhance the metabolism of A. sojae in SSF, I pursued to evaluate fermentations of this fungal strain at a larger scale. In Chapter 4 of this thesis I show that the fungal strain A. sojae vgb+ retained its improved protein titers over the wild-type strain when scaled-up (100 times) fermentations were carried out. In
comparison to the flask cultures, pectinases contents of both fungal strains were enriched up to 40% in fermentations at reactor scale. I could also show that the enzymatic extracts of the *A. sojae* vgb+ strain are better suited for beverage processing applications in comparison to the enzymatic extracts of the *A. sojae* wt strain, as demonstrated by clarification of apple juice and blood orange juice studies. Overall, the results of this investigation, discussed altogether in more detail in Chapter 5, show that this genetic engineering approach is well-suited to improve the biological productivities of *A. sojae* in SSF. Such an optimized bioprocess may increase the cost-effectiveness of industrial processing of food and beverages.

**Future research**

The fungal strains, molecular tools, and knowledge generated during the development of this study open up new possibilities for further research on bioprocesses with *A. sojae*, some of which are listed below:

- The developed ATMT could be applied for heterologous expression of other relevant proteins in *A. sojae*. As demonstrated with VHb, a particular enzyme or molecular element could be incorporated into this fungus to explore its function. For example, the protein complex of *A. sojae* could be further enriched with cellulases, amylases or other pectinases activities from different organisms, which could result in technological advantages.

- Although SSF systems were previously identified to favor protein production of *A. sojae* wt, the modified *A. sojae* vgb+, which has an enhanced metabolism, may yield promising amounts of protein in SmF systems. Thus, the performance of the created recombinant fungal strain could be assessed for protein production in SmF systems.
A. sojae vgb+ produced up to 38% more enzymatic units of pectinases compared to the A. sojae wt in a substrate mixture composed of wheat bran and sugar beet pulp, which is an optimized medium for the production of pectinases. As A. sojae vgb+ has an enhanced metabolism, it is expected that this engineered fungal strain will likely yield higher levels of protein in other substrate formulations. Thus, the study of A. sojae vgb+ in other economical substrate mixtures is expected to be of great interest for the production of valuable biomolecules.

The clarification efficiencies of enzymatic extracts of A. sojae vgb+ were up to 50% higher than the enzymatic extracts of A. sojae wt in fruit juices. However, the content of exo-pectinases, endo-PG and un-specific protease were comparable between both enzymatic complexes. This indicated that other relevant enzymatic activities in the enzymatic extracts of A. sojae vgb+ contribute to clarify fruit juices more efficiently in comparison to the enzymatic extracts of A. sojae wt. Thus, the protein complex of the recombinant fungal could be further investigated to discern wheter other relevant activies were enhanced in this fungal strain.

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## Appendix

**Detailed experimental protocols and technical notes**

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DNA sequences
(Created by Rodrigo Mora-Lugo)

- T-DNA sequence length: 4996 bp.
- Plasmid sequence length (complete): 8414 bp
- The figure A shows the circular form of the pRM-eGFP plasmid and B shows an enlargement of its T-DNA region with all the target sites for oligonucleotide primers and restriction enzymes highlighted.
- The enhanced green fluorescent protein (EGFP) gene (egfp) is green highlighted in the DNA sequence.
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T-DNA from pRM-vgb plasmid

- T-DNA sequence length: 5515 bp
- Plasmid sequence length (complete): 8933 bp
- The figure A shows the circular form of the pRM-vgb plasmid and B shows an enlargement of its T-DNA region with all the target sites for oligonucleotide primers and restriction enzymes highlighted.
- The *Vitreoscilla* hemeoglobin (VHb) gene (vgb) is pink highlighted in the DNA sequence.
Culture media and stock solutions
(Recipes compiled by Rodrigo Mora-Lugo)

Here are listed and described in detail the preparation of reagents, stock solutions and culture media used in this work. Most of the recipes were prepared according to known sources, e.g. scientific publication, manufacturer directions or the laboratory molecular cloning manual of Sambrook and Russell (2001). All chemicals used were of analytical grade and were purchased from commercial sources, unless otherwise stated.

Reagents and stock solutions

Phosphate-buffered Saline (PBS)
137 mM NaCl
2.7 mM KCl
10 mM Na$_2$HPO$_4$
2 mM KH$_2$PO$_4$

Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na$_2$HPO$_4$, and 0.24 g of KH$_2$PO$_4$ in 800 ml distilled H$_2$O. Adjust the pH to 7.4 with HCl. Add H$_2$O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 psi (1.05 kg/cm$^2$) on liquid cycle or by filter sterilization. Store the buffer at room temperature.

10X Tris EDTA (TE) pH 7.4
100 mM Tris-Cl (pH 7.4)
10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 minutes at 15 psi (1.05 kg/cm$^2$) on liquid cycle. Store the buffer at room temperature.

Tris-Cl (1 M)
Dissolve 121.1 g of Tris base in 800 ml of H$_2$O. Adjust the pH to the desired value by adding concentrated HCl <!>. Afterwards, add H$_2$O to 1 liter. Sterilize solution by autoclaving. Store the solution at room temperature.

<table>
<thead>
<tr>
<th>pH</th>
<th>HCL</th>
<th>ml</th>
</tr>
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<tr>
<td>7.4</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

10X Tris-buffered Saline containing Tween-20 (TBS-T) (BioLegend)
Dissolve 80 g of NaCl, 2 g of KCl, 30 g of Tris base and 10 ml Tween-20 in 800 ml distilled H$_2$O. Adjust the pH to 7.4 with HCl. Add H$_2$O to 1 liter. Store the buffer at room temperature.

Blocking buffer
1X TBS-T
5% nonfat dry milk
Dissolve 5 g of nonfat milk in up to 100 ml of 1X TBS-T. Prepare a fresh solution for each Western Blot determination.

HCl (2.5 N)
Add 20.5 ml of concentrated HCl (!) (37% = 12.1 N) to 79.5 ml of sterile H$_2$O. Never do the other way around! Store the solution at room temperature.

50X TAE buffer
Dissolve 242 g of Tris base, 57.1 ml of glacial acetic acid (!) and 100 ml of 0.5 M EDTA (pH 8.0) in up to 1 liter of distilled H$_2$O. Dilute the concentrated stock solution to 1X (working solution) with distilled H$_2$O before use.

6X DNA loading dye
10 mM Tris-Cl (pH 7.6),
0.03% bromophenol blue,
0.03% xylene cyanol FF,
60% glycerol,
60 mM EDTA
Dispense the solution into aliquots and store them at room temperature or at 4°C up to 12 months. For longer periods, store at -20°C.

2X SDS Gel-loading Buffer
100 mM Tris-Cl (pH 6.8)
4% (w/v) SDS (electrophoresis grade)
0.2% (w/v) bromophenol blue
20% (v/v) glycerol
200 mM dithiothreitol or β-mercaptoethanol (!)
Dispense the solution into aliquots and store them at -20°C.

