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Study program: Microbiology

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Integrated development of a bioprocess: From the soil enzyme to the yeast production platform

Integrovaný vývoj bioprocesu: Z půdního enzymu do kvasinkové produkční platformy

Doctoral thesis

Supervisor: RNDr. Pavel Kyslík, CSc.

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Prohlášení:

Prohlašuji, že jsem tuto disertační práci vypracovala samostatně pod vedením RNDr. Pavla Kyslíka, CSc. s použitím pramenů, které cituji a uvádím v seznamu použité literatury. Tato práce ani podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Declaration:

I declare that I wrote the doctoral thesis myself. All the information sources used in the thesis have been cited. Neither this thesis, nor its substantial part were used for gaining another or the same academic degree.

V Praze, ……………………………………… RNDr. Martina Borčinová

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Abstract

For a sustainable future, there is a call to increase the market share of bio-based technologies and materials. Microbial-based technologies have the potential and the ability to contribute substantively on many levels to global efforts to achieve sustainability. Development and utilization of microbial technologies is, however, an extensive process involving numerous steps, including the discovery of novel technologies and the development of industrially viable production systems. In the presented thesis, individual steps of microbial biotechnology development were addressed.

In the first part of the study, a variety of methodological approaches were employed in order to study the effect of the anthropogenic activity (i.e. decades lasting production of penicillin G) on the structure of soil microbial communities. Moreover, both cultivable and non-cultivable fractions of populations were subjected to functional screening in order to unravel the biotechnological potential of the microorganisms in terms of production of enzymes involved in biotransformation of beta-lactam antibiotics: penicillin G acylase (PGA) and alpha amino acid ester hydrolase (AEH). Our results indicated that the impacted communities harbour a microbial community with increased diversity and richness. However, on the composition level, these communities differ significantly from the control samples, thus evidencing the grave impact of the industrial activity. Consequent analyses of biotechnological potential proved that this environment is a rich source of microorganisms with PGA- and AEH- like activity that could hold the potential to increase the portfolio of industrially important enzymes.

The second part of the study was concerned with the further steps of biotechnology development: the upstream development. The experimental plan aimed at the construction of the *Pichia pastoris* strain producing PGA with a particular focus on determining the optimum cultivation strategy leading to maximum extracellular concentrations of PGA, as well as on defining the physiological and genetic limitations of the production system. Fed-batch cultivations with the constructed strain showed a potential to extracellularly produce fully active PGA, however, a serious secretion bottleneck was also observed, as only around 40% of the produced enzyme was found outside of the cells. Consequently, it was revealed that the secretion limitations can be attributed to the cellular stress caused by intracellular accumulation of the produced enzyme that results in substantial up-regulation of the unfolded protein response pathway. This leads to translational arrest that on one hand relieves the cellular stress after which the system could reach its secretion maxima, although at the same time it significantly decreases the specific productivity of the system.

Overall, this unfortunately means that even after multiple cultivation-optimization trials the constructed strain failed to achieve the desired biotechnological potential, thus leading to the

conclusion that the strain construction process ought to be repeated while taking into account the knowledge established by this thesis.

To facilitate future research aimed at unravelling the true potential of PGA-*P.pastoris* system, an extra pilot study regarding rational strain design was performed. This study showed that the limitations of PGA production and secretion can be successfully overcome by rational design of the production strain and suitable cultivation strategy and evidenced that PGA-*P. pastoris* production platform indeed has a great potential for industrial biotechnology.

Abstrakt

V zájmu udržitelné budoucnosti se stupňuje tlak na zvýšení podílu biotechnologií a biomateriálů na trhu. Mikrobiální biotechnologie mají potenciál a schopnost na mnoha úrovních podstatně přispět k tomuto celosvětovému úsilí o dosažení udržitelnosti. Vývoj a využití mikrobiálních technologií je však komplexní proces zahrnující řadu kroků, včetně objevování nových technologií a vývoje průmyslově realizovatelných výrobních systémů. V předložené práci byly řešeny jednotlivé kroky vývoje mikrobiální biotechnologie.

V první části studie byla využita řada metodických přístupů ke studiu vlivu antropogenní aktivity (tj. desetiletí trvající produkce penicilinu G) na strukturu půdních mikrobiálních společenstev. Kultivovatelné i nekultivovatelné frakce těchto populací byly zároveň podrobeny funkčnímu screeningu za účelem odhalení biotechnologického potenciálu mikroorganismů ve smyslu produkce enzymů podílejících se na biotrasformaci beta-laktamových antibiotik: penicilin G acylázy (PGA) a esterázy alfa-aminokyselin (AEH). Naše výsledky ukázaly, že společenstva zasažených půd ukrývají mikrobiální komunitu se zvýšenou biodiverzitou a druhovou bohatostí. Na úrovni složení se však tyto komunity významně liší od kontrolních vzorků, co je důkazem zásadního dopadu průmyslové činnosti na tato společenstva. Následné analýzy biotechnologického potenciálu prokázaly, že toto prostředí je však bohatým zdrojem mikroorganismů s aktivitou podobnou enzymům PGA a AEH, které by mohly mít potenciál k rozšíření portfolia těchto průmyslově významných enzymů.

Druhá část studie se zabývala dalšími kroky vývoje biotechnologie: tzv. upstream development. Experimentální plán byl zacílen na konstrukci kmene *Pichia pastoris* produkujícího PGA, se zvláštním zaměřením na stanovení optimální kultivační strategie vedoucí k maximálním extracelulárním koncentracím PGA, jakož i na identifikaci fyziologických a genetických limitací produkčního systému. Fedbatch kultivace (tj. kultivace s postupným přidáváním dávek živin) s konstruovaným kmenem ukázaly potenciál extracelulární produkce plně aktivní PGA v *P. pastoris*, současně však byla také pozorována výrazná limitace v sekreci. Následné experimenty ukázaly, že tuto limitaci lze připsat buněčnému stresu způsobenému intracelulární akumulací produkovaného enzymu vedoucí k podstatné upregulaci UPR dráhy, která představuje buněčnou odpověď na nesbalené nebo špatně sbalené proteiny, které se hromadí v lumenu endoplazmatického retikula. Tato upregulace vedla k translačnímu arestu, který na jedné straně zmírnil buněčný stres, načež systém mohl dosáhnout maxima sekrece, současně však také významně snížil celkovou specifickou produktivitu systému.

V souhrnu výsledky této práce znamenají, že i přes veškeré pokusy o optimalizaci kultivace se konstruovanému kmeni nepodařilo dosáhnout požadovaného biotechnologického potenciálu. Konstrukci kmene je tedy třeba zopakovat se zohledněním poznatků získaných v této práci.

Pro usnadnění budoucího výzkumu zaměřeného na odhalení skutečného potenciálu systému PGA-*P. pastoris* byla provedena dodatečná pilotní studie týkající se racionalizace designu produkčního kmene. Tato studie ukázala, že omezení produkce a sekrece PGA lze překonat optimální konstrukcí produkčního kmene a vhodnou strategií kultivace, a prokázala, že produkční platforma PGA-*P. pastoris* má skutečně velký potenciál pro průmyslovou biotechnologii.

Contents

1. List of abbreviations

|	
α-MF	alpha-mating factor
AEH	alpha amino acid ester hydrolase
AOX1	alcohol oxidase 1
AOX2	alcohol oxidase 2
API	active pharmaceutical ingredient
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated protein degradation
GA	Golgi apparatus
his4	histidine dehydrogenase gene
Mut-	methanol utilizing minus
Mut+	methanol utilizing plus
MutS	methanol utilizing slow
NHEJ	non-homologous end joining pathway
Ost1	open stomata 1 signal sequence
pAOX1	alcohol oxidase 1 promoter
pAOX2	alcohol oxidase 2 promoter
pDC	catalase promoter
pFLD1	formaldehyde dehydrogenase promoter
p*FMD*	formate dehydrogenase promoter
PGA	penicillin G acylase
pGAP	glyceraldehyde-3-phosphate dehydrogenase promoter
POI	protein of interest
q_p	specific rate of product formation (U.g^{-1}.h^{-1})
SNARE	Snap receptor proteins
μ	specific growth rate (h^{-1})
UPR	unfolded protein response
VACV	valacyclovirase

2. Introduction

The encompassing definition of biotechnology is "the integrated use of microbiology, biochemistry and engineering sciences in order to achieve the technological application of the capacities of microorganisms and cultured tissue cells" (Gray, 1984).

2.1 Milestones in biotechnology development

Development and utilization of bio-based technology is an extensive process involving numerous steps (Fig. 1). In the discovery phase, detailed assessment of the microbial community of the selected ecosystem needs to be performed first in order to estimate the potential of the sample to harbour the desired characteristics/ protein of interest (POI). In the second step, functional screening needs to be performed to reveal such POIs. This step can be performed both by using cultivable organisms from the sample or by using whole isolated environmental DNA to prepare metagenomic library, each of which is consequently screened. Positive isolates/ clones are subjected to gene mining with the aim of obtaining the DNA sequence of the POI that is consequently cloned into the basic production strain and thoroughly analysed. The final step in the discovery phase is the assessment of industrial viability of the relevant POI.

In the next phase, the upstream development begins. First, a suitable host organism is chosen according to the product/ planned biotechnology and both the host and the gene encoding the POI are optimized. Created clones are analysed by using the high through-put method and the most promising ones are then used for the high-cell density cultivations in a bioreactor. In this step, the modelling of the process (including its performance) and throughout analyses are both performed in order to identify the optimal cultivation conditions for maximizing the process outcomes. Should this step fail and the outcomes are not industrially viable, the development needs to start again with the optimization of the strain itself.

Apart from discovery and upstream part of the development, scale-up and downstream of the technology also needs to be planned and optimized. However, this part is beyond the scope of presented thesis and will not be further discussed in detail.
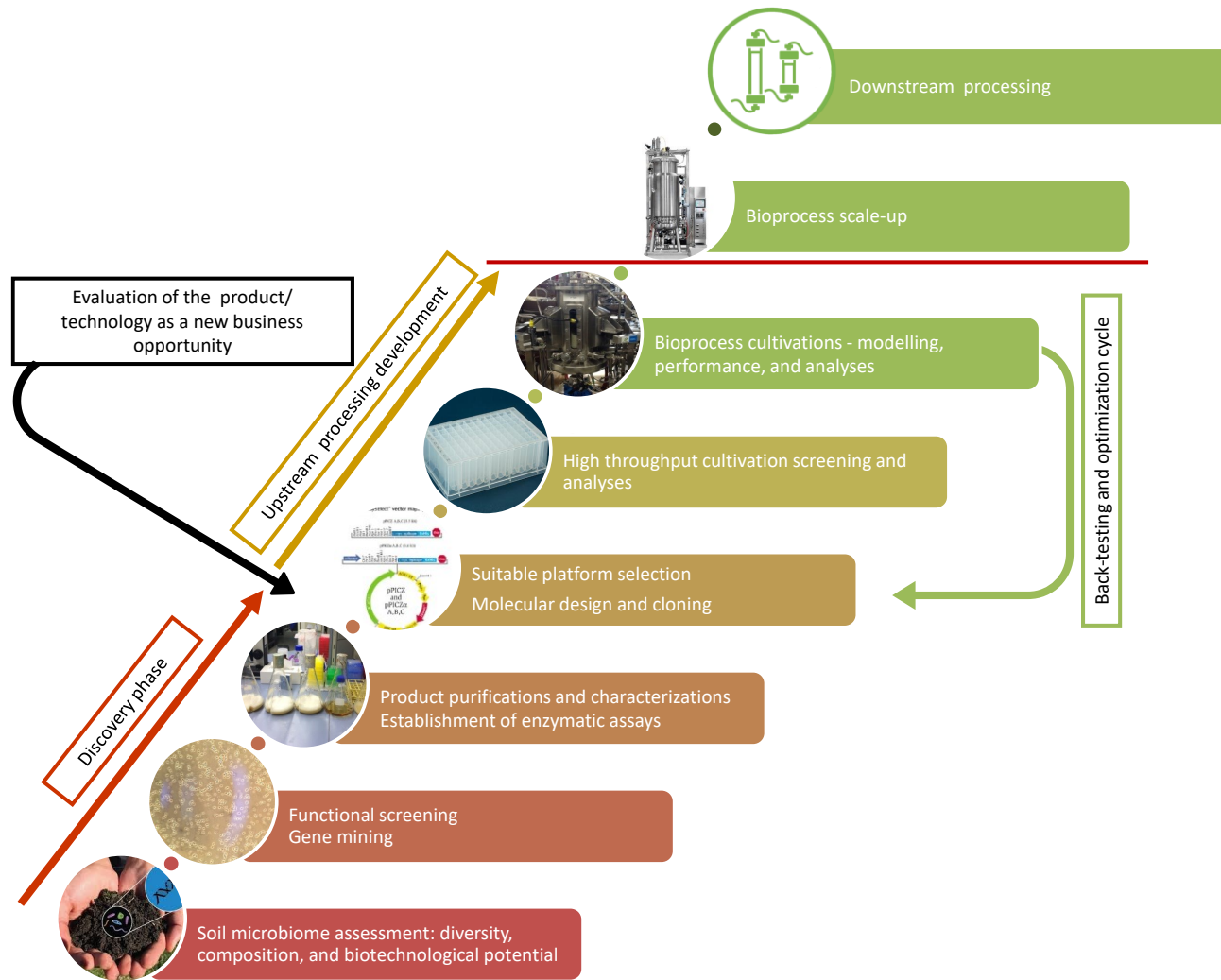
Fig. 1 Value chain in the biotechnology development (up-stream)

2.2 Soil microbial communities

2.2.1 Antibiotic production and environmental pollution

Antibiotics and antimicrobials account for the majority of the pharmaceuticals produced and used all around the globe. Between 2000 and 2015, global antibiotic consumption increased by 65% from 21.1 billion to 34.8 billion defined daily doses annually, with the expected further increase of 200% by 2030 (Eurosurveillance editorial, 2019). Additionally, antibiotics are not only intended for the human use, but a smaller fraction is also produced for veterinary purposes and agriculture (Singh *et al.*, 2017, Sneeringer *et al.*, 2019). However, since the global regulation and good manufacturing practice guidelines for the environmentally safe production are yet to be implemented, pharmaceuticals are now major environmental pollutants, and are ubiquitous in waters and soils (Kamba *et al.*, 2017).