10X SDS PAGE (stock solution for running buffer)
250 mM tris base
1.92 M glycine
1% (w/v) SDS
Dissolve 300 g of tris base, 144.1 f of glycine and 10 g of SDS (electrophoresis grade) in up to 1 l of distilled H$_2$O. The pH of the buffer should be 8.3 and no pH adjustment is required. Store the buffer at room temperature.

Acrylamide Solution (45% w/v)
Acrylamide (DNA-sequencing grade) (!) 434 g
$N,N'$-methylenebisacrylamide (!) 16 g
H$_2$O to 600 ml

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Heat the solution to 37°C to dissolve the chemicals. Adjust the volume to 1 liter with distilled 
H₂O. Filter the solution through a nitrocellulose filter (e.g., 0.45-µm pore size), and store the filtered 
solution in dark bottles at room temperature.

**Ethidium Bromide (0.5 µg/ml)**

Prepare a 0.5 µg/ml working solution by adding 10 µl of 10 mg/ml ethidium bromide solution 
to 200 ml of distilled H₂O. Soak the gel for 15 minutes with gentle agitation. Longer staining times 
will result in high background. Rinse the gel with distilled H₂O and destain with fresh distilled H₂O for 
15 minutes with gentle agitation. At this point, the gel should be ready for photographing, although 
another 15 minute rinse with fresh distilled H₂O will reduce background further

**Ammonium Acetate (10 M)**

To prepare a 1-liter solution, dissolve 770 g of ammonium acetate in 800 ml of H₂O. Adjust 
volume to 1 liter with H₂O. Sterilize by filtration (e.g., 0.22-µm pore size). Store the solution at room 
temperature or at 4°C. Ammonium acetate decomposes in hot H₂O and solutions containing it should 
not be autoclaved.

**Coomassie Staining Solution**

Dissolve 0.25 g of Coomassie Brilliant Blue R-250 in 90 ml of methanol:H₂O <!> (1:1, v/v) 
and 10 ml of glacial acetic acid <!>. Filter the solution through a Whatman No.1 filter to remove any 
particle matter. Store at room temperature.

**EDTA (0.5 M, pH 8.0)**

Add 186.1 g of disodium EDTA.2H₂O to 800 ml of H₂O. Stir vigorously on a magnetic 
stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets <!>). Dispense the solution into 
 aliquots and sterilize them by autoclaving. Store the buffer at room temperature. The disodium salt of 
EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by addition of NaOH.

**KCl (4 M)**

Dissolve an appropriate amount of solid KCl in H₂O, autoclave for 20 minutes on liquid cycle 
and store at room temperature. Ideally, this solution should be divided into small (~100 µl) aliquots in 
sterile tubes and each aliquot thereafter used one time.

**NaOH (10 N)**

The preparation of 10 N NaOH <!> involves a highly exothermic reaction, which can cause 
breakage of glass containers. Prepare this solution with extreme care in plastic bakers. To 800 ml of 
H₂O, slowly add 400 g of NaOH pellets <!>, stirring continuously. As an added precaution, place the 
beaker on ice. When the pellets have dissolved completely, adjust the volume to 1 liter with H₂O. Store 
the solution in a plastic container at room temperature. Sterilization is not necessary.

**Culture media**

**LB Medium (Luria-Bertani Medium)**

To 950 ml of deionized H₂O, add:
Appendix

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Tryotone 10 g
Yeast extract 5 g
NaCl 10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 ml). Adjust the volume of the solution to 1 liter with deionized H₂O. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on liquid cycle.

Minimal Medium (MM)
10 mM K₂HPO₄,
10 mM KH₂PO₄,
2.5 mM NaCl,
2 mM MgSO₄.7H₂O,
0.7 mM CaCl₂,
9 µM FeSO₄.7H₂O,
4 mM (NH₄)₂SO₄,
10 mM glucose

Mix 100 ml of each of the 10X salt-stock solutions, and adjust the volume of the solution to 1 liter with sterile deionized H₂O.

Minimal Medium 10X salt-stock solutions
Dissolve each of the following salts in deionized H₂O to a final volume of 1 liter, and sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. The salts with * can be prepared and autoclaved together. Prepare the MgSO₄.7H₂O, CaCl₂ and FeSO₄.7H₂O solutions separately, sterilize by autoclaving, and add the solutions at the end. Sterilize the glucose solution by passing it through a 0.22-µm filter.

*K₂HPO₄ 17.42 g
*KH₂PO₄ 13.60 g
*NaCl 1.46 g
*(NH₄)₂SO₄ 5.28 g
MgSO₄.7H₂O 4.93 g
CaCl₂ 0.66 g
FeSO₄.7H₂O 0.014 g
Glucose 18.01 g

MES (0.4 M)
Dissolve 7.808 g of 2-N-morpholino ethanesulfonic acid in 80 ml of distilled H₂O. Adjust pH to 5.3 with 5 M KOH. Adjust the volume of the solution to 100 ml with distilled H₂O. Sterilize the solution by passing it through a 0.22-µm filter. Dispense the solution into aliquots and store them at -20°C.

Acetosyringone (AS) 20 mM
Dissolve 1.011 g of AS in 10 ml of 95% ethanol. Adjust the volume of the solution to 50 ml with distilled H₂O. Sterilize the solution by passing it through a 0.22-µm filter. Dispense the solution into aliquots and store them at -20°C.
**Induction Medium with Acetosyringone (IMAS)**
Dissolve in MM each compound to the following final concentrations. Prepare a fresh solution before use.
- 40 mM MES
- 0.5% (w/v) glycerol
- 200 µM Acetosyringone

**LB, MM or IMAS Agar plates**
Dissolve 2 g of agar per 100 ml of liquid media. Pour the media into petri dishes.

**Antibiotic stock solutions**
Prepare the antibiotics stock solution at the following concentrations in distilled H₂O. Sterilize the solutions by passing them through a 0.22-µm filter. Dispense the solutions into aliquots and store them at -20°C.
- 50 mg/ml Ampicillin
- 100 mg/ml Cefotaxime
- 50 mg/ml Hygromycin B
- 50 mg/ml Kanamycin
- 100 mg/ml Phleomycin
- 50 mg/ml Streptomycin
- 100 mg/ml Zeocin

**Molasses Agar Medium (MAM)**

\[
\begin{align*}
\text{*Molasses} & \quad 45 \text{ g} \\
\text{*Glycerol} & \quad 45 \text{ g} \\
\text{*Peptone} & \quad 18 \text{ g} \\
\text{*NaCl} & \quad 5 \text{ g} \\
\text{*KCl} & \quad 0.5 \text{ g} \\
\text{*Agar} & \quad 20 \text{ g} \\
\text{*KH}_2\text{PO}_4 & \quad 0.06 \text{ g} \\
\text{CuSO}_4\cdot5\text{H}_2\text{O} & \quad 12 \text{ mg} \\
\text{MgSO}_4 & \quad 50 \text{ mg} \\
\text{MnSO}_4\cdot\text{H}_2\text{O} & \quad 15 \text{ mg} \\
\text{FeSO}_4\cdot7\text{H}_2\text{O} & \quad 15 \text{ mg} 
\end{align*}
\]

Dissolve each of the following salts in deionized H₂O to a final volume of 1 liter, and sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. The salts with * can be prepared and autoclaved together. Prepare the CuSO₄•5H₂O, MgSO₄, MnSO₄•H₂O and FeSO₄•7H₂O solutions separately, sterilize by autoclaving, and add the solutions at the end. Pour the media into petri dishes.

**Potato Dextrose Agar (PDA) Medium**
Dissolve 4.8 g of potato dextrose broth in 150 ml of deionized H₂O. Adjust the pH to 6.5 with 5 M KOH (~0.1 ml). Adjust the volume of the solution to 200 ml with deionized H₂O. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. Pour the media into petri dishes.
Preparation and transformation of electrocompetent Top10-<i>Escherichia coli</i> cells

(Protocol modified by Rodrigo Mora-Lugo)

**Preparation procedure**
1. Streak out frozen glycerol stock of Top10-<i>E. coli</i> cells onto a LB agar plate (no antibiotics!) and incubate it at 37°C, overnight.
2. Select a single-isolated colony of <i>E. coli</i> and inoculate it in 10 ml of LB media (no antibiotics!). Incubate culture at 37°C, 250 rpm, overnight.
3. Inoculate 500 ml of LB media (no antibiotics!), contained in a 2 L-Erlenmeyer flask, with the overnight-pre-culture (all volume). Incubate under the same conditions (37°C, 250 rpm) until OD<sub>600</sub> equals 0.6-0.9 (log phase growth).
4. Once the desired OD is reached, place the flask on ice. IMPORTANT: From this step onwards keep the cells always at 4°C (or on ice).
5. Distribute the bacterial culture into two pre-chilled 500-ml-centrifuge bottles, and centrifuge them at 4000 rpm, 20 min, at 4°C.
6. Remove supernatant immediately as cell pellet begins to lift off quickly.
7. Resuspend gently each pellet in 200-ml ice-cold water.
8. Centrifuge at 4000 rpm, 20 min, at 4°C.
9. Repeat the wash step (step 6-8) once or twice.
10. Remove supernatant and resuspend the total biomass in 40 ml of pre-chilled-10% glycerol. Centrifuge at 4000 rpm, 20 min, at 4°C.
11. Repeat the last step one more time.
12. Remove supernatant and resuspend the total biomass in 2 ml of pre-chilled-10% glycerol.
13. Prepare 100 µl-aliquots in pre-chilled 1.5ml-eppendorf tubes.
14. Freeze tubes in liquid nitrogen, and store aliquots-cells at -80°C.

**Transformation procedure**
1. Take competent cells out of -80°C and thaw on ice (approximately 20-30min).
2. Take agar plates (containing the appropriate antibiotic) out of 4°C to warm up to room temperature or place in 37°C incubator.
3. Mix 1 or 2 µl of DNA (usually 10pg to 10ng) into the competent cells. GENTLY mix by flicking the bottom of the tube with your finger a few times.
4. Transfer competent cell/DNA mix to electroporation cuvette (0.1 cm). Avoid introducing air bubbles into the cell mix in the cuvette as this can cause arcing during electroporation.
5. Transform cells with multiporator by electric pulse at 1,200 V and add immediately 500-1000µl LB or SOC media (without antibiotic).
6. Cultivate cells in shaking incubator at 37°C for 45min.
7. Plate serial dilution of the transformed cells on selective medium.
8. Incubate plates at 37°C overnight.
9. Calculate Transformation Efficiency in transformants/μg

Reference:

Transformation efficiency values of commercial strain “One Shot® TOP10 Electrocomp™ *E. coli* (Life Technologies)” is >1 x 10⁹ cfu/μg.
Preparation of *Aspergillus sojae* conidia
(Protocol modified by Rodrigo Mora-Lugo)

Procedure
1. Streak out *Aspergillus sojae* ATCC 20235 from a frozen glycerol stock or a fresh culture onto a PDA plate and incubate it at 28°C until conidiation (5-8 days).
2. Add 10 ml of 0.02 % (w/v) Tween 80.
3. Rub the fungal growth with a loop to resuspend the spores in the solution.
4. Filter the fungal solution through cotton to remove hyphae.
5. Prepare 20% (w/w) glycerol stocks in 1.5ml-ependorf tubes.
6. Store at -80°C.

Reagents and materials
Note: The following materials are required sterile:
- PDA plate
- 0.02 % (w/v) Tween 80
- Glycerol
- Disposable inoculation loops
- 1.5 ml-ependorf tubes

Filtered spore solutions of *Aspergillus sojae* strains
Counting of Aspergillus sojae conidia
(Protocol modified by Rodrigo Mora-Lugo)

Procedure
1. Prepare 1-ml-serial-dilutions (1:10, 1:100, 1:1000, …, etc.) of A. sojae spore solution with water in 1.5ml-eppendorf tubes.
2. Put the glass cover on the hemocytometer chamber central area.
3. Load 10 µl of the fungal serial dilution into the hemocytometer chamber by placing the pipette tip close to the glass cover edge.
4. Place the hemocytometer on the microscope stage and focus the central big square.
5. Count the number of spores contained in the central big square.
6. Calculate number of spore/ml with the following equation:

\[
\text{Concentration (spore/ml)} = \#\text{spores} \times 10,000 \times \text{DF}
\]

Where:
\#spores = number of spores contained in the central big square
Factor = 10,000
Sample’s dilution factor = DF

Materials
- Hemocytometer (Thoma, Germany)
- Cover glass
- Optical microscope
- Micropipette with disposable tips
- 1.5 ml-eppendorf tubes
- Distilled water

Microscope view of Hemocytometer counting area.
The central big square is highlighted with a dotted red square.
Extraction of genomic DNA from *Aspergillus sojae* (Protocol modified by Rodrigo Mora-Lugo)

*Aspergillus sojae* ATCC 20235 was broken up to release its genomic DNA (gDNA) according to a described method by Melo et al. (2006) with some modifications. The released gDNA was then isolated with a phenol-chloroform mixture according to standard procedures (Sambrook and Russell 2001).