Since the 1980s, indications about the burden connected to industrial manufacturing of antibiotics started to appear (Lee & Arnold, 1983). Even so, it was generally thought that antibiotics are primarily reaching environment through their usage or inappropriate disposal. In 2007, however, a study dealing with the release of huge amounts of antibiotics from the industrial plants was published (Larsson *et al.*, 2007) and changed the perception about the main origin of the problem. It was proven that the contamination resulting from industrial production of active pharmaceutical compounds (APIs) unprecedentedly spreads in the environment, polluting surface, ground and drinking water, as well as the soil (Fick *et al.*, 2009, Rutgersson *et al.*, 2014). Even though it is still expected that only a small fraction of the produced APIs is entering the environment, this amount can still be greater than therapeutic concentrations (Larsson, 2014). Also, it was shown that even very low doses of antibiotics can pose enough selection pressure to promote changes in microbial communities, especially when lasting for prolonged periods (DeVries *et al.*, 2015, Bengtsson-Palme & Larsson, 2016). Comprehensive review of the pollution caused by drug manufacturing was published by Joakim Larsson (Larsson, 2014), while the portfolio of newer studies has been increasing ever since (Lubbert *et al.*, 2017, Chen *et al.*, 2020, Milakovic *et al.*, 2020).

In the Czech and Slovakian context, antibiotics (penicillin G, erythromycin, sulfamethoxazole, trimethoprim, chloramphenicol, and clarithromycin) were detected in the wetlands in the water-shed of a drinking water reservoir Švihov (Vymazal *et al.*, 2017) and sulphonamide antibiotic pollution was also detected in rivers and the water bodies in the region of South Moravia (Járová *et al.*, 2015). Wide variety of antibiotics was found in the effluent of health care institutions both in Czechia and Slovakia (Mackuľak *et al.*, 2019) as well as in the influent of eight waste water treatment plants around Slovakia (Fáberová *et al.*, 2017) and six around Czechia (Tylová *et al.*, 2013).

2.2.2 Microbial ecology in context of environmental antibiotic pollution

Soil microorganisms are suggested to be the major contributors to soil health (Doran, 2002). They have an irreplaceable role, carrying key ecological functions in global element cycling, such as carbon and nitrogen; they are involved in turnover processes of organic matter, breakdown of xenobiotics, sustaining plant growth, or stabilization of the soil structure (Ducklow, 2008, Gattinger *et al.*, 2008, Martínez, 2017).

The homeostasis of the soil is dependent on both biotic and abiotic factors, including the presence of antibiotics. Selection pressures associated with antibiotic pollution can act on the overall microbial community composition by reducing taxa diversity or by shifting microbial composition (Cycon *et al.*, 2019). According to the exposition level, antibiotics can alter the population structure of the microbiome with (i) the immediate effect which decreases the diversity of the culture and (ii) the long term effect which allows the overall structure of the community to recover, yet not reach the equivalent genomic structure (Martínez, 2017). In the presence of antibiotics, bacterial populations are able to exert changes in gene expression or perform fluctuations in critical physiological traits, which reduce the diversity in microbial populations by favouring the growth of more tolerant or more resistant microbial lineages (Sanchez-Romero & Casadesus, 2014, El Meouche & Dunlop, 2018). In general, bacteria harbour wide range of processes altered by the presence of antibiotics, such as gene expression adjustments, SOS induction, conjugation, biofilm formation, increased uptake of extracellular DNA, or quorum sensing (Maiques *et al.*, 2006, Skindersoe *et al.*, 2008, Zhang *et al.*, 2013, Sanchez-Romero & Casadesus, 2014, Slager *et al.*, 2014, Penesyan *et al.*, 2020), all of which increase the available pool of genetic & phenotypic diversity; reviewed in Kraemer *et al.* (2019).

The changes in the microbial community composition can consequently alter the ecological functionality of the soil (Molaei *et al.*, 2017). Numerous studies confirm this assumption. For example, exposition to sulphonamides in high concentrations resulted in decrease of basal respiration, nitrification rate, and measured enzymatic activity of beta-glucosidase, protease and urease (Liu *et al.*, 2016), while ultralow-dose of the same antibiotic caused increased nitrate reduction and changes in N_2O fluxes (DeVries *et al.*, 2015). On the other hand, natural microbial communities have a large functional redundancy, because of the presence of multiple species able to carry out the same process. That means that the process can continue even during the antibiotic exposition despite modifications in the community structure (Roose-Amsaleg & Laverman, 2016).

In the case of beta-lactam antibiotics, the exposition was described to lead to the activation of SOS response and horizontal transfer of virulence factors (Maiques *et al.*, 2006). Vertical gene transfer was also described to be one of the most important drivers for the spread of resistance in the

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microbial community in the presence of amoxicillin (Meng *et al.*, 2017). Moreover, presence of beta-lactam antibiotics increases the frequency of conjugation and DNA transport between cells (Barr *et al.*, 1986, Nesme & Simonet, 2015) and also changes expression of genes responsible for protective effect against perturbation of bacterial wall (Rogers *et al.*, 2007). In the work of Akimenko *et al.* (2015), exposition to penicillin G lead to a decrease in the activity of catalase, dehydrogenase, phosphorylase, and invertase enzymes (20-70% of control). Similar results were also obtained by Telesiński *et al.* (2018), whereby penicillin G caused temporal decrease in the activity of both acid and alkaline phosphatase, urease, and dehydrogenase. However, this effect was only temporal and was slowly diminishing over the cultivation time (overall 56 days). Presence of amoxicillin led to the decrease in abundance of genera responsible for carbohydrate, amino acids and glucose degradation (Meng *et al.*, 2017). Overall, presence of beta-lactam antibiotics significantly affects composition of the microbial community and its function through spread of resistance and emergence of non-beta-lactam-antibiotics-sensitive bacteria (Singh *et al.*, 2013, Cai *et al.*, 2019, Das *et al.*, 2019).

Finally, pollution by antibiotics not only disrupts the structure and the function of microbial communities but also presents a substantial risk due to the development of drug-resistant microorganisms which could pose a significant health risk to both humans and animals, should they spread (Grenni *et al.*, 2018, Bengtsson-Palme *et al.*, 2019).

2.2.3 Assessment of biotechnological potential of microbiome of polluted environments

It is widely accepted that microorganisms represent unique source of enzymes with potential biotechnological importance (Sleator *et al.*, 2008, Simon & Daniel, 2011). Moreover, microbial consortia of harsh environments (extreme conditions, polluted environments) had adapted themselves to reside with those environments, developing mechanisms that tamper the extrinsic influence or help them to nutritionally profit on those conditions (Ferrer *et al.*, 2007, Dantas *et al.*, 2008, Sayed *et al.*, 2020). Such "specialized" microorganisms and their enzymes are biotechnologically important, because they often have characteristics more suitable for the industrial usage, such as higher temperature optimum and stability (Escuder-Rodríguez *et al.*, 2018, Thamer & Pravej, 2020), lower/ increased pH optimum (Steele & Streit, 2005), increased salt tolerance (Cretoiu *et al.*, 2015), increased substrate specificity (Huo *et al.*, 2018), etc.

With the development of culture-independent methods for functional screening and gene mining, such as preparation of the metagenomic libraries, it is now possible to reach the potential of the uncultivable microorganisms which account for vast majority of the whole microbiome (Daniel, 2004, Ngara & Zhang, 2018). This is even more important for the mentioned microorganisms from

the extreme environments, which are very difficult to cultivate due to their specific cultivation demands (Bodor *et al.*, 2020). Such libraries theoretically provide access to the entire genetic information of the studied sample (genome, total environmental DNA, DNA obtained from enriched culture etc.) (Dias *et al.*, 2014). The presence of the gene encoding POI is assessed by the targeted functional screening of created library clones from metagenomic DNA, thus not requiring knowledge about the structure of the gene (Almeida *et al.*, 2020). Whole sequence of POI gene is consequently "mined" from the positive clones either by using sequence homology methods (when conserved parts of the gene are expected) or by whole insert sequencing (Dias *et al.*, 2014).

Such approach of metagenomic potential mining already lead to the discovery of various interesting products, such as new melanin-like pigment from industrially polluted metagenomic library (Amin *et al.*, 2018), alkaline protease isolated from oil-polluted mud flat metagenome (Gong *et al.*, 2017), or lipase and foldase obtained from fat-contaminated soil (Almeida *et al.*, 2019). Gabor *et al.* (2005) isolated new penicillin G acylase (PGA) using the plasmid library from DNA of sand soil enrichment culture and cosmid library from the Antarctic soil sediment was also the source of the new thermostable PGA (Zhang *et al.*, 2014). Moreover, selection pressure was described to drive spontaneous mutation in the *pga* gene causing change in substrate specificity of the enzyme resulting in an increased survival rate of the organism of origin (Roa *et al.*, 1994), thus confirming the influence of "harsh" conditions on the function of exposed organisms.

Nevertheless, screening of the cultivable fraction of the microorganisms should not be completely overlooked either. Even though metagenomic approach brings novel ways of exploiting hidden potential of the microbial consortia, there are still certain limitations to be tackled. The probability of recovering a certain gene depends on its abundance in the environmental DNA used for the library construction, on the length of the target gene, and on the presence of expression signals that are functional in the host organism (Gabor *et al.*, 2004). Moreover, the production of enzyme not only requires gene expression, but also proper folding, intracellular transport, and secretion. Therefore, exploring the biotechnological potential among the cultivable organisms can overcome the stated limitations; such organism can be potentially also used itself as a whole cell catalyst, without the need of molecular optimization steps (de Carvalho, 2017).

2.3 Enzymes biotransforming beta-lactam antibiotics

While beta-lactam antibiotics remain the most important specific therapeutics for treatment of bacterial diseases (Eurosurveillance editorial, 2019), their extensive use has led to spread in bacterial resistance (Sawa *et al.*, 2020). Generic technologies to efficiently synthesise novel effective antibiotics are therefore being sought. Biocatalytic processes using penicillin G acylase (PGA) and alpha amino acid ester hydrolase to produce such semi-synthetic beta-lactam antibiotics (i.e. ampicillin, amoxicillin, cephalexin) represent a more environmentally-friendly alternative to the established manufacturing technologies using chemical synthesis. The success of the current trend of "bio-pures" is dependent on the availability of enzymes of desired quality at a reasonable price. Therefore, optimization of enzymes to improve their catalytic functions as well as their production platform to maximize the yields is of immense importance.

2.3.1 Penicillin G acylase EC 3.5.1.11

PGA is an important industrial enzyme, produced by various organisms, including bacteria, yeast, and fungi. It belongs to the N-terminal nucleophile hydrolase (Ntn) superfamily, which is characterized by the four-layer alpha/beta structure around the active site with a single N-catalytic residue (McVey *et al.*, 1997, Done *et al.*, 1998).

The mature PGA is a heterodimeric enzyme with lighter alpha-subunit and heavier beta-subunit (McVey *et al.*, 1997). Like all Ntn hydrolases, PGA is expressed as an inactive precursor peptide which undergoes post-translational processing. This pre-pro-protein contains an N-terminal leader sequence and proenzyme, consisting of alpha- and beta-subunits that are separated by a short spacer of 30–50 amino acid residues (Hewitt *et al.*, 2000). Processing of the enzyme precursor begins in the cytoplasm, where the pre-pro-protein is stabilized by Ca^{2+} and the beta-subunit is released by intra-molecular autocatalytic cleavage (Ignatova *et al.*, 2005). The signal sequence of the precursor is cleaved upon crossing the cytoplasmic membrane and followed by sequential removal of the spacer, generating the C-terminus of the alpha-subunit (Kasche *et al.*, 1999).

The physiological role of PGA remains poorly understood. It is hypothesised that *in vivo* PGA functions as a scavenger enzyme acting on alternative carbon sources containing phenylacetyl group (Valle *et al.*, 1991); being related to the hydroxyphenylacetic acid catabolic pathway (Kim *et al.*, 2004). This hypothesis is supported by the fact that, at transcriptional level, *pga* gene is repressed by glucose but induced by phenylacetic acid (Merino *et al.*, 1992). Moreover, it is hypothesized that PGAs can be involved in bacterial cell signalling as they are structurally related to acyl homoserine lactone acylases (Matsuda *et al.*, 1987, Bokhove *et al.*, 2010) which are widely

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studied quorum quenching enzymes in Gram-negative bacteria (Utari *et al.*, 2017). Therefore, PGAs can also potentially be involved in mediating antibiotic resistance, as quorum quenching in bacteria was shown to control antibiotic resistance (Evans *et al.*, 2018).