**Detailed procedure**

1. Using a sterile toothpick or inoculating loop, scrap *Aspergillus* spores or hyphae (0.1-1.0 mg) from a 3-10-day-old molasses agar plate, and transfer it to a 1.5-ml-Eppendorf tube.
2. Add 0.3 g (roughly 0.3 ml) of prepared glass beads (see preparation procedure below!), 200 µl of lysis buffer and 200 µl of 1:1 phenol-chloroform mix.
3. Vortex the tube at top speed for 2-5 min.
4. Add 200 µl of TE buffer and vortex again for a few seconds.
5. Centrifuge at top speed (≥13,000 rpm) for 5-10 min (room temperature).
6. Transfer 200 µl of the aqueous (upper) phase to a fresh Eppendorf tube. Use a new pipette tip for each sample. Discard the tube with the glass beads.
7. Add 400 µl (~2 volumes) of 100% ethanol at room temperature. Mix thoroughly.
8. Centrifuge for 5-10 min and discard the supernatant. Take care not to dislodge the pellet, which sometimes is not well visible.
9. Add 400 µl of TE, 3 µl of 10 mg/ml RNAse and incubate for 5 min at 37°C.
10. Add 10 µl of 4 M ammonium acetate, 400 µl of absolute ethanol and mix gently.
11. Centrifuge immediately at top speed for 10-30 min, and discard the supernatant.
12. Dry the DNA pellet using SpeedVac (15-60 min), and resuspend it in 50 µl of TE buffer.
13. Analyze DNA sample by nanodrop and agarose-gel electrophoresis.
14. Store DNA sample at -20°C. Note: DNA degradation increases with the number of thawing/refreezing cycles!

**Preparation of glass beads**

1. Use **212-300 µm** beads. Sigma G-9143 may work as well according to reference.
2. Pour the beads (0.5 kg bottle) into a 1 litre beaker.
3. Fill the beaker with concentrated HCl until the beads are fully submerged. Let stand in a fume hood for 15 min.
4. Wash with tap-distilled water until the pH is neutral. It helps to run the water through glass tubing jammed to the bottom of the beaker so that the water flows up from the bottom of the beaker.
5. Transfer the beads to a baking dish and bake (overnight is good).
6. Pour the beads into a sterile bottle.

**Materials:**
- Autoclaved milli-Q water
- 1.5 ml-epppendorf tubes
- 10 mg/ml RNAse
- Glass beads
- Vacuum concentrator 5301 (SpeedVac)

**Reagents:**

**4 M ammonium acetate**
\[ \text{MM: 77.08 g/mol.} \]
AppliChem
Dissolve 3.1 g of \( \text{C}_2\text{H}_7\text{NO}_2 \) in up to 10 ml of distilled water.
Store at -20°C.

**TE buffer**
10 mM Tris, pH 8.0
1 mM Na$_2$EDTA

**Lysis buffer**
10 mM Tris, pH 8.0
1 mM Na$_2$EDTA
100 mM NaCl
1% SDS
2% Triton X-100

**Phenol-chloroform mix (1:1)**

**References:**
Melo SCO, Pungartnik C, Cascardo JCM, Brendel M (2006) Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa* Genetics and Molecular Research 5:851-855
Moisture content
(Protocol modified by Rodrigo Mora-Lugo)

The quantity of water of fermented samples was determined as moisture content on wet basis (MC\textsubscript{wb}) according to the following equation:

**Formula:**

\[
MC_{wb} (\%) = \frac{\text{weight}_{wet}}{\text{weight}_{wet} - \text{weight}_{dry}} \times 100
\]

**Where:**

weight\textsubscript{wet} = the initial weight of the moisturized culture samples in grams

weight\textsubscript{dry} = the final weight of the culture samples after a drying treatment of 70°C for 3 days.

Different enzymatic yields (e.g. protease, exo-PG, endo-PG and exo PMG) were calculated considering the amount of dried solid in fermented samples (weight\textsubscript{dry}) and expressed as enzymatic unit per gram (U/g). Hence, after experimental determination of weight\textsubscript{wet} and MC\textsubscript{wb}, of each fermented sample, weight\textsubscript{dry} values were calculated as follow:

**Formula:**

\[
\text{weight}_{dry} (g) = \text{weight}_{wet} - \frac{MC_{wb} \times \text{weight}_{wet}}{100}\%
\]

**Reference:**

Protein soluble (total) determination
(Protocol modified by Rodrigo Mora-Lugo)

Total soluble protein was measured in enzymatic samples from SSF according to modified Bradford method (Bradford 1976), using Coomassie Plus™ Protein Assay Kit (Thermo Scientific, Germany). The assay was performed in a microplate by determining the absorbance at 595 nm using bovine serum albumin (BSA) as a standard. Determinations were performed in duplicate.

Formula:
Protein (µg/ml) = (ΔA_{595}* DF_{sample})/(slope*0.02)

Where:
ΔA_{595} = A_{595 (sample)} - A_{595 (average blank)}
Slope = ΔA_{595} / µg/ml
Sample volume = 0.02 ml
Sample’s dilution factor = DF_{sample}

Procedure
1. Prepare a set of albumin standards for the working range 100-1500 µg/ml according to the manufacturer directions.
2. Prepare dilutions of the unknowns’ enzymatic samples (e.g. 1:10 and 1:100 dilutions).
3. Pipette 20µL of each standard or unknown sample into the appropriate microplate wells.
4. Add 250µL of the Coomassie Plus Reagent to each well and mix with plate shaker or vortex for 30 seconds.
5. Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).
6. Read absorbance at 595 nm.
7. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
8. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.
Materials and Reagents:
- Vortex
- 2 mg/ml Albumin Standard
- Coomassie Plus (Bradford) Reagent
- MTP spectrophotometer (Biotrak II, Amersham Biosciences)
- Disposable clear-flat-bottom 96-well micro plate (reaction and lecture plate)

Typical curve with BSA as a standard. The standard curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve should be generated each time the assay is performed. Note: the linearity of this standard curve was up to 500 mg of albumin.

Reference:

Exo-polygalacturonase (exo-PG) activity assay
(Protocol modified by Rodrigo Mora-Lugo)

Exo PG activity was measured in enzymatic samples according to an adapted method from (Miller 1959; Silva et al. 2005). The assay was performed in a microplate by mixing 10 µl of enzymatic sample and 90 µl of polygalacturonic acid (substrate) in a 100 mM citrate-Na biphosphate buffer pH 5.0. The reduced galacturonic acid (GA) released by the reaction after 30 minutes of incubation at 30°C, was quantified by the DNS method at 575 nm (Miller 1959) and compared to a galacturonic acid (GA) standard curve. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of GA (product) per minute at standard assay conditions (in this case pH 5.0 and 30°C). Determinations were performed in duplicate. Exo-PG activity was calculated in terms of U per ml of enzymatic sample (supernatant), as follow:

Formula:

Exo-PG Activity (µmolGA min⁻¹ ml⁻¹ = U/ml) = (ΔA₅₇₅* Dilution factor)/ (Slope* 0.01*30)

Where:
ΔA₅₇₅ = A₅₇₅ (sample) – A₅₇₅ (sample blank)
Slope = ΔA₅₇₅/ µmol galacturonic acid
Sample volume = 0.01 ml
Reaction time = 30 min

Procedure
1. Book the thermocycler and the centrifuge (eppendorf 5810R) for the reaction plate.
2. Prepare all the necessary reagents.
3. Prepare enzymatic samples by thawing a -20°C aliquot and make a 1:10 and a 1:20 dilution with buffer. Note: Good values falling into the linear standard curve were obtained with dilution 1:10.
4. Prepare the standard mixtures (standard curve) according to the table below, and then continue with these samples from step 6.
5. For each enzymatic sample (unknown) or standard mixture, prepare a set of wells with 90 µl of substrate and 90 µl of buffer (blanks), respectively.
6. Add 10 µl of each enzymatic sample (unknown) or standard mixture to both the substrate wells and corresponding blank wells.
7. Immediately, seal with adhesive film, vortex and centrifuge the microplate for 30 sec at maximum speed (≥3,220 x g).
8. Incubate immediately at 30°C for 30 minutes in the thermocycler.
9. Place the reaction plate in the pre-chilled eppendorf rack.
10. Finish the reaction by addition of 100 µl of DNS reagent to all the samples.
11. Seal tightly with adhesive film, vortex and centrifuge the microplate for 30 sec at maximum speed.
12. Incubate at 98°C for 5 minutes in the thermocycler.
13. Place the reaction plate in the pre-chilled eppendorf rack.
14. Transfer 100 µl of each well sample into the read plate.
15. Dilute each sample with 100 µl of distilled water.
16. Read absorbance at 575 nm. For each well calculate the change in absorbance at 575nm (ΔA<sub>575</sub>) by subtracting the A<sub>575</sub> of the blank from that of the corresponding substrate well. The ΔA<sub>575</sub> in the enzymatic samples is the absorbance generated by their pectinase activity.
17. Dispose reaction mixtures in inorganic waste. Wash with distilled water and ethanol 70% the reaction and lecture plate for reuse.

### Sample standards: solution mixtures

<table>
<thead>
<tr>
<th>Standard</th>
<th>100 mM GA µl</th>
<th>Assay Buffer µl</th>
<th>GA µmol</th>
<th>ΔA&lt;sub&gt;575&lt;/sub&gt;</th>
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### Materials:
- Vortex
- Eppendorf thermocycler
- Eppendorf 96 plate freezer
- Eppendorf multipipette plus
- Eppendorf centrifuge 5810R
- Adhesive cover film for 96-plate
- 96 Multiply PCR-plate natural (reaction plate)
- Disposable clear-flat-bottom 96-well micro plate (lecture plate)
- MTP spectrophotometer (Biotrak II, Amersham Biosciences)

### Reagents:
**Reaction buffer: 100 mM Potassium acetate**
MM: 98.14 g/mol. AppliChem
Dissolve 1.962 g of acetate in up to 150 ml of distilled water. Adjust to pH 5.0 with glacial acetic acid. Add distilled water to 200 ml final volume. Store at RT.

**100 mM Citric acid**
MM: 210.14 g/mol. AppliChem
Dissolve 1.055 g of citric acid monohydrate in up to 50 ml of distilled water. Store at RT.

**200 mM Di-sodium hydrogen phosphate**
MM: 141.96 g/mol. Roth
Appendix

Version 2015 by R. Mora-Lugo

Dissolve 1.434 g of Na$_2$HPO$_4$ in up to 50 ml of distilled water. Store at RT.

**Substrate (PGA): 0.5 % (w/v)** Polygalacturonic acid sodium salt from citrus fruit. Sigma

Add 5 ml of citric acid solution in 50 ml of distilled water (pH 2.5). Weight 0.5 g of PGA, and add it slowly to the previous solution until pH reaches 3.4. Add 5 ml of citric acid solution (pH 3). Add again PGA until pH reaches 3.4. Add 5.2 ml of citric acid solution (pH 3). Add the rest of PGA. If pH is higher than 3.5, add citric acid solution until pH reaches 3.3. Add slowly 16.75 ml of Na$_2$HPO$_4$ solution until pH reaches 5. Bring volume up to 100 ml with distilled water. Filter solution through 3-5 µm if visible particles present. Prepare 10 ml-aliquots and store at -20°C.

**Reaction stopper and Color developer: DNS reagent. 3,5-dinitrosalicil acid**

Dissolve about 1 g of DNS in 50 ml of distilled water. To this solution add about 30 g of sodium potassium tartrate tetrahydrate in small lots, the solution turns milky yellow in color. Then add 20 ml of 2N NaOH, which turns the solution to transparent orange yellow color. The final volume is made to 100 ml with the distilled water. This solution is stored in an amber colored bottle at RT.

**Product (standard curve): 100 mM D-(-)-Galacturonic acid (GA)**

MM 212.16, Assay 97%, Fluka

Dissolve 0.02187 g of GA in up to 1 ml of acetate buffer. Prepare a fresh solution each time the assay is performed!

![Graph](image)

Typical curve with galacturonic acid as a standard. The standard curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve should be generated each time the assay is performed.

**References:**


Exo-polymethylgalacturonase (exo-PMG) activity assay
(Protocol modified by Rodrigo Mora-Lugo)

Exo PMG activity was measured in enzymatic samples according to an adapted method from (Blandino et al. 2002; Heerd et al. 2012) The assay was performed in a microplate by mixing 10 µl of enzymatic sample and 90 µl of citrus pectin (substrate) in a 100 mM acetate buffer pH 5.0. The reduced galacturonic acid (GA) released by the glycosidic hydrolysis of esterified pectin after 10 minutes of incubation at 45°C, was quantified by the DNS method at 575 nm (Miller 1959) and compared to a GA standard curve. One unit of exo-enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of GA (product) per minute at standard assay conditions (in this case pH 5.0 and 45°C). Determinations were performed in duplicate. Exo-PMG activity was calculated in terms of U per ml of enzymatic sample (supernatant), as follow:

Formula:
Exo-PMG Activity (µmol$_{GA}$ min$^{-1}$ ml$^{-1}$ = U/ml)
= ($\Delta$A$_{575}$*dilution factor)/(slope*0.01*10)

Where:
$\Delta$A$_{575}$ = A$_{575}$ (sample) – A$_{575}$ (sample blank)
Slope = $\Delta$A$_{575}$/ µmol$_{galacturonic}$ acid
Sample volume = 0.01 ml
Reaction time = 10 min

Procedure
1. Book the thermocycler and the centrifuge (eppendorf 5810R) for the reaction plate.
2. Prepare all the necessary reagents.
3. Prepare the standard mixtures (standard curve) according to the table below, and then continue with these samples from step 7.
4. Place the reaction plate in the pre-chilled eppendorf rack.
5. For each enzymatic sample (unknown) or standard mixture, prepare a set of wells with 90 µl of substrate and 90 µl of buffer (blanks), respectively.
6. Get ready the thermocycler at 45°C.
7. Add 10 µl of each enzymatic sample or standard mixture to both the substrate wells and corresponding blank wells, and start recording the time from the addition of the first sample.
8. Immediately, seal with adhesive film, vortex and centrifuge the microplate for 30 sec at maximum speed (≥3,220 x g).
9. Incubate immediately at 45°C for 10 minutes in the thermocycler.
10. Place the reaction plate in the pre-chilled eppendorf rack.
11. Get ready the thermocycler at 98°C.
12. Finish the reaction by addition of 100 µl of DNS reagent to all the samples.
13. Seal tightly with adhesive film, vortex and centrifuge the microplate for 30 sec at maximum speed.
14. Incubate at 98°C for 5 minutes in the thermocycler.
15. Place the reaction plate in the pre-chilled eppendorf rack.
16. Transfer 100 µl of each well sample into the read-plate.
17. Dilute each sample with 100 µl of distilled water.
18. Read absorbance at 575 nm. For each well calculate the change in absorbance at 575 nm (ΔA<sub>575</sub>) by subtracting the A<sub>575</sub> of the blank from that of the corresponding substrate well. The ΔA<sub>575</sub> in the enzymatic samples is the absorbance generated by their pectinase activity.
19. Dispose reaction mixtures in inorganic waste. Wash with distilled water and ethanol 70% the reaction and lecture plate for reuse.