The main industrial potential of PGA lies in its ability to catalyse the hydrolysis of amidic bonds of penicillin G and cephalosporin G, producing amine nucleophiles 6-aminopenam (6-amino penicillanic acid, 6-APA) and 7-aminocephem (7-amino deacetoxy cephalosporanic acid, 7-ADCA) (Bruggink *et al.*, 1998). PGA also catalyses kinetically-controlled syntheses of semisynthetic antibiotics such as ampicillin, amoxicillin, cephalexin, etc. (Bruggink & Roy, 2001, Maresova *et al.*, 2014). Moreover, further applications of PGAs have been described, such as catalysis of peptide syntheses and the resolutions of racemic mixtures for the production of enantiopure active pharmaceutical ingredients. PGA also rank among promiscuous enzymes because they also catalyse reactions such as trans-esterification, Markovnikov addition or Henry reaction; reviewed in Grulich *et al.* (2013). Summary of the PGA biocatalytic potential is depicted in Fig. 2.

However, to fully utilize its potential on an industrial level, certain limitations of the enzyme need to be overcome. PGA has to be improved in terms of its stability (temperature, pH, tolerance to organic solvents) and sensitivity to inhibition by reactants. Also, the recognition of further beta-lactams as substrates or the acquisition of better catalytic traits (e.g. increased ratio of the initial rate of the product synthesis to the initial rate of the hydrolysis of activated acyl donor) is necessary to improve its competitiveness with chemical processes (Becka *et al.*, 2014).

For sake of completeness, it should be noted that one of the other potential strategies to improve the productivity of PGA-catalysed reactions is by way of enzyme immobilization (Kallenberg *et al.*, 2005, Datta *et al.*, 2013). For instance, catalysts based on the immobilized PGAs are already used for large-scale production of semisynthetic β-lactam antibiotics in kinetically controlled syntheses (Becka *et al.*, 2014, Maresova *et al.*, 2014).
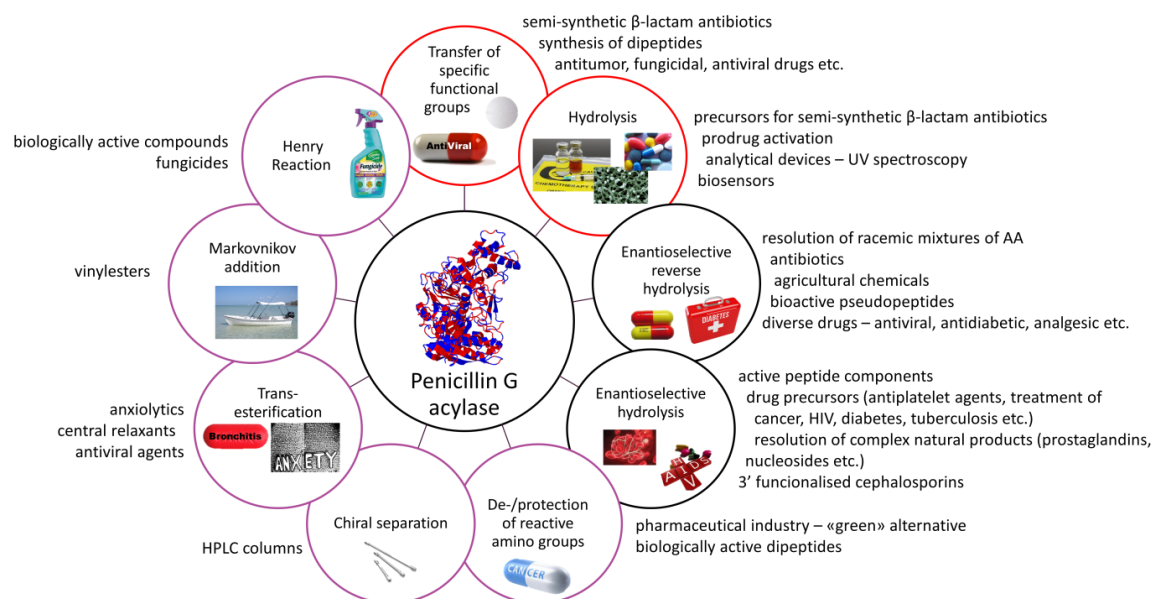
Fig. 2 Reactions catalysed by PGA (in circles) and the area/ final products of respective usage

2.3.2 Alpha amino acid ester hydrolase EC 3.1.1.43

Alpha amino acid ester hydrolases form a family of enzymes capable of synthesis and hydrolysis of dipeptide-esters and semi-synthetic beta-lactam antibiotics (Barends & Dijkstra, 2003). Currently, the class of alpha amino acid ester hydrolases (AEH) was enriched with the structurally and functionally related subgroup, valacyclovirases (VACVases). Even though they differ in number of traits, they have similar active-centre structure and thus similar substrate specificity and activity (Kurochkina *et al.*, 2013).

AEHs are composed of two or four identical subunits (70-72 kDa) that form a dimer or dimer of dimers with 81 residues that make up the subunit interfaces (Kim & Byun, 1990). VACVase is a basic monomeric protein of low molecular mass (27-30 kDa) (Kim *et al.*, 2003), though the presence of dimers was also observed (Lai *et al.*, 2008). Both AEHs and VACVases share the same structure of the catalytic domain with classical catalytic serine hydrolases triad Ser-Asp-His and possess two important catalytic centre elements responsible for the recognition and binding of amino-group containing substrates (Barends *et al.*, 2003, Kim *et al.*, 2004).

Native role of AEH is not completely clear; however they do not share PGA's synthesis/expresion induction by phenylacetic acid when cultivated on rich or minimal medium (Polderman-Tijmes, 2004), thus indicating no native functional relationship with PGA. All the open reading frames found upstream of the AEH genes encode proteins that are involved in the biosynthesis of amino acids, suggesting AEHs supportive role in general amino acid biosynthesis (Polderman-Tijmes *et al.*, 2002a, Polderman-Tijmes *et al.*, 2002b). The alternative theory suggests that AEHs may have a role in

hydrolysis of misfolded oligoproteins, due to its multimeric structure. Moreover, AEH from *Xanthomonas campestris* pv*. campestris* have been categorized in the cluster of proteins involved in pathogenicity, virulence and adaptation, more specifically in toxin production and detoxification (Blum & Bommarius, 2010). As for VACVases, their native role is yet to be revealed. However, promoter region of human VACVase conform to those of typical constitutively expressed housekeeping gene, indicating an important physiological role of this enzyme (Puente *et al.*, 1998).

AEHs are known to be able to catalyse the transfer of the acyl group from alpha-amino acid esters to amine nucleophiles such as 7-aminocephem and 6-penam compounds (synthesis) or to water (hydrolysis) (Polderman-Tijmes *et al.*, 2002a). Their main biotechnological potential lies in their ability to catalyse one-pot syntheses of semi-synthetic aminopenicillins and aminocephalosporins (Sklyarenko *et al.*, 2015). When compared to PGAs, AEHs have increased ability to catalyse beta-lactam synthesis with minimal secondary hydrolysis of synthesized product and are not inhibited by phenylacetic acid (Blinkovsky & Markaryan, 1993, Blum & Bommarius, 2010). Additionally, AEHs are exhibiting stereospecificity, allowing the antibiotic syntheses from racemic mixture (Fernandez-Lafuente *et al.*, 2001); and are also able to synthesize dipeptides, yet the yields of such dipeptides are still inadequate for industrial utilization (Hossain *et al.*, 2018).

Studies of human VACVases demonstrated that the enzyme hydrolyses esters of alpha-amino acids and displays a broad specificity spectrum of aminoacyl moiety (Lai *et al.*, 2008). They catalyse (*in vivo)* hydrolytic activations of alpha-amino acid ester prodrugs of a broad range of antiviral and anticancer nucleoside analogues (Kim *et al.*, 2004, Lai *et al.*, 2008). Therefore, they possess potential not only for utilization as an activation target but can also be used in the planning and design of those prodrugs (Sun *et al.*, 2010).

The available portfolio of genes encoding enzymes of alpha-amino acid ester hydrolase family is still rather restricted, limiting full utilization of those enzymes in the industrial processes. Therefore, identification of novel metagenomic AEH and VACVase sequences can potentially reveal novel catalytic functions and leverage the utilization of this catalyst.

2.4 Heterologous protein production with *Pichia pastoris*

P. pastoris is budding yeast belonging to the order *Saccharomycetales*, family *Phaffomycetaceae*, and only distantly related to better-known yeasts such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Candida albicans*. In the case of *P. pastoris*, genome sequencing analysis led to the change in the classification of commonly applied *P. pastoris* strains into the

closely related genera *Komagataella phaffii* and *K. pastoris* (Kurtzman, 2009). However, the original classification had in-built itself in the scientific community. Therefore, it is still common practice to use the name *P. pastoris* instead of *K. phaffii* or *K. pastoris* in scientific publications. The rest of this thesis will adhere to this principle.

It was discovered in 1969 by Koichi Ogata (Ogata *et al.*, 1969) as one of the first yeast able to utilize methanol as a sole source of carbon; and for the past 40 years, over which it has been used for commercial production, *P. pastoris* has been proven as a substantial workhorse for biotechnology (Shay LK, 1984, Schmidhalter & Meyer, 2014).

It is one of the most effective and versatile host systems for the production of heterologous proteins. It has already been used for the production of more than five hundred different proteins, and the number is expected to increase (Ahmad *et al.*, 2014, Juturu & Wu, 2018, Baghban *et al.*, 2019). Also, in 2009, the first FDA and EMA approval for recombinant production of kallikrein inhibitor protein by *P. pastoris* was granted for human use (Ahmad *et al.*, 2014), thus paving the way for this host in the industrial production of pharmaceuticals. Currently, over 70 products are on the market or at the late stages of development (https://pichia.com/science-center/commercialized-products/).

Moreover, *P. pastoris* is not only used to produce single proteins, but further diverse products are also emerging, ranging from industrial enzymes (Valero, 2018) to multi-enzyme pathways (Geier *et al.*, 2015). Although there are some examples of *P. pastoris* expression in the ≥1 g/L range, there are also few examples of expression ≥10 g/L, ranking the *P. pastoris* expression systems as one of the most productive eukaryotic expression systems available (Cregg *et al.*, 2000, Ahmad *et al.*, 2014, Karbalaei *et al.*, 2020).

2.4.1 *P. pastoris* as a production host

Among the hosts utilized for heterologous protein production, *Escherichia coli* is still widely preferred due to the vast information availability about this host, its rapid growth, and simple cultivation techniques (Vieira Gomes *et al.*, 2018). Nevertheless, depending on the properties of produced protein (such as posttranslational modifications) and demands concerning its harvest and down-stream processing, utilization of this host can be limited. Moreover, usage of bacterial production hosts has got a number of disadvantages when compared to yeast production systems, especially with *P. pastoris.* Comparison of the commonly used microbial production hosts are summarized in Tab. 1.

Tab. 1 Comparison of key characteristics of commonly applied microbial hosts for recombinant protein expression (Fernandez & Hoeffler, 1999, Brondyk, 2009, Vieira Gomes *et al.*, 2018).

	E. coli	*S. cerevisiae*	*P. pastoris*
Viral infections	Bacteriophages	No	No
Endotoxins	Lipopolysaccharides	No	No
Secretion	Low	Medium	Medium to high
Growth rate	High	Medium	Medium
Medium complexity	Low	Low	Low
Product titre	Medium to high	Low to medium	Medium to high
Process complexity	Low	Low	Low to medium
Post-translational modifications			
Protein folding	Low	Medium	Medium to high
Glycosylation	No	Yes (high mannose; hyperglycosylation)	Yes (high mannose)
Phosphorylation	No	Yes	Yes
Acetylation	No	Yes	Yes
Acylation	No	Yes	Yes
γ-Carboxylation	No	No	No

Yeast production systems combine the simplicity of unicellular organism with the ability to realize most of the required posttranslational modifications essential for bioactive products (Mattanovich *et al.*, 2012). They are more robust than the bacterial systems, due to the higher stability of recombinant strains, non-susceptibility to the bacteriophages and higher metabolic capacity (Meehl & Stadheim, 2014). They also provide strengths in terms of large portfolio of molecular tools, can be grown in very high cell densities, and are more adaptable to the harsh industrial-scale conditions (Vieira Gomes *et al.*, 2018).

In comparison to other yeast systems, *P. pastoris* is advantageous especially through its ability to secrete a high quality protein product with a minor basal secretion of its own proteins (Delic *et al.*, 2013). Thus, downstream processing is reduced, having a substantial beneficial impact on manufacturing costs. Moreover, *P. pastoris* is known for giving higher recombinant titres, when compared to conventional yeast host *S. cerevisiae*, since *P. pastoris* is Crabtree-negative and does not produce ethanol under aerobic conditions, which results in higher biomass formation and consequently in more recombinant protein (Mattanovich *et al.*, 2012). One of the main features of *P. pastoris* is the usage of tightly regulated promoters, especially alcohol oxidase promoter (*pAOX1*) allowing for uncoupling of the growth from the production phase, i.e., biomass is accumulated prior to protein expression. Therefore, cells are not stressed by the accumulation of recombinant protein during the growth phase (Ahmad *et al.*, 2014).

The recombinant product production and secretion in *P. pastoris* starts with protein folding at the endoplasmic reticulum (ER), where also post-translational modifications are initiated (Hammond *et al.*, 1994, Delic *et al.*, 2013). As every secretory protein carries signal sequences for its final destination, folded proteins aimed at secretion are recognized there, packed in COP-II vesicles and transported into the Golgi apparatus (GA) (Montegna *et al.*, 2012). GA in *Pichia* is closer to the mammalian morphology; it is arranged in ordered stacks, close to transitional ER sites where COP-II vesicles bud-off (Rossanese *et al.*, 1999). In GA, further processing of the glycans takes place (Delic *et al.*, 2013), signal sequence is cleaved, and protein undergoes final "quality check" (Coughlan *et al.*, 2004). The final step in the secretory pathway is the transport of the cargo in light and dense vesicles that bud-off at the trans-GA and fuse with the plasma membrane. The exocytosis is performed by the complex called exocyst, where many different proteins functions together (Harsay & Schekman, 2002). Beside the exocyst, snap proteins (SNAREs) are also involved in exocytosis (Aalto *et al.*, 1993, Damasceno *et al.*, 2012).

2.4.2 *P. pastoris* cellular bottlenecks and limitations

As described before, many proteins can be produced in *P. pastoris* at high levels. However, production of (especially) complex or multimeric proteins of heterologous origin has proven to be a challenge, leading to low production yields (Puxbaum *et al.*, 2015). Production of secretory proteins can be hampered at all levels of protein synthesis, maturation, and secretion.

Overproduction of heterologous proteins can lead to accumulation of misfolded product due to the insufficiency or over-saturation of ER, consequently causing stress in the production cells. The key cellular mechanisms triggered by the accumulation of unfolded/misfolded proteins are the unfolded protein response (UPR) (Mattanovich *et al.*, 2004) and endoplasmic reticulum associated protein degradation (ERAD) (Zahrl *et al.*, 2017). Those two cellular mechanisms are causing adverse reactions in terms of protein folding. UPR response leads to positive feedback loop, enhancing protein folding by transcriptional activation of genes encoding chaperons and related factors (Cox & Walter, 1996, Wu *et al.*, 2014). In case that this response is not sufficient and the unfolded proteins are not being successfully repaired, they are eliminated within the ERAD proteolytic pathway (Zahrl *et al.*, 2017). Proteolysis of the produced protein is also caused by their misdirection to the cell's vacuoles/lysozymes (Sinha *et al.*, 2005, Yang *et al.*, 2013, Heiss *et al.*, 2015), or by proteolysis caused by membrane proteases (Silva *et al.*, 2011, Wu *et al.*, 2013). The proportion of such degraded protein can be very high, reaching up to 60% of total produced protein (Pfeffer *et al.*, 2011).

Furthermore, secretion of the active product can be hampered by limitations in the secretory pathway itself. It was shown that every step in protein secretion, as described in the chapter "2.4.2 *2.4.*1 P. pastoris as a production host", can be rate limiting (Puxbaum *et al.*, 2015). Numerous studies focused on overexpression of individual components of secretory pathway revealed improvements in the secretion of the product, therefore showing that the produced amount, as well as recycling of those components, can significant limit the productivity of the system (Juturu & Wu, 2018, Fischer & Glieder, 2019). In particular, such limitations were proposed at the level of protein translocation into ER (Fitzgerald & Glick, 2014), ER-GA trafficking via COP-II vesicles (Barrero *et al.*, 2018), secretory targeting in GA (Zheng *et al.*, 2016), exocytosis via exocyst (Marsalek *et al.*, 2019) and via SNAREs (Gu *et al.*, 2015).

The major recently discussed bottlenecks and limitations of *P. pastoris* hosts are highlighted in the Fig. 3.
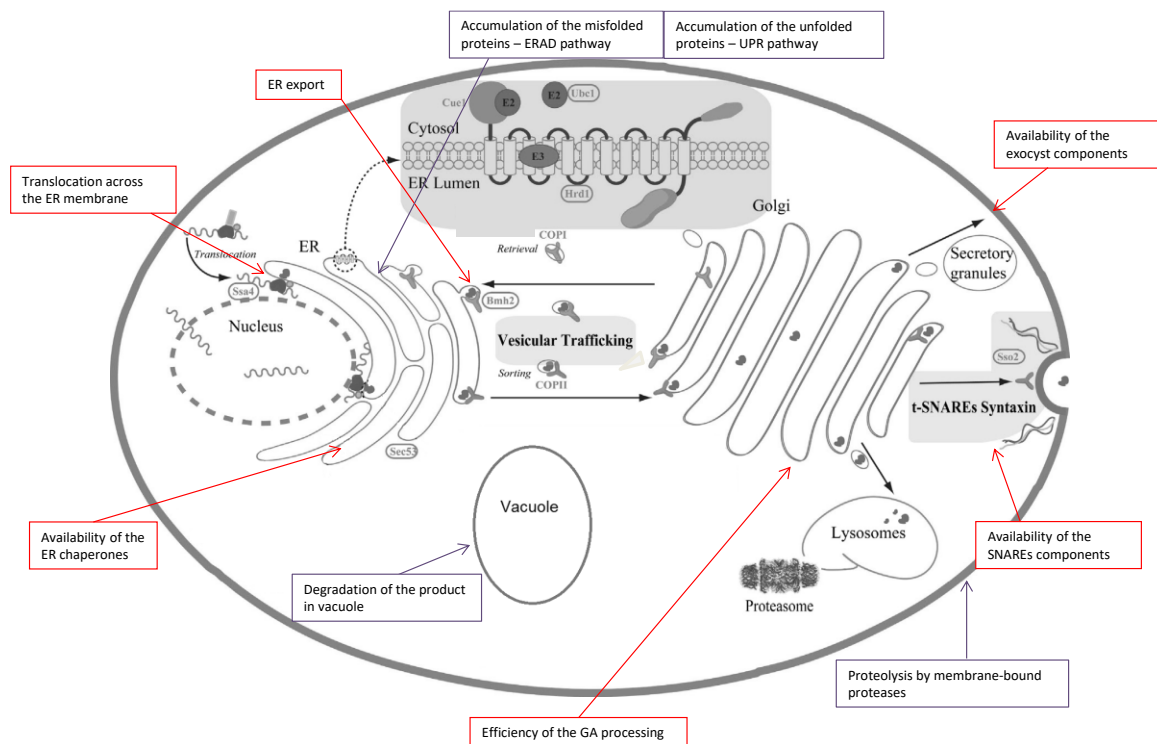
Fig. 3 Secretion in *P. pastoris*. Red squares: Major bottlenecks of the production and secretion production of product. Purple squares: Most important physiological responses to the stress caused by overproduction. Figure adapted from Gu *et al.* (2015).

2.4.3 *P. pastoris* and PGA

Development of an efficient process for industrial production of penicillin G acylase (PGA) would be of great relevance, since there is an emerging interest in leveraging the intrinsic versatility of this enzyme for multiple organic syntheses and the demand for PGA at an affordable price is growing rapidly (Grulich *et al.*, 2013, Maresova *et al.*, 2014).

The best results in the production of PGA have so far been obtained by using bacterial hosts. The highest activity and productivity of PGA (*E. coli* origin) that has been documented in production using *E. coli* was obtained by Velez *et al.* (2014) with volumetric activity up to 100.000 U/L. However, this result was obtained by utilizing conditions that are difficult to be transferred into the industrial environment (e.g. low temperature, sudden changes in the cultivation process). Despite those results, the bacterial hosts also have several features which make them less attractive for the industrial production of PGA, such as plasmid instability (Valesova *et al.*, 2004), formation of inclusion bodies (Orr *et al.*, 2012) or accumulation of the product in periplasmic space (Scherrer *et al.*, 1994) among others.

PGA has not yet been produced efficiently by either *S. cerevisiae* (Ljubijankic *et al.*, 1999, Ljubijankic *et al.*, 2002) or *P. pastoris* (Sevo *et al.*, 2002, Senerovic *et al.*, 2006, Maresova *et al.*, 2010). PGA maturation is a complex process spanning from the bacterial cytoplasm to the periplasmic space (as described in chapter 2.3.1 Penicillin G acylase EC 3.5.1.11). However, it was shown that bacterial periplasm has got very similar functional qualities to yeast endoplasmic reticulum (Miller & Salama, 2018) and therefore it can be hypothesized that the maturation of PGA should be successfully performed in this yeast compartment; as was shown for a variety of other heterologously produced proteins (Delic *et al.*, 2014).

The first study dealing with the production of PGA in *P. pastoris* (GS115 *his4*) was performed by Sevo *et al.* (2002). Using *pga* gene from *Providencia rettgeri*, up to 180 U/L of PGA was secreted in the cultivation medium (1L shake-flask culture) – which was two order higher result than the same production in *S. cerevisiae* (Ljubijankic *et al.,* 1999). Intracellular production of PGA from *P. rettgeri* in *P. pastoris* LN 5.5 *pep4* was also studied by Senerovic *et al.* (2006). The final achieved volumetric activity of PGA in supernatant (150 mL shaking flasks, 6 days induction) was up to 3800 U/L. In the consequent high-cell density cultivation in 9 L bioreactor (5 days methanol induction), PGA activity reached 26500 U/L - so far the highest titre obtained while producing PGA of this origin in any host. The enzyme was also N- and O- glycosylated which led to the improvement of its stability (Senerovic *et al.*, 2006, Senerovic *et al.*, 2009).

Secretory production of PGA (*E. coli* origin) was only studied in *S. cerevisiae*, although the production was described as "poor" (Ljubijankic *et al.*, 1999). On the other hand, intracellular

production of PGA (*E. coli* origin) was studied in the work of Maresova *et al.* (2010). Leader-less *pga* gene was cloned into *P. pastoris* X33 and the evaluation of the activity was done in 10 L stirred reactor (6 days methanol induction). Under optimal conditions the average volumetric activity of 25900 U/L was obtained. However, cell destruction was necessary in order to obtain the enzyme.

These results indicate that the production of PGA in *P. pastoris* presents a promising path towards the development of appropriate industrial production process. However, to develop the biotechnological use of a yeast system for PGA production, it is necessary to consider the integrated process by identifying essential properties of developed strains in a production procedure and, simultaneously, to use this knowledge to optimise the expression system.

### 2.4.4 Rational strain and production platform design

For successful recombinant protein expression in *P. pastoris*, it is important to prepare a suitable recombinant system that includes the design of expression cassette and the choice of suitable host strain. Strain design needs to suit the produced heterologous protein both in terms of its structure and the chosen production strategy (e.g., intracellular production, secretory production, membrane protein production).

A: *Preparation of Expression cassette*

The initial step of the production of the heterologous proteins in *P. pastoris* is the construction of expression cassette which is consequently used for chromosomal integration. Such cassette is delivered into the *P. pastoris* cells in the form of expression vector (Fig. 4)

When the expression vector is delivered inside the *P. pastoris* cells, the non-homologous end joining (NHEJ) pathway mediates integration of DNA (Klinner & Schäfer, 2004). Integration of the expression cassette results in random, non-targeted, chromosomal insertion. In *P. pastoris* this NHEJ pathway is more pronounced than homologous recombination (Emerson & Bertuch, 2016) and, depending on the target locus, approx. 1000 bp long homologous sequences are needed for efficient targeting (Schwarzhans *et al.*, 2016). Therefore, functionality of frequently present short homologous sequences on expression vectors is disputable and explains differences between the created clones (Schwarzhans *et al.*, 2016). On the other hand, knock-out strains have been created to improve targeted integration. For example, knockout of *ku70* gene (encoding ATP-dependent DNA helicase II subunit 1) increased efficiency of targeted integration by 85% (Naatsaari *et al.*,

2012); simultaneous knockout of *dnl4* gene (encoding DNA ligase IV) further improved this efficiency (Ito *et al.*, 2018).
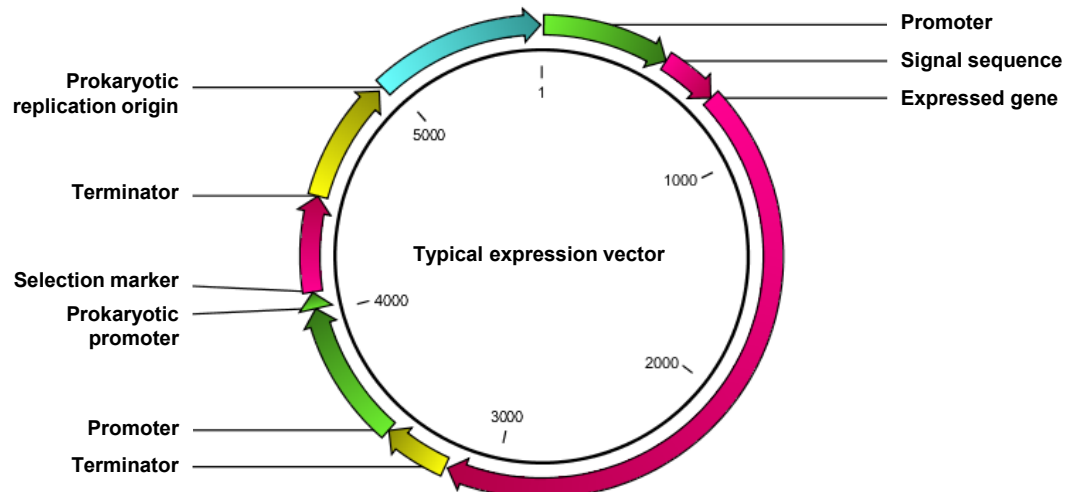
Fig. 4 Structure of a typical expression vector for extracellular protein expression in *P. pastoris*

The first step in production cassette preparation is customization of the expressed gene. *Pichia* species display a non-random pattern of synonymous codon usage and show general bias towards a subset of codons, leading to different heterologous expression efficiencies (Oberg *et al.*, 2011). Thus, codon optimization of the gene encoding product has to be performed by replacing rarely used codons with frequently used ones, as well as by considering the effect of codon sequences on mRNA secondary structure (Wang *et al.*, 2015).