### Sample standards: solution mixtures

<table>
<thead>
<tr>
<th>Standard</th>
<th>100 mM GA µl</th>
<th>Assay Buffer µl</th>
<th>GA µmol</th>
<th>ΔA&lt;sub&gt;575&lt;/sub&gt;</th>
</tr>
</thead>
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<td>100</td>
<td>0</td>
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</tr>
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</table>

### Materials:
- Vortex
- Eppendorf Thermocycler
- Eppendorf 96 plate freezer
- Eppendorf centrifuge 5810R
- Adhesive cover film for 96-plate
- 96 Multiply PCR-plate natural (reaction plate)
- Disposable clear-flat-bottom 96-well micro plate (lecture plate)
- MTP spectrophotometer (Biotrak II, Amersham Biosciences)

### Reagents:
**Reaction buffer: 100 mM Potassium acetate**
MM: 98.14 g/mol. AppliChem
Dissolve 1.962 g of acetate in up to 150 ml of distilled water. Adjust to pH 5.0 with glacial acetic acid. Add distilled water to 200 ml final volume. Store at RT.

**Substrate : 0.5% (w/v) Pectin, esterified potassium salt from citrus fruit**
20-34% esterified. Sigma-Aldrich
Dissolve 0.5 g of pectin in up to 100 ml of acetate buffer.
Reaction stopper and Color developer: DNS reagent, 3,5-dinitrosalicil acid
Dissolve about 1 g of DNS in 50 ml of distilled water. To this solution add about 30 g of sodium potassium tartrate tetrahydrate in small lots, the solution turns milky yellow in color. Then add 20 ml of 2N NaOH, which turns the solution to transparent orange yellow color. The final volume is made to 100 ml with the distilled water. This solution is stored in an amber colored bottle at RT.

Product (standard curve): 100 mM D-(+)-Galacturonic acid (GA)
MM 212.16, Assay 97%, Fluka
Dissolve 0.02187 g of GA in up to 1 ml of acetate buffer. Prepare a fresh solution each time the assay is performed!

Figure. Typical curve with galacturonic acid as a standard. The standard curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve should be generated each time the assay is performed.

Reference:
Appendix

Endo-polygalacturonase (endo-PG) activity assay
(Protocol modified by Rodrigo Mora-Lugo)

Endo PG -PG was measured in enzymatic samples according to an adapted method from (Ortiz et al. 2014). The assay was performed in a microplate by mixing 8 µl of enzymatic sample and 8 µl of 5 µg/µl polygalacturonic acid (substrate; PGA) in a 100 mM citrate-Na biphosphate buffer pH 5.0. The hydrolyzed PGA unable to precipitate with ruthenium red dye (RR) after 20 minutes of reaction at 40°C, was measured and compared against a PGA standard curve (from 0 to 36 µg). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µg of PGA in smaller fragments unable to precipitate with RR per minute at the standard assay conditions (in this case pH 5.0 and 40°C). Determinations were performed in duplicate. Endo-PG activity was calculated in terms of U per ml of enzymatic sample (supernatant), as follow:

Formula:
Endo-PG activity (µgPGA min⁻¹ ml⁻¹ = U/ml)
= (ΔA₅₃⁵* dilution factor/slope*0.008*20)

Where:
ΔA₅₃⁵ = A₅₃⁵ (sample blank) − A₅₃⁵ (sample)
Slope = ΔA₅₃⁵/ µgPGA
Sample volume = 0.008 ml
Reaction time = 20 min

Procedure
1. Book the thermocycler and the centrifuge (eppendorf 5810R) for the reaction plate.
2. Prepare all the necessary reagents.
18. Prepare enzymatic samples by thawing a -20°C aliquot and make a 1:10 and a 1:20 dilution with buffer. Note: Good values falling into the linear standard curve were obtained with dilution ≥1:20.
3. Place 16 µl of each standard-curve solution mixture in the reaction plate.
4. Get ready the thermocycler at 40°C.
5. Mix 8 µl of 0.5% PGA (substrate) and 8 µl of enzymatic sample.
6. Seal with adhesive film, vortex and centrifuge the microplate for 30 sec at maximum speed (≥3,220 x g).
7. Incubate at 40°C for 20 minutes in the thermocycler.
8. Place the reaction plate in the pre-chilled eppendorf rack.
9. Add 40 µl of 1.125 mg/ml RR aqueous solution to each well.
10. Add 100 µl of 8 mM NaOH solution.
11. Seal with adhesive film, vortex vigorously and centrifuge the microplate for 10 min at 4°C at maximum speed (≥3,220 x g).
12. Transfer 25 µl of the supernatant to the lecture plate containin g 175 µl of water.

1 of 3
13. Read absorbance at 535 nm. For each well calculate the change in absorbance at 535nm ($\Delta A_{535}$) by subtracting the $A_{535}$ of the samples to the $A_{535}$ of the samples blank ($A_{535}$ (sample blank)). This $\Delta A_{535}$ is the absorbance generated by the pectinase activity. 

**Note 1:** Measure the activity of some nonincubated samples as blank ($A_{535}$ (sample blank)) for activity calculations and to detect the presence of interferences in the sample. For screening procedures involving samples from the same medium and strain (i.e., mutant screening), this value could be considered as constant after a short validation step to save space in the plate and then to increase the throughput of the assay. 

**Note 2:** The measurements can alternatively be performed at 492 nm by using 50 µl of supernatant and 150 µl of water. 

**Note 3:** Measure promptly absorbance values as they change quickly and constantly with time. 

**Note 4:** The smaller precipitated pellet the more enzymatic activity. 

14. Dispose reaction mixtures in inorganic waste. Wash with a saturated NaCl solution, distilled water and 70% ethanol the reaction and lecture plates for reuse.