In the second step, suitable signal sequence needs to be chosen. The protein secretion pathway in yeast starts from recognition of a signal peptide sequence of nascent peptide by the signal recognition particle and their translocation co- or post-translationally into the ER lumen (Delic *et al.*, 2013). Although many types of signal peptide sequences have been developed, the most commonly used signal sequence is α-mating factor pre-pro signal from *S. cerevisiae* (Lin-Cereghino *et al.*, 2013) which directs posttranslational translocation to ER. This type of translocation could be inefficient due to the complex secondary structure of the protein, as was proven for the monomeric superfolded GFP (Fitzgerald & Glick, 2014). Therefore, usage of the signal sequence directing co-translational cytosol-ER translocation, such as open stomata 1 signal sequence (Ost1) from *Arabidopsis thaliana*, could overcome the translocation bottleneck and be advantageous for more complex proteins (Barrero *et al.*, 2018).

The choice of the right promoter is another crucial point for efficient gene expression, as most regulations in productivity take place at the transcriptional level (Weinhandl *et al.*, 2014). Generally, promoters can be divided into several classes according their regulation:

i. Constitutive promoters – unregulated promoters that allow for continual transcription of its associated gene.

ii. Inducible promoters – characterized by strong and tight controllable regulation, cost-efficient induction, and effective expression of the gene of interest being placed downstream of the inducible promoter sequence. Chemically regulated promoters are induced or repressed by the presence or absence of chemical compounds, such as carbon sources.

iii. Repressed/ de-repressed promoters – regulated at the level of presence/ absence of the repressor; typically carbon source, such as glycerol. The expression of the protein of interest does not start during cell growth, when the carbon source is typically abundant, but only at the late exponential phase, allowing *de facto* regulated gene expression without external induction step.

iv. Combination-regulated promoters – certain promoters can be regulated using more regulation strategies. An example can be the catalase promoter (*pCat*) - this promoter is both de-repressed upon glycerol depletion and further induced by the presence of methanol. This characteristic allows for switching between regulation strategies according the needs of the strain.

The most popular promoters used in *P. pastoris* for foreign gene expression are the methanol inducible alcohol oxidase 1 promoter p*AOX1* and the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter p*GAP* (Vogl & Glieder, 2013). p*AOX1* offers tight regulation under repressed conditions, and exceptionally high activity when induced with methanol (Cereghino & Cregg, 2000). p*GAP,* as a constitutive promoter, has got stable activity throughout the cultivation which is comparable to the one of p*AOX1* (Waterham *et al.*, 1997). Each of the promoter type has its own pros and cons and their respective use needs to be tested in order to suit the prepared production strain. Since p*AOX1* is induced by methanol, handling of such carbon source can be of concern and cultivation conditions need to be adapted due to increased oxygen consumption and heat generation of high cell density culture grown on methanol (Looser *et al.*, 2015). On the other hand, p*GAP* promoter is not suitable for expression of toxic compounds as by using this promoter the growth and the production cannot be uncoupled.

In the recent years, large portfolio of both natural and synthetic promoters for the use in *P. pastoris* has been created; comprehensively reviewed in Vogl & Glieder (2013) and Türkanoğlu Özçelik *et al.* (2019). Those include alternative promoters with different expression strengths and regulatory properties, such as methanol inducible promoters of the methanol utilization pathway (Vogl *et al.*, 2016); as well as other constitutive or repressible promoters (Türkanoğlu Özçelik *et al.*, 2019). For example, formaldehyde dehydrogenase (p*FLD1*) inducible promoter, when induced by methanol or methylamine, was found to have similar/ higher strength to p*AOX1* (Shen et al., 1998), and *S. cerevisiae* p*CUP1* promoter can actively work in *P. pastoris* and be induced at desired levels by different Cu^{2+} concentrations (Koller *et al.*, 2000). In addition to natural promoters there has been a growing interest in synthetic promoters driving enhanced protein expression (Vogl *et al.*, 2013). Using directed evolution approach, variants of both p*AOX1* and p*GAP* with adjusted transcriptional activity have been developed to enable advanced, fine-tuned expression profiles (Hartner *et al.*, 2008, Qin *et al.*, 2011). Using the knowledge on the basis of the sequence/function relationship of natural core promoters, nucleosome occupancy and the presence of short motifs, completely synthetic core promoter sequences were designed by Portela *et al.* (2017). Such core promoters were fused to the *P. pastoris* p*AOX1 cis*-regulatory modules, and the resulting activity spanned more than a 200-fold range of the wild type p*AOX1* (Portela *et al.*, 2017).

Finally, in order to ensure sufficient level of transcription of the heterologous gene, strategies involve not only choosing the right promoter strength but also gene copy number (i.e. number of integrated expression cassettes). Gene copy number generally is described as "early" production bottleneck affecting heterologous product productivity. In numerous examples, the isolation of multicopy integrants resulted in dramatically higher yields, so gene dosage can be critical for maximal expression (Romanos *et al.*, 1998, Daly & Hearn, 2005, Parashar & Satyanarayana, 2017). On the other hand, it was proved that correlation between increased gene dosage and productivity is not linear for all products (Puxbaum et al., 2015); in some cases, increased copy-number of cassettes was observed to lead to upregulation of anti-oxidative genes and changes in expression levels of methanol metabolic pathway genes, leading to transcriptional attenuation of the methanol metabolism. This change was concomitant with reduced levels of secreted heterologous product (Zhu *et al.*, 2011, Cámara *et al.*, 2017). Therefore, increasing copy number in the created strain also needs to be carefully planned and tested.

B: Strain choice

The parental strains for the most used *P. pastoris* protein expression hosts are CBS7435 and DSMZ70382 (deposited in the former Centraalbureau voor Schimmelcultures, now Westerdijk Institute, Netherlands), some of the very first discovered methanol utilizing yeast stains. The first step in methanol utilization is its oxidation to formaldehyde by the enzyme alcohol oxidase (AOX, EC 1.1.3.13) (Hartner & Glieder, 2006). Production of this enzyme is significantly induced by the presence of methanol; in methanol grown cells up to 30% of the total cellular protein is transcribed from the *pAOX* promoters, being regulated at the level of transcription involving two mechanisms: repression/ de-repression and induction (Ellis *et al.*, 1985, Vogl & Glieder, 2013). There are two genes in *P. pastoris* that code for alcohol oxidase – *aox1* and *aox2* – but *aox1* is responsible for the vast majority of the alcohol oxidase activity in the cells. The enzymes are produced from respective promoters, *pAOX1* and *pAOX2*, out of which *pAOX1* is almost 10 times stronger (Cregg *et al.*, 1989). Even though methanol-induced expression is highly useful in the production, it is also desirable to minimize methanol´s use because of its high flammability. Additionally, its metabolism requires increased amount of oxygen (Arnau *et al.*, 2010), causes excessive heat generation (Krainer *et al.*, 2012) as well as formation of toxic products such as formaldehyde and hydrogen peroxide (Jungo *et al.*, 2007, Jungo *et al.*, 2007).

The solution to these problems can be either strict control of the growth rate of the culture (and therefore minimization of the methanol feed) or creation of strains with limited utilization of methanol (whereby methanol is mainly used as an inducer of gene expression rather than as a carbon source).

Cells having both functional *AOX* genes are called "methanol utilizing plus (Mut+)". Cells having only functional *AOX2* are utilizing methanol more slowly, therefore are referred to as "methanol utilizing slow (MutS)". Cells in possession of no alcohol oxidase activity are called "methanol utilizing minus (Mut-)". Mut- as well as MutS strains need to be provided with a secondary carbon source, such as sorbitol, mannitol, trehalose or alanine, all of which were found not to repress *pAOX* induction (Inan & Meagher, 2001). MutS and Mut- strains can only tolerate low levels of methanol and therefore Mut+ strains are less likely to become poisoned by methanol, though are more likely to become oxygen limited (Daly & Hearn, 2005). Both Mut+ and MutS strains are currently used in preparation of the production platforms and no consensus of the scientific community about their superiority / inferiority has been reached yet (Singh & Narang, 2019). On the other hand, lack of any recent literature indicates that Mut- strains are not currently used.

Apart from the methanol utilization phenotype, a wide range of genetically different *P. pastoris* strains is currently available and their use is determined by the required application. For example, strains deficient in protease activity have been widely popular (Zhang *et al.*, 2007). Several protease-deficient strains, such as SMD1163 (*his4, pep4, prb1*), SMD1165 (*his4, pep4*), and

SMD1168 (*his4, pep4*) have been found to be effective in enhancing the yield and the quality of various heterologous proteins (Cereghino & Cregg, 2000). Such strains have been successfully used for production of insulin-like growth factor I (Brierley, 1998) or laccase (Jonsson *et al.*, 1997). However, Cereghino & Cregg (2000) pointed out that protease-deficient strains exhibit lower specific growth rates, are difficult to transform, and have lower viability. Therefore, they are recommended to be used only if the other methods reducing proteolysis fail.

Certain genotypes were also introduced in order to simplify the selection of the positive transformants – for example strains GS115 or SMD1168 are defective in histidine dehydrogenase gene (*his4*) and therefore can be selected on histidine-free media if the inserted production cassette contains functional *his4* as a selection marker. Commonly used X33 strain (Invitrogen, Life Technologies; USA) is considered wild type, even though random mutagenesis of this strain has been historically performed (based on personal communication with prof. Cregg). Still, the phenotype of X33 is seemingly not differing from the parental strain.

C – Cultivation method

In order to prepare a successful production platform using *P. pastoris,* an integrated process of genetic alterations as well as physiological manipulation needs to be considered. Construction of the production strains thus needs to be done with respect to the appropriate handling in the cultivation process.

Shake-flask small scale cultivations of constructed strains are often the first stage in optimizing protein levels as well as in selecting culture conditions (Daly & Hearn, 2005). However, the levels of produced product are generally 10-fold lower than in cultivations using controlled conditions, mainly due to the concentration of the dissolved oxygen which becomes growth-limiting (Cereghino & Cregg, 2000). Thus, in order to obtain heterologous product on the large scale, fed-batch high-cell-density cultivation using bioreactors with strictly controlled conditions (aeration, pH, temperature, agitation, pressure, foam formation, exhaust air analyses, feeding) is established. This is performed with the ultimate goal of maximizing the amount of product in the minimum process time. Therefore, specific productivity q_p (U.g^{-1}.h^{-1}; units of product per gram of biomass produced per hour), biomass (g), and production time (h) are critical bioprocess variables.

Fundamental step in identifying the physiological constraints of *P. pastoris* is the development of a suitable feeding strategy (i.e. carbon and energy substrates addition) (Spadiut *et al.*, 2014, Looser *et al.*, 2015, Liu *et al.*, 2019). The relationship between q_p and specific growth rate μ (h^{-1}) reflects the equilibrium between cellular processes until the product is maturated/ secreted (i.e. induction

of gene expression, translation, protein folding and degradation in the endoplasmic reticulum, flux of folded protein out of the ER, and trafficking through the secretory machinery) (Looser *et al.*, 2015). Therefore, it is essential to design production strategy with the strict control of the desired μ_{set} using controlled addition of the carbon source.

Among the traditional feeding strategies belong (Liu *et al.*, 2019):

    i.   Dose feeding strategy: an indirect feedback of on-line measurement of dissolved oxygen concentration. Increase in the dissolved oxygen signalizes depletion of the C-source (e.g. methanol) which is automatically supplemented in the response. However, dose feeding strategy does not allow retaining constant growth rate and methanol concentration in the culture is varying. Therefore, this strategy does not allow uncoupling of the influence of those variables on the production outcomes.

   ii.   Methanol non-limited strategy: methanol concentration is on-line monitored and automatically supplied to the designed level. However, the concentration of methanol fluctuates around the methanol set-point and such non-linear conditions can again have an important impact on the studied variables.

  iii.   Exponential feeding strategy: methanol addition is pre-planned in order to maintain designed μ. A feeding rate profile is calculated using mass balance equations and μ of the culture is constant. Therefore, the yield of biomass to substrate is expected to be constant with a quasi-exponential accumulation of biomass as well as concentration of methanol should be close to zero at any given point.

Keeping the constant μ accelerates process reproducibility and allows to study μ-related effects on heterologous protein production (Liu *et al.*, 2019). Therefore, exponential feeding strategy (iii) represents the best design for the studies aimed at understanding the production and secretion of difficult-to-produce products, such as PGA.

Fed-batch process of *P. pastoris* producing heterologous proteins under the control of inducible promoter (*pAOX1*) comprises a phase of biomass growth in both the batch and the fed-batch modes and a production phase in the fed-batch mode. The process is initiated with a batch culture containing glycerol as a substrate. At the point of substrate depletion, the exponential addition of glycerol feed is initiated in order to reach the desired biomass concentration.   A methanol feed is exponentially added during the subsequent production phase, Fig. 5 (Hyka *et al.*, 2010, Looser *et al.*, 2017).
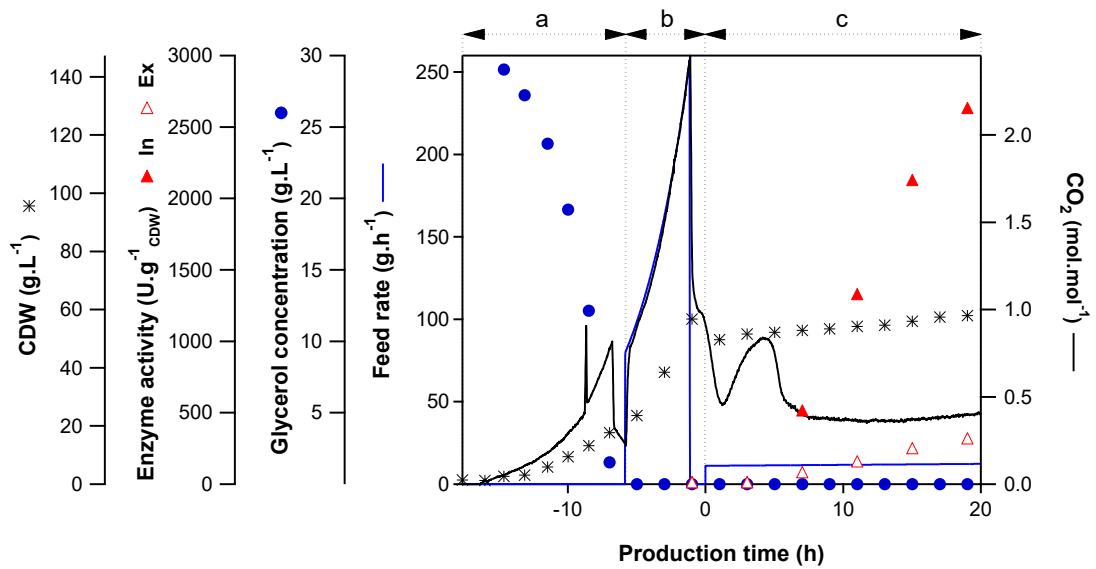
Fig. 5 Key characteristics of the cultivation strategy. Asterisks: cell dry weight CDW (grams per litre); Blue dots: Concentration of glycerol in medium (grams per litre); Blue line: Feed rate of glycerol/ methanol (grams per hour); Black line: CO_2 efflux (moll per moll); Triangles: PGA production intracellular (full), extracellular (empty). Dashed lines distinguish the process into the three phases: **a** Batch phase, **b** Growth fed-batch on glycerol, **c** Production fed-batch on methanol.

Apart from the process control strategy, other physical parameters affecting the production levels of heterologous proteins in *P. pastoris* include nutrient supply, pH, temperature, and oxygen quantity (Looser *et al.*, 2015). One of the most important parameters is suitable pH. *P. pastoris* can grow in wide range of pH from 3.0 to 7.0 with little to no effect on its growth (Stratton *et al.*, 1998). pH optimum is therefore mostly dependent on the properties of product, especially its stability (Macauley-Patrick *et al.*, 2005). Cultivation temperature can also have significant impact on product yields (Chen *et al.*, 2000). Even though temperature optimum for *P. pastoris* is 30 °C, lower cultivation temperature can have positive effect on protein folding and decreased activity of proteases (Li *et al.*, 2001, Hong *et al.*, 2002).

Choosing the most appropriate operational mode and the feeding profile significantly improves cell performance and decreases the burden caused by heterologous protein production, thus pushing the productivity of the system to its peak. Such an optimized process can be further scaled and transferred into the industrial process.

3. Aims of the study

This thesis contains data from two main projects I participated in during my PhD study. Both of these projects are focused on the microbial biotechnology development and are divided into "discovery" and "upstream development" parts.

Project 1 "discovery": Biotechnological potential of soil microbiomes affected by industrial production of antibiotics

i. Analysis of the microbial consortia exposed to long-term selection pressure resulting from industrial production of penicillin G and other pharmaceuticals;

ii. Exploration of the biotechnological potential of the microbial consortia exposed to long-term selection pressure caused by industrial production of penicillin G in question and discovery of the new enzymes involved in biotransformation of beta-lactam antibiotics.

Project 2 "upstream development": Relationship between yeast cell physiology, molecular design of expression system, and secretion of heterologous penicillin G acylases

i. Analysis of the biotechnological potential of Penicillin G acylase;

ii. Preparation of the yeast-based penicillin G acylase production platform;

iii. Detailed characterization of the yeast-based penicillin G acylase production process and in-depth analyses of intracellular protein fluxes.

4. List of methods

During the experimental part of the thesis preparations following methods were employed:

- Microbial community studies
    - o Metagenomic DNA isolation
    - o 16s rRNA V4 hypervariable region analyses using Illumina MiSeq platform
    - o Preparation of fosmid libraries from the metagenomic DNA
    - o Gene-of-interest mining (homology-based strategy, phenotype qualitative screening)
    - o Whole genome sequencing and analyses (Illumina HiSeq platform)

- Product-related studies
    - o *In vitro* and *in vivo* hydrolytic/ synthetic experiments
    - o Establishment of enzymatic assays including high throughput variant

- Production platform preparation and screening
    - o DNA manipulations – editing codon usage, gene optimizations
    - o Production cassette design and construction
    - o Vector construction, cloning
    - o Production strain optimization
    - o Selection and screening of the clones
        - ▪ High throughput cultivations and enzymatic assays
        - ▪ Shake flask cultivations and enzymatic assays

- Cultivation/ strain physiology studies
    - o Bioprocess modelling
    - o Bioreactor cultivations (Batch, fed-batch mode)
    - o Product characterisations (enzymatic assays, SDS-page, Western Blot, glycosylation assays)
    - o Strain characterizations – flow cytometry, fluorescence microscopy, transcriptome analyses (*q*PCR)

5. Results

Publications related to the thesis

5.1 Project 1

Biotechnological potential of soil microbiomes affected by industrial production of antibiotics

List of the publications related to this thesis' sub-project and the respective author statements.

Paper I

Characteristics of microbial community of soil subjected to industrial production of antibiotics.

In this study, we focused on characterization of microbial consortia exposed to long-term selection pressure caused by penicillin G production with the aim of examining the diversity and composition of these communities and exploring their biotechnological potential.

For the analyses, the soil from the area of pharmaceutical plant Biotika, a.s. (Slovenská Ľupča, Slovakia), which has been producing penicillin G since 1956, was sampled. For control purposes we also sampled the soil from the same geographical area outside the mentioned plant. Metagenomic DNA from both on-site and control samples was used to analyse and compare the composition of the respective microbial communities by analysing V4 hypervariable region of 16S rRNA gene by using Illumina MiSeq platform.

Consequently, metagenomic DNA from the on-site samples was also used for creation of *E. coli* T1R-based fosmid library. The aim was to unravel the biotechnological potential of the communities in terms of enzymes involved in biotransformation of beta-lactam antibiotics, i.e. penicillin G acylase and alpha amino acid ester hydrolase.

This study offers new insights into the changes in microbial communities of soils exposed to anthropogenic activity and indicates that those soils may represent a hotspot for biotechnologically interesting targets.

Author statement: Contribution of the author: 70%. For this publication, I was involved in all of the steps of the publication preparation. I conceptualized the study and collected the respective samples. Subsequently, I planned the experimental part of the study and worked on the sequencing analyses of the samples. I was also responsible for the statistical analyses of the data and for the interpretation of the results. I also drafted the manuscript.

Paper II

Draft genome sequence of *Pantoea agglomerans* JM1, a strain isolated from soil polluted by industrial production of beta-lactam antibiotics that exhibits valacyclovir-like hydrolase activity.

Zahradník, J.; Plačková, M.; Palyzová, A.; Marešová, H.; Kyslíková, E.; Kyslík, P. Genome Announcements 2017, 5, e00921-00917, doi:10.1128/genomeA.00921-17.

IF$_{2018}$ 0.89

In this study, we were screening cultivable organisms from the on-site soil samples within the area of pharmaceutical plant Biotika, a.s. (Slovenská Ľupča, Slovakia) for the presence of microorganisms exhibiting PGA- or AEH- like activity.

Out of the screened isolates, one was weakly positive for AEH activity. Genome of this strain was sequenced, assembled and described. Genes with a predicted PGA- or AEH-like activity were identified in the genome of this strain and were cloned and expressed in *E. coli* BL21. Using this approach, we discovered a new protein with alpha/beta hydrolase fold that was remotely homologous to human valacyclovirase gene (member of AEH-enzyme family).

The study brought new information on genes encoding novel enzymes with industrial potential and further supported the theory that microbial consortia from soils polluted by antibiotics are a potent source of microorganisms with industrially usable characteristics.

Author statement: Contribution of the author: 30%. For this publication I was responsible for the selection of the strain. I performed the cultivation experiments and screened for the strains in possession of the desired characteristics (production of enzyme biotransforming beta-lactam antibiotics; i.e. PGA or AEH). After I selected the strain in question, I also performed pilot PCR gene mining experiments using the method of sequence homology. I was also involved in the manuscript corrections.

5.2 Project 2

Relationship between yeast cell physiology, molecular design of expression system, and secretion of heterologous penicillin G acylases

List of the publications related to this thesis' sub-project and the respective author statements.

<u>**Paper III**</u>

Current state and perspectives of penicillin G acylase-based biocatalyses.

Maresova, H.; <u>Plackova, M.</u>; Grulich, M.; Kyslik, P. Applied Microbiology and Biotechnology 2014, 98, 2867-2879, doi: 10.1007/s00253-013-5492-7.

IF$_{2019}$ 3.530

This publication is a review article focused on the enzyme Penicillin G acylase (PGA) and its potential for the industrial biocatalyses. In the course of more than 60-year history, PGA has gained a unique position among the enzymes used in bioprocesses and for biotransformation of beta-lactam antibiotics, especially in the production of beta-lactam nuclei from penicillin G and glutaryl-7-aminocephalosporanic acid. A portfolio of other PGA traits required for enzymes with high industrial potential was summarized and discussed in terms of the current industrial utilization of these traits and their potential for other biotechnological applications. PGA was also compared with enzymes competing with PGA in the syntheses of semisynthetic beta-lactam antibiotics (alpha amino acid ester hydrolases, penicillin V acylases, and cephalosporin acylases).

The performed review of literature showed that PGA has a great potential to go beyond the beta-lactam biocatalyses and has the potential to be used in synthetic reactions, in the production of achiral and chiral compounds, or in the pro-drug activation. On the other hand, our review also revealed that even though a great number of PGAs of different origins has been described, only a limited number of production strains was at the time available for industrial-scale production of PGA; all of them based on prokaryotic host, namely *E. coli*.

<u>Author statement:</u> Contribution of the author: 30%. For this publication, I participated in the literature review and in writing the manuscript, namely I drafted the section concerning the description of the enzymes competing with PGA for syntheses of semisynthetic beta-lactam antibiotics. I was also involved in the manuscript corrections.

Potential of *Pichia pastoris* for the production of industrial penicillin G acylase.

In this study we focused on construction and characterization of two *P. pastoris* based production systems for intracellular and extracellular production of PGA from *Achromobacter* sp. CCM 4824. Prokaryotic *pga* gene was codon optimized for the use in the yeast host and was cloned into the commercial vectors p*PICZ* and p*PICZα* for intracellular and extracellular production, respectively.

P. pastoris X33 was consequently transformed with the prepared plasmids and the created transformants were screened for those with the best PGA-production performance and characterized. A set of fed-batch 6 L stirred bioreactor cultivations with the prepared strains was consequently performed using the in-study optimized media.

The performed bioreactor fed-batch cultivations revealed that the strain producing PGA intracellularly yielded a comparable amount of enzyme as industrially established *E. coli* production systems. On the contrary, equivalent bioreactor cultivation with the strain constructed for extracellular production of PGA revealed secretory bottleneck of the production strain, whereby only approx. 40% of the produced enzyme was secreted into the culture supernatant while the majority was retained intracellularly.

This study laid, for the first time, the basis for extracellular PGA production in *P. pastoris*. Even though the potential of *P. pastoris* as a production host for PGA was established in principle, the secretory bottleneck needed to be addressed in further studies.

Author statement: Contribution of the author: 20%. During the experimental part of this study, I was working on the bioreactor cultivations and I was performing the enzymatic assays. I was also involved in the data analyses and in the manuscript preparation and corrections.

In this study we continued with the development of the *P. pastoris* production system for extracellular production of PGA. We performed a detailed study dealing with the optimization of the production process as well as with the quantification of the time-dependent specific rate of PGA secretion and its interdependence with intracellularly retained PGA and biomass growth.

The strain producing PGA extracellularly (developed in the Paper IV, Maresova *et al.*, 2017) was cultivated in a series of 6 L stirred bioreactor fed-batch cultivations. Those cultivations were performed at different specific growth rates, which were maintained by exponentially increasing the feeding of methanol. Detailed analyses of the production process as well as of cells and their analysts were performed, including substrate analyses, protein analyses, and cell viability and lysis analyses. In order to study the evolution of specific productivity of the system over the course of cultivation in great detail, a descriptive mathematical model was developed. This advanced data interpolation and fitting tool allowed us to describe dynamic changes in specific productivity (q_p) and specific rate of product secretion ($q_{p,extra}$) in considerable depth.

The key achievement of the study is a description of the temporal change in the rate of specific product formation during the production phase of *P. pastoris* fed-batch cultivation, when producing PGA under the control of the p*AOX1* promoter. We also showed that the stress caused by heterologous PGA production induced cellular imbalance leading to the selective translational arrest as a response to the oversaturation of the secretory pathway.

The study represents a significant contribution to understanding the dynamic changes in q_p over time and may generate opportunities for expanding the biotechnological application potential of the *Pichia-pAOX1* system for difficult-to-produce products.

Author statement: Contribution of the author: 80%. For this publication, I was involved in all of the steps of the publication preparation. I conceptualized the study and I planned the experimental part of the study. I performed all of the bioreactor cultivations, including sample analyses and interpretation. I was also responsible for the analyses of the resulting data and for writing the manuscript.

Paper VI

Single-cell approach to monitor the unfolded protein response during biotechnological processes with *Pichia pastoris*

Raschmanova, H.; Zamora, I.; Borcinova, M.; Meier, P.; Weninger, A.; Machler, D.; Glieder, A.; Melzoch, K.; Knejzlik, Z.; Kovar, K. Frontiers in Microbiology 2019, 10, 335, doi:10.3389/fmicb.2019.00335.