### Sample standards: solution mixtures

<table>
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<tr>
<th>Standard</th>
<th>0.5% PGA µl</th>
<th>Distilled H₂O µl</th>
<th>PGA µg</th>
<th>$\Delta A_{535}$</th>
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</tbody>
</table>

**Typical curve with polygalacturonic acid as a standard.** The standard curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve should be generated each time the assay is performed.
Appendix

Version 2015 by R. Mora-Lugo

Materials:
- Vortex
- Eppendorf Thermocycler
- Eppendorf 96 plate freezer
- Eppendorf Multipipette Plus
- Eppendorf centrifuge 5810R
- Adhesive cover film for 96-plate
- 96 Multiply PCR-plate natural (reaction plate)
- Disposable clear-flat-bottom 96-well micro plate (lecture plate)
- MTP spectrophotometer (Biotrak II, Amersham Biosciences)

Reagents:

8 mM NaOH
Dilute 800 µl of 100 mM NaOH in up to 10 ml of distilled water.

100 mM Citric acid
MM: 210.14 g/mol. AppliChem
Dissolve 1.055 g of citric acid monohydrate in up to 50 ml of distilled water. Store at RT.

200 mM Di-sodium hydrogen phosphate (reaction buffer)
MM: 141.96 g/mol. Roth
Dissolve 1.434 g of Na₂HPO₄ in up to 50 ml of distilled water. Store at RT.

0.5% (w/v) Polygalacturonic acid sodium salt from citrus fruit (PGA)
Assay: ≥75%. Sigma-Aldrich
Add 5 ml of citric acid solution in 50 ml of distilled water (pH 2.5). Weight 0.5 g of PGA (substrate), and add it slowly to the previous solution until pH reaches 3.4. Add 5 ml of citric acid solution (pH 3). Add again PGA until pH reaches 3.4. Add 5.2 ml of citric acid solution (pH 3). Add the rest of PGA. If pH is higher than 3.5, add citric acid solution until pH reaches 3.3. Add slowly 16.75 ml of Na₂HPO₄ solution until pH reaches 5. Bring volume up to 100 ml with distilled water (final conc. 5 µg/µl). Filter solution through 3-5 µm if visible particles present. Prepare 10 ml- aliquots and store at -20°C.

1.125 mg/ml Ruthenium red (RR)
Dissolve 0.01125 g of RR in up to 10 ml of distilled water. Store solution at RT!

Reference:

3 of 3
The activity of proteases (non-specific) was measured in enzymatic extracts from SSF samples according to an adapted method from Pierce Protease Assay Kit (Thermo Scientific). The assay was performed in a microplate by mixing 50 µl of enzymatic sample and 100 µl of succinylated casein (substrate) in a 50 mM borate buffer pH 8.5. The released tyrosine (product) by the reaction after 20 minutes of incubation at 37°C, was measured at 450 nm and compared to a TPCK-trypsin (protease) standard curve. Determinations were performed in duplicate. One unit of protease activity was defined as the amount of enzyme that catalyzes as much substrate as 1 µg of TPCK-trypsin per minute at the above standard assay conditions. Protease activity was calculated as follow:

**Formula:**

Protease Activity (µg\textsubscript{trypsin} min\textsuperscript{-1} ml\textsuperscript{-1} = U/ml)  
= ([e^((\Delta A_{450} - b)/m)]* DF\textsubscript{sample}) / (0.05*20)

Where:

\(\Delta A_{450} = A_{450} \text{ (sample)} - A_{450} \text{ (sample blank)}\)

\(e^((\Delta A_{450} - b)/m) = \mu g_{\text{trypsin}}\)

Standard curve values (logarithmic fit) = b & m

Sample volume = 0.05 ml

Reaction time = 20 min

Sample’s dilution factor = DF\textsubscript{sample}

**Procedure**

1. Book the thermocycler and the centrifuge (eppendorf 5810R) for the reaction plate.
2. Prepare all the necessary reagents.
3. Prepare enzymatic samples by thawing a -20°C aliquot and make a 1:20 and a 1:50 dilution with buffer. Note: Good values falling into the linearity of exponential regression were obtained with dilution ≥1:20.
4. Place by duplicate 50 µl of the standard curve mixtures (see table below) and enzymatic samples (unknowns) in the lecture plate.
5. Add with multipipette 100 µl of buffer to the blanks wells.
6. Add with multipipette 100 µl of substrate to the samples wells.
7. Immediately, seal with adhesive film, vortex and centrifuge the microplate for 30 sec at maximum speed (≥3,220 x g).
8. Incubate at 37°C for 20 minutes.
9. Finishing the reaction by addition of 50 µl of TNBSA to all the samples.
10. Incubate at room temperature for 20 minutes.
11. Read absorbance at 450 nm. For each well calculate the change in absorbance at 450nm ($\Delta A_{450}$) by subtracting the $A_{450}$ of the blank from that of the corresponding substrate well. This $\Delta A_{450}$ is the absorbance generated by the proteolytic activity of the protease.
12. Dispose reaction mixtures in organic waste. Wash with distilled water and ethanol 70% the 96-well plate for reuse.

**Sample standards: solution mixtures**

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<tr>
<th>Standard</th>
<th>0.5 mg/ml Trypsin µl</th>
<th>Assay Buffer µl</th>
<th>LogScale Trypsin</th>
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</table>

**Figure. Typical curve with TPCK-trypsin as a standard (logarithmic fit).** The standard curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve should be generated each time the assay is performed.
Materials:
- Vortex
- Eppendorf Multipipette plus
- Eppendorf centrifuge 5810R
- Adhesive cover film for 96-plate
- Disposable clear-flat-bottom 96-well micro plate (lecture plate)
- MTP spectrophotometer (Biotrak II, Amersham Biosciences)

Reagents:
**Reaction buffer: 50 mM borate pH 8.5**
Dissolve the salt content of the BupHTM Borate Buffer Pack (included in the manufacturer kit box!) in up to 500 ml of distilled water. Store at RT.

**Substrate (casein): 2 mg/ml Succinylated Casein**
Dissolve 1 vial (10mg) of lyophilized Succinylated Casein (included in the manufacturer kit box!) in 5ml of Assay Buffer. Let the vial stand for 5 minutes after addition of buffer, then gently swirl the vial to fully dissolve the protein. Five milliliters of succinylated casein solution will be sufficient to assay 48 samples in a 96-well microplate. **Note:** It is normal for the succinylated casein solution to be slightly opalescent.

**Reaction stopper and color developer: TNBSA (2,4,6-trinitrobenzene sulfonic acid)**
Dilute 50 µl of the supplied TNBSA stock solution (included in the manufacturer kit box!) in up to 7.5 ml of assay buffer.

**Standard protease: 0.5 mg/ml TPCK-trypsin**
MM: 23,800 g/mol. Thermo Scientific
TPCK-trypsin is included in the manufacturer kit box! 50 mg/ml aliquots were prepared and stored at -20°C. Dilute 10 µl of a 50 mg/ml TPCK-trypsin stock solution in up to 1 ml of assay buffer (0.5 mg/ml final conc.).