IF$_{2019}$ 4.235

In this study, production and secretion of three different recombinant proteins (PGA from *E. coli*, lipase B from *Candida Antarctica*, and xylanase A from *Thermomyces lanuginosus*) by *P. pastoris* was investigated along with up-regulation of the unfolded protein response pathway (UPR) and cell viability, which were assessed at a single-cell level in living cells and at-line with flow cytometry.

For the purpose of the study, a new strain carrying PGA production cassette as well as UPR-reporter cassette was constructed (analogously for lipase B and xylanase A). The constructed strain was cultivated in 6 L stirred bioreactor fed-batch cultivation and intracellular and extracellular product concentrations were measured along with measurements of the cell viability and the upregulation of UPR using flow-cytometry. Subsequently, the cells´ viability and the levels of UPR up-regulation were put in the context of production patterns.

The resulting data brought novel insight into the development of heterogeneity in a recombinant *P. pastoris* population during a biotechnological process. They also provided us with important information about UPR upregulation in context of the dynamic changes of specific productivity of PGA-*P. pastoris* system described in the previous study (Paper V, Borcinova *et al.*, 2020).

This study represents a first trial in which UPR up-regulation was studied at a single-cell level and in a non-invasive manner. By understanding the relationship between the protein production/secretion and the tuning of the UPR, this monitoring system based on fluorescence measurement can be utilized in future bioprocess control and optimizations.

Author statement: Contribution of the author: 30%. For this publication, I was involved in the PGA production strain design and preparation. I performed the respective bioreactor cultivations with the created strain and I was involved in sample analyses. I was also involved in the manuscript preparation and corrections.

Pilot study VII

Effect of multivariate engineering and co-expression of helper factors on an efficient production of penicillin G acylase in *Pichia pastoris*

Borčinová, M.; Krainer, F.; Kyslík, P.; Glieder, A. Concept study performed for the European Research Council H2020 grant application.

This concept study dealt with the development and studying of various *P. pastoris* strains for PGA production. By employing an integrated process of identifying essential properties of the production strains, the aim was to acquire an in-depth view of the bottlenecks and limitations of PGA production by *P. pastoris*.

Gene codon usage, promoter choice and regulation, the type of transport to endoplasmic reticulum, the effect of increasing gene dosage, and finally the influence of overproduction of the proteins involved in the protein maturation and secretion were analysed. Overall, 40 different constructs were prepared during the course of the study. In a series of cultivation experiments performed by the high throughput method in 96-deep well plates, the uncoupled effect of the mentioned studied parameters was quantified and putative activity landscapes were generated.

This study represents a pilot analysis of the rate limiting steps in the maturation and secretion of PGA in *P. pastoris* that evidences the potential of this host for PGA production. The obtained knowledge should give base for the development of a tailored strain capable of efficient production and secretion of PGA.

Author statement: Contribution of the author: 80%. For this study, I was involved in all of the steps of the study preparation. I conceptualized the study and planned the experimental part of the study. I constructed all of the studied strains and performed all of the cultivations, including sample analyses and interpretation. I was also responsible for the analyses of the resulting data.

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RNDr. Pavel Kyslík, CSc., the supervisor

6. Discussion

The presented thesis consists of two sub-projects dividing the thesis into the "discovery" and the "upstream" parts of biotechnology development.

In the "discovery" part of the thesis, the impact of industrial production of beta-lactam antibiotics on microbial consortia was studied along with the analyses of the biotechnological potential of those communities in terms of enzymes involved in bio-transformation of beta-lactam antibiotics, namely penicillin G acylase (PGA) and alpha-amino acid ester hydrolase (AEH). The second "upstream" part of the thesis was aimed at the exploration of the biotechnological potential of PGA production using yeast organism *Pichia pastoris* by studying the limitations and bottlenecks of this production system.

Biotechnological potential of soil microbiomes affected by industrial production of antibiotics

In the "discovery" sub-project of this thesis, we studied the impact and consequences of industrial production of beta-lactam antibiotics on soil microbiomes. Soil microbial communities are an integral part of soil health and soil-related processes. However, with the expansion of industrial manufacturing of pharmaceutical compounds without clear guidelines for environmentally safe production, increasing amounts of xenobiotics are polluting the environment and are influencing the composition and functions of such communities (Cycon *et al.*, 2019). Soil samples for our analyses (Paper I, II) were collected from the area of a pharmaceutical plant operating since 1953. Even though a wide variety of products had been produced there, our main interest laid in the impact of over 60 years of the production of penicillin G which started in 1956 and has intermittently been continuing to this day. In order to assess the change in diversity and composition of the consortia, control samples from the same geographical location outside the plant site were analysed along with the soil samples from the plant site.

As expected, our analyses revealed that the communities were significantly affected by penicillin G production. However, contrary to the initial hypothesis, the on-site samples exhibited significantly higher bacterial diversity and richness than the control samples. The Shannon diversity index (H) of the on-site samples equalled 4.7 and 5.2 respectively; for the control samples the H' index equalled 3.2 and 3.1 respectively. Our results are in accordance with the previously described long-term effect of the pollutants that allows for the overall structure of the community to recover, but not to reach the equivalent genomic structure (Martínez, 2017). The same observations were described in an analysis of bacterial communities in water exposed to penicillin G-plant effluent, in which

higher levels of diversity were observed compared to those in non-exposed water (Li *et al.*, 2011). Our results therefore confirm the theory that the selection pressure inhibits a certain, more susceptible, part of the community, which consequently allows the remainder to increase in abundance, and therefore minor taxons can emerge (Cleary *et al.*, 2016).

The linear discriminant analysis effect size method (LefSe method) was further employed to detect phyla, orders and genera explaining the difference between the samples and acting as "biomarkers" of the respective microbial community (Segata & Huttenhower, 2011). It was shown that a significant decrease in Proteobacteria, especially Gammaproteobacteria and Actinobacteria, occurred among the on-site samples. On the other hand, enrichment in Betaproteobacteria, Deltaproteobacteria, Gemmatimonadetes, Acidobacteria and Planctomycetia was observed. Decrease in Proteobacteria and enrichment in Acidobacteria is of particular importance because it points out to the reduced nutritional status of the environment (Smit *et al.*, 2001). This finding is further supported by the fact that phyla Actinobacteria was, at the same time, an important determinant of the control samples and was therefore significantly underrepresented in the on-site samples. Actinobacteria were described to be highly sensitive to the C/N nutrient status of the soil (Wolińska *et al.*, 2019). Actinobacteria have a key role in soil communities in terms of organic carbon recycling and the cycling of nitrogen, phosphorous and potassium; they also produce a variety of hydrolytic enzymes enabling them to degrade natural polymers, including lignin, cellulose, chitin and other organic compounds, reviewed in Zhang *et al.* (2019). Therefore, their decrease in the environment also indicates disrupted metabolic and enzymatic functions of the soil.

At class level, the enrichment of the on-site samples was mostly in accordance with the previously published study of rivers receiving treated penicillin G and oxytetracycline production wastewater (Li *et al.*, 2011). However, we encountered one surprising finding which was the decrease in the Gammaproteobacteria class, previously described as one of the most abundant classes on the global level (Ghannam *et al.*, 2020). Numerous studies showed that Gammaproteobacteria are one of the dominant classes in antibiotic-contaminated environments (Petrovich *et al.*, 2020, Shen *et al.*, 2020). Also Zhang *et al.* (2017) indicated the prevalence of Gammaproteobacteria in the soil treated with penicillin G. Gammaproteobacteria were shown to be positively correlated with the "multifunctionality" of the soil (measures of enzymatic activity and respiration) (Delgado-Baquerizo *et al.*, 2017) and we can therefore hypothesize that their decrease again indicates a reduction in the functionality of the on-site soil.

These findings led us to the hypothesis that the nutritionally poor soil with potentially disrupted functionality could give rise to bacteria able to adapt to such conditions. The PGA enzyme is thought to assist in the production of phenylacetic acid via degradation of phenylacetylated compounds,

such as penicillin G, whereas phenylacetic acid may in turn be used as a carbon source for bacteria when in a free-living mode (Done *et al.*, 1998, Tishkov *et al.*, 2010). Therefore, we hypothesized that the possession of PGA enzymatic activity can provide a nutritional advantage to the bacteria present in the on-site soil and thus the abundance and diversity of PGA could be increased.

For that reason, isolated metagenomic DNA from the on-site samples was also used for the preparation of a fosmid library that was subjected to functional screening with the aim of assessing the presence and diversity of PGA-like enzymes. The screening of the created library (2000 clones tested) brought forth 48 positive clones, out of which 14 were proven to contain PGA-like enzyme. By way of comparison, Zhang *et al.* (2014) constructed a cosmid metagenomic library using the soil metagenome of sediment microbial consortia in Antarctica. Their screening revealed 9 clones, out of 3000 tested, that carried the gene for PGA. Therefore, we can conclude that our library yielded high hit rates and confirmed our hypothesis about the abundance of PGA enzymes.

Out of the 14 obtained sequences, only one was 100% identical (comparison of amino acids) to the sequence deposited in the NCBI database. All of the remaining 13 sequences have a great potential to significantly increase the portfolio of industrially usable PGAs. The work of Deaguero *et al.* (2012) showed that mutation in a single amino acid can change the enantioselectivity of the PGA enzyme and thus change/ increase its usability. The importance of single nonsynonymous mutations was also comprehensively analysed by Wrenbeck *et al.* (2017). Therefore, when fully characterized, the identified metagenomic PGA sequences can potentially reveal novel catalytic functions and leverage the utilization of this catalyst.

Nevertheless, screening of the cultivable fraction of the microorganisms should not be overlooked either. Even though metagenomic approach brings novel ways of exploiting hidden potential of the microbial consortia, there are still certain limitations of this method, such as uncertainty of finding the genes of interest due to their potentially low abundance in the metagenomic sample as well as its structure and processing requirements (Gabor *et al.*, 2004, de Carvalho, 2017). Thus, in the subsequent study (Paper II), we also screened cultivable fraction of microorganisms from the on-site soil samples for isolates exhibiting activities analogous to PGAs and AEHs. The strain that showed positive phenotype, later identified by genome sequencing as *Pantoea agglomerans* JM1 (Czech Collection of Microorganisms, CCM 8766), was isolated. Members of genus *Pantoea* were not previously described as producers of either PGAs or AEHs. By identifying the prospective genes in the genome sequence of this organism we identified a protein with alpha/beta hydrolase fold that was remotely homologous (22%) to human valacyclovirase gene, which belongs to the alpha amino acid ester hydrolase family of enzymes (Kurochkina *et al.*, 2013). Valacyclovirases were described to activate number of clinically important precursors with a wide range of antiviral and

anticancer action and are predicted to play an important role in the medical field in prodrug activation (Sun *et al.*, 2010, Kurochkina *et al.*, 2013). This enzyme has not yet been identified in prokaryotes and the one revealed in our study can therefore hold the potential for further biocatalytic conversions.

In conclusion, this "discovery" sub-project represents the first holistic approach to studying the ecology and the biotechnological potential of soils exposed to decades' lasting production of penicillin G. The study revealed novel patterns in the impact of the antibiotics' production on the composition and function of microbial communities that in response can hold interesting biotechnological potential, as was also declared by our results.

Relationship between yeast cell physiology, molecular design of expression system, and secretion of heterologous penicillin G acylases

In parallel to the first sub-project, we also concentrated on the second part of the thesis which aimed at "upstream" development of an efficient production platform for penicillin G acylase.

Many industrial-scale *E. coli*-based PGA production systems are already available (Paper III), however this prokaryotic platform has numerous limitations, especially in terms of downstream processing. In *E. coli,* PGA preproprotein is translocated to the periplasmic space; the process during which the protein folds. Thus the mature enzyme needs to be mechanically released and consequently purified (Velez *et al.*, 2014). Therefore, we decided to test and utilize the emerging *P. pastoris* platform which recently gained significant attention in production of other industrial enzymes (Ahmad *et al.*, 2014). This organism is able to secrete a high-quality product with a low basal secretion of its own proteins (Delic *et al.*, 2013), which has a substantial impact on lowering the manufacturing costs by reducing downstream processing.

For the first study concerning this objective (Paper IV), *P. pastoris* X33 strains producing PGA from *Achromobacter* sp. CCM 4824 were constructed using the commercial vectors p*PICZ* and p*PICZα* (Thermo Fisher Scientific, USA). In the first created strain, PGA was produced intracellularly, while in the second strain, α-mating signal sequence from *S. cerevisiae* was employed for secretory production of the enzyme. Performed bioreactor fed-batch cultivations revealed that the strain producing PGA intracellularly yielded comparable amount of enzyme as *E. coli* production systems (Becka *et al.*, 2014). On the other hand, equivalent bioreactor cultivation with the strain constructed for extracellular production of PGA revealed secretory bottleneck of the production

strain, whereby only approx. 40% of the produced enzyme was secreted into the culture supernatant while the majority was retained intracellularly. Similar observation about intracellular retention of the enzymes aimed at secretory production in *P. pastoris* was previously described for numerous other products such as for alkaline phytase from *Lilium longiflorum* (Yang *et al.*, 2015), glucose oxidase from *Aspergillus niger* (Bankefa et al., 2018), or methyl parathion hydrolase from *Ochrobactrum* sp. (Wang *et al.*, 2014). However, there is only limited information available on the production of PGA using *P. pastoris* and thus the reasons for the described secretory limitation of this system needed to be further experimentally studied.