Reference:
Pierce Protease Assay Kit (Thermo Scientific)
The biomass in SSF samples was measured in terms of the glucosamine (GlcN) released by acid hydrolysis of chitin present in the cell wall of the filamentous fungus. Samples of 0.1 g of dried fermented substrate (dfs) were assessed for glucosamine content according to an adapted method (Zamani et al. 2008) with slight modifications, and compared to a standard curve of D-(+)-glucosamine hydrochloride (Sigma-Aldrich, Germany). Fungal biomass was expressed in terms of mg of glucosamine (mg\text{GlcN}) per g of dfs, and calculated as follow:

**Formula:**

\[
\text{Biomass (mg}\text{GlcN}\cdot\text{g}^{-1}) = \left( e^{\left[\frac{(\Delta A_{650} - b)}{m}\right]} \right) \times \text{DF_{sample}} / (0.01)
\]

Where:

\[
\Delta A_{650} = A_{650}\text{ (sample)} - A_{650}\text{ (sample blank)}
\]

\[
e^\left[\frac{(\Delta A_{650} - b)}{m}\right] = \text{mg}\text{GlcN}
\]

Standard curve values (logarithmic fit) = \(b\) & \(m\)

Sample’s amount = 0.01 g of dfs

Sample’s dilution factor = \text{DF}_{\text{sample}}

**Procedure**

1. Dry SSF samples with lyophilizer for 1-2 days (see figure below).
2. Prepare all the necessary reagents.
3. Grind dried samples to a fine powder with electric grinder or mortar and pestle.
   
   **Day 1:**
   
   4. Place 0.1 g of each grained sample into 15 ml screw cap glass tubes. Note: original article says 0.01 g, however, after I tested out different amounts 0.05, 0.1 and 0.2 g, the best reproducibility was obtained with 0.1 g.
5. Add 500 µl of 72% (v/v) H\text{2}SO\text{4} , close the tubes tightly and incubated for 90 min at room temperature with occasional vortexing (every ~20 minutes). Note: this step can be prolonged overnight!
6. Add 8.4 ml of distilled water and close tightly the tubes.
7. Prepare the standard solutions according to the table below.
8. Autoclave for 20 minutes at 121°C for hydrolysis.
9. Split two samples (sample and sample blank) of each 0.5 ml into clean glass tubes while solutions are still warm after autoclaving (important!) and let them cool at room temperature. Dispose remaining autoclaved mixtures in organic waste.
10. Add 500 µl of 1 M NaNO\textsubscript{2} and 500 µl of distilled water to the samples and the sample blanks tubes, respectively (Important: don’t mix it up!).

11. Mix and let tubes standing \textit{overnight} under the hood to complete depolymerization. Note: the bubbling observed is due to the NO\textsubscript{2} released as a byproduct in the reaction mixture (deamination reaction).

\textbf{Day 2:}

12. Apply to all the tubes (sample, sample blanks, sample standards, etc.) the following steps:

13. Add 500 µl of 12% (w/v) NH\textsubscript{4}SO\textsubscript{4} and vortex for 1-4 minutes.

14. Add 500 µl of 0.5% (w/v) MBTH and let the tubes at room temperature for 1 h without mixing.

15. Add 500 µl of 0.5% (w/v) FeCl\textsubscript{3}, vortex and incubate at room temperature for 1 h. Solutions turn blue color at this step!

16. Transfer 20 µl to the lecture plate containing 180 µl of water (10 times dilution).

17. Read absorbance at 650 nm. For each well calculate the change in absorbance at 650nm (Δ\textsubscript{A650}) by subtracting the A\textsubscript{650} of the blank from that of the corresponding sample or standard well. This Δ\textsubscript{A650} is the absorbance generated by the glucosamine content.

18. Dispose reaction mixtures in organic waste. Wash with saturated NaCl solution, distilled water and ethanol 70% the lecture plate for reuse.

\textbf{Materials:}

- Vortex
- Autoclave
- 5 ml glass tubes
- 15 ml glass tubes with screw caps
- Electric grinder or mortar and pestle
- Freeze dryer Chris Alpha 1-2/LD plus
- MTP spectrophotometer (Biotrak II, Amersham Biosciences)
- Disposable clear-flat-bottom 96-well micro plate (lecture plate)

\textbf{Reagents:}

\textbf{72\% (v/v) Sulfuric acid}
Assay: 95-98\%. MM: 98.08 g/mol. AppliChem
Mix 75 ml of H\textsubscript{2}SO\textsubscript{4} in up to 100 ml of distilled water. Store solution in amber flask at RT!

\textbf{1 M Sodium nitrite}
Assay: ≥97\%. MM: 69 g/mol. Sigma-Aldrich
Dissolve 6.9 g of NaNO\textsubscript{2} in up to 100 ml of distilled water. Store solution in amber flask at RT!

\textbf{2 of 4}
12% (w/v) Ammonium sulfamate
Assay: ≥98%. MM: 114.12 g/mol. Fluka
Dissolve 1.2 g of NH₄SO₃NH₂ in up to 100 ml of distilled water. Store solution in amber flask at RT!

0.5% (w/v) 3-methyl-2-benzothiazolinone-hydrazonehydrochloride hydrate
Assay: ≥97%. Sigma-Aldrich (Note: stored in toxic cabinet!)
Dissolve 0.5 g of MBTH in up to 100 ml of distilled water. Store clear colorless-solution in amber flask at RT! Prepare a fresh solution if solution turns blue!

0.5% (w/v) Iron (III) chloride
Assay: ≥97%. MM: 162 g/mol. Sigma-Aldrich
Dissolve 0.5 g of FeCl₃ in up to 100 ml of distilled water. Store clear yellow-solution in amber flask at RT! Prepare a fresh solution if solution turns cloudy!

12 mg/ml D-(-)-Glucosamine hydrochloride
Assay: ≥99%. MM: 215.63 g/mol. Sigma-Aldrich
Dissolve 0.3 g of GlcN•HCl in up to 25 ml of distilled water. Prepare a fresh solution each time the assay is performed!

Sample standards: solution mixtures

<table>
<thead>
<tr>
<th>Standard</th>
<th>12 mg/ml GlcN•HCl ml</th>
<th>distilled H₂O ml</th>
<th>72% H₂SO₄ µl</th>
<th>GlcN mg</th>
<th>ΔA₆₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>8.4</td>
<td>500</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.42</td>
<td>7.98</td>
<td>500</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.84</td>
<td>7.56</td>
<td>500</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.68</td>
<td>6.72</td>
<td>500</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.52</td>
<td>5.88</td>
<td>500</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.36</td>
<td>5.04</td>
<td>500</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.2</td>
<td>4.2</td>
<td>500</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Note: the linearity of this standard curve is up to 50 mg of glucosamine.

Reference:
Drying of fermented samples with lyophilizer (freeze dryer Chris Alpha 1-2/LD plus, Germany).

Typical curve with D-(+)-glucosamine hydrochloride as a standard (logarithmic fit). The standard curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve should be generated each time the assay is performed.

\[ y = 0.4875 \ln(x) - 0.4159 \]
\[ R^2 = 0.9927 \]
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