For this purpose, a detailed study dealing with optimization of the production process as well as with the quantification of the time-dependent specific rate of PGA secretion and its interdependence with intracellularly retained PGA and biomass growth of the previously created secretory strain was performed (Paper V). Knowledge of product formation kinetics, i.e. the relationship between the specific rate of protein production and the specific growth rate of culture, is imperative for bioprocess development and optimization. Using a mathematical fitting tool for data analysis, formation kinetics of PGA in *P. pastoris* was investigated, with the focus on the kinetics of not only PGA secretion, but also its intracellular retention, which has not yet been described in the literature. Generally, three main phases (Fig. 7, originally published in Paper V) with respect to changes in specific productivities of secreted and intracellularly retained PGA were distinguished in each of the performed fed-batch cultivations: (I) An initial steady-state phase characterized by quasi stable specific productivity (q_p) and specific rate of product secretion ($q_{p,extra}$), followed by (II) a transition period coinciding in time with reaching the intracellular saturation maxima when overall q_p was declining while $q_{p,extra}$ increased, and the final (III) saturation phase, again characterised by stable q_p and $q_{p,extra}$. In contrast to initial steady-state phase (I), where q_p of product retaining inside the cells is about 6-times higher than $q_{p,extra}$. $q_{p,extra}$ was twice as high as q_p of retained product during steady-state saturation phase (III), i.e. the majority of the synthesized product was secreted.
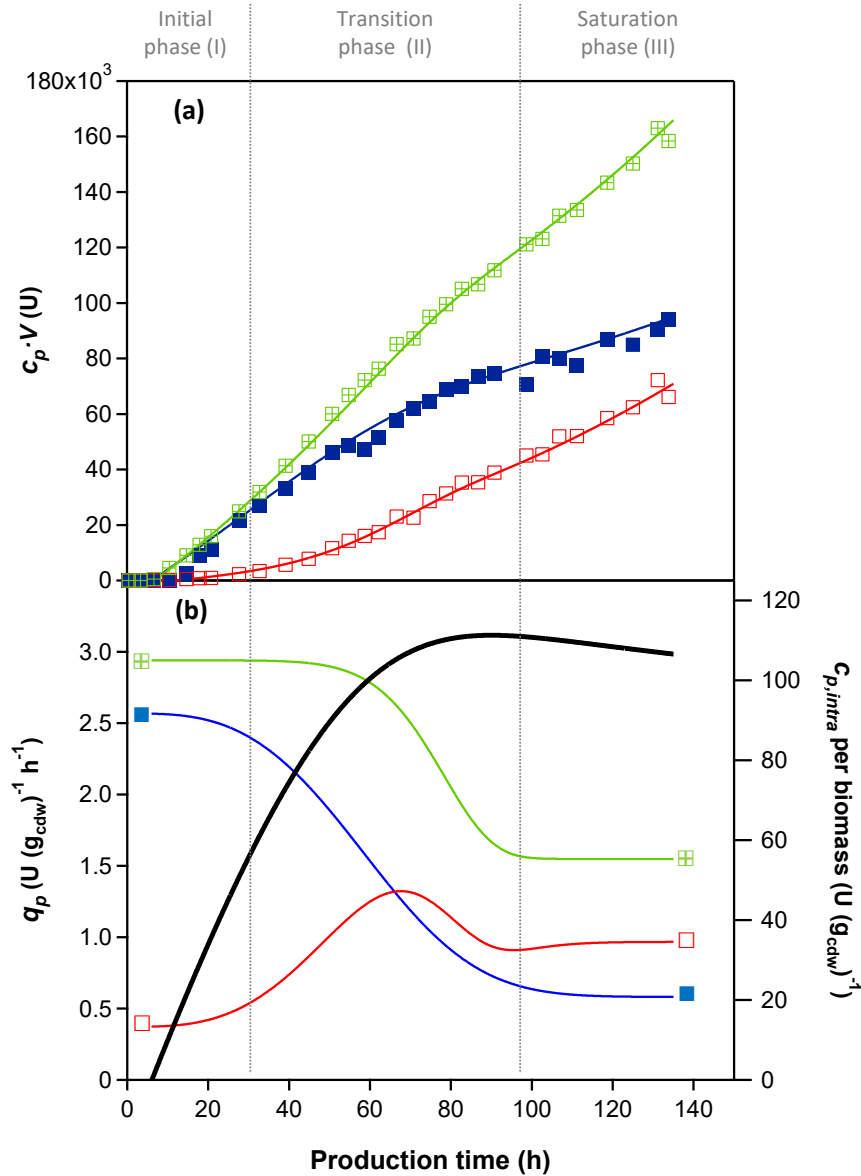
Fig. 7 Time course of PGA production with distinguished phases with respect to q_p. The displayed data represent the typically observed trend in PGA formation in all cultivations enlisted within Paper V (Borcinova *et al.*, 2020). **a:** Amount of PGA (in U) during the production phase of cultivation. The symbols represent measured enzyme: crossed squares – total amount; full squares – intracellular amount; empty squares – extracellular amount. The solid lines represent the calculated theoretical values for the respective measured enzyme (U). **b**: The coloured lines marked by respective square symbols represent calculated $q_p(t)$ values: green (crossed square) – $q_{p,total}$, blue (full square) – $q_{p,intra}$, red (empty square) – $q_{p,extra}$ (U $(g_{cdw})^{-1}$ h^{-1}). The black bold line represents the time development of intracellular PGA activity per gram of cell dry weight (U $(g_{cdw})^{-1}$), which indicates the saturation of the cell with product. The time course of the specific production rate of PGA $q_p(t)$ was divided into three phases as indicated by the vertical dotted lines: initial, transition, and saturation phase. (Paper V, Borcinova *et al.,* 2020)

The most probable reason behind the significant proportional increase in secreted PGA in phase III is partial translational arrest, as previously described by Edwards-Jones *et al.* (2015). The most likely stress factor that could promote translational arrest is a nutritional imbalance, resulting from demands of cells undergoing nutritional limitation and synthesising high level of proteins, leading to a redox imbalance between the cytosol and mitochondria (Edwards-Jones *et al.*, 2015). This state consequently significantly affects heterologous protein production. In their work, Hesketh *et al.* (2013) observed significant changes in the levels of transcripts predicted to encode proteins associated with mRNA processing and translation. This may reflect general changes in protein synthesis during recovery from cellular stress, as indicated by the down-regulation of genes associated with ribosome biogenesis (Hesketh *et al.*, 2013). Moreover, they also showed that intracellular protein aggregation was followed by up-/ down-regulation of transcription of genes involved in responses to intracellular stress. Several of these genes were also found to be antisense to genes associated with cell membrane biosynthesis and metabolism. This confirmed the inference from the "sense" transcriptome that overproduction of a misfolded or over-accumulated protein has a significant impact on cell wall-associated processes and can therefore significantly affect the secretory abilities of the cell (Hesketh *et al.*, 2013). Similar results were also described in the work of Barrigón *et al.* (2013) who suggested that the down-regulation of transcription happens in response to activation of unfolded protein response (UPR) pathway (Barrigón *et al.*, 2013).

We assume that translational arrest may have helped to relieve the stress, facilitating restoration of the secretory pathway in phase III, although at the same time it also resulted in lower overall productivity of the system.

The study also revealed the significant shift in the relationship between q_p and specific growth rate (μ). Our results indicated that initial product formation is related to growth in a bell-shaped manner, as previously described in Looser *et al.* (2015), while after the transition period the $q_p(\mu)$ relationship shifted towards a linear production kinetics, which is usually found for constitutive promoters. After the described shift to phase III, the specific secretion rate of the cells was up to three times higher. Rebnegger *et al.* (2014) described significant changes in the regulation of important groups of genes at high μ. Specifically, this involved up-regulation of translational and UPR genes such as those involved in translocation of nascent proteins to the ER, enhancement of protein folding in the ER and the synthesis of cytosolic chaperones. High μ also led to the down-regulation of genes involved in proteolytic degradation of proteins in the secretory pathway and exocytosis (Rebnegger *et al.*, 2014). Such regulation of genes at high μ could explain the linear relationship between $q_{p,extra}$ and μ_{set} observed in this work.

Overall, based on the results of Paper V, it was hypothesized that the underlying cause for the strain´s behaviour was intracellular stress caused by the accumulation of the protein in the intracellular secretory pathway leading to UPR stress – consequently causing translational arrest. Therefore, in order to test the hypothesis, up-regulation of the UPR pathway was studied during the course of the cultivation (Paper VI).

Firstly, in order to confirm the transferability of the results from Paper V, we tested the observed trends on other *P. pastoris* systems (Paper V) as well as on *Ec*PGA strain that was newly developed for this purpose. The *Ec*PGA strain was constructed in the same way as strain from Paper IV and Paper V: *pga* gene under the control of the p*AOX1* promoter was integrated into the *P. pastoris* X33 genome. The behaviour of the strain during fed-batch cultivations was proven to follow the same pattern of the three phases described in Paper V (unpublished data). Therefore, we used this newly constructed strain for the consequent analyses in which plasmid $P_{KAR2(FL)}$-*sf*GFP was co-integrated to the *Ec*PGA *strain*. This plasmid for monitoring UPR carried a 324bp upstream region of the *KAR2* coding sequence (encoding ER chaperone BiP) containing one copy of the unfolded protein responsive element before the super folded green fluorescent protein (*sf*GFP) coding sequence. It was used for UPR up-regulation monitoring based on *sf*GFP production upon the activation of *KAR2* promoter.

The constructed strain was cultivated in bioreactor fed-batch cultivations and intracellular and extracellular product concentrations were measured along with measurements of cell viability and the up-regulation of UPR using flow-cytometry measurements. It was shown that proportion of the cells with up-regulated UPR steeply increased right after the induction of production and, after 30 hours of production fed-batch, approx. 60% of the cells had up-regulated UPR. UPR up-regulation correlated with the previously described phases of PGA production, plateaued at the end on phase I just before the maximal saturation of the cells with intracellular product, and remained at approx. 60% till the end of the cultivation. This confirms that maximal UPR up-regulation correlated with the switch in specific productivity of the system and thus confirming the previously discussed translational arrest caused by UPR up-regulation (Paper V).

Surprisingly, only 13% of the cells had impaired viability which was the rate comparable to the non-producing control strain. Therefore, we can conclude that even though UPR up-regulation was strong, it did not promote cell lysis, contrary to the results of (Ron & Walter, 2007) whom observed significant cell lysis due to extensive UPR up-regulation.

Using the principal component analysis of the flow cytometric data, four sub-populations of cells were identified in all the cultivation processes: smaller and less complex viable cells with no UPR

up-regulation (on average constituting 30% of the cells), larger and more complex viable cells with no UPR up-regulation (8%), viable cells with up-regulated UPR (up to 60%), and cells with an impaired viability (at maximum 13%). In the study of Dragosits *et al.* (2010), it was shown that the non-producing cells were larger than the cells producing the recombinant product. On the other hand, according to work of Aw *et al.* (2017), *P. pastoris* strains secreting a higher amount of a recombinant protein were shown to have larger cells than the strains producing the same product with lower titres. Even though we did not measure the secretion of PGA at a single-cell resolution, based on the results of the population heterogeneity in relation to the production and secretion of PGA we can conclude that the cell cluster most probably responsible for PGA secretion were the viable cells with up-regulated UPR. This result is mostly in agreement with the results of Love *et al.* (2010) who, by using microengraving experiments, described the heterogeneity of *P. pastoris* population with regard to the secretion of human Fc fragment: one cluster of the cells yielded no significant secretion over the course of the experiment (35%), one cluster (32%, consisting of two subpopulations) consistently secreted protein, and one cluster (33%, three subpopulations) exhibited a significant change in their rates of secretion during the experiment (Love *et al.*, 2010).

In conclusion, as discussed in Paper IV and Paper V and as evidenced by the results of Paper VI, production of PGA leads to its incorrect maturation and accumulation in ER which results in ER stress. Consequently, ER stress negatively influences PGA production and secretion. Since Papers IV and V showed that the same behaviour of the strain can be observed irrespective of specific growth rate of the culture and other cultivation conditions, we have to conclude that in order to develop an industrially viable *P. pastoris*-PGA platform, it is necessary to take a step back and further optimize the production strain on the construction/ molecular level.

In order to analyse whether a rational strain design could reduce cellular stress responses and improve PGA production and secretion, we performed the pilot study concerned with the development of a library of PGA-producing *P. pastoris* constructs (Pilot study VII). Overall, 40 different *P. pastoris* strains were constructed for the purpose of the study.

Generally, the major challenge for heterologous protein production in *P. pastoris* is inefficient protein folding and secretion. However, this process is rather complicated as numerous interrelated mechanisms and stress responses need to be in equilibrium (Gu *et al.*, 2015).

In the first part of the study, we performed series of experiments in which the uncoupled effect of gene codon optimization, signal sequence, promoter, and gene dosage were studied. The most important finding of this part of the study was the observation that increasing transcript availability

significantly improved production of PGA, especially when strong promoter was combined with the multicopy insertion of the production cassette. The yield of PGA of the resulting created strain, pPpB1_*pFMD*_Da-PGA, was 2.74-fold higher in comparison to the benchmark strain (strain *Ec*PGA, Paper VI). This result is rather surprising in the context of the previous studies (namely Paper VI), where overproduction of PGA was proven to be the main reason for cellular stress. The explanation can lie in the fact that a different type of the host strain was utilized in this study. In contrast with the previously used Mut+ strain X33, the strain used in this study, BSYBG11, was MutS strain. The slow growth and lower methanol consumption of MutS strains may have some advantages for production processes such as lower demand for oxygen and reduced heat formation (Schotte *et al.*, 2016). Moreover, fast methanol oxidation is also linked to by-product formation of hydrogen peroxide formation, which is known to cause cellular stress and potentially induce cell death (Kern *et al.*, 2007). In that respect, MutS strain could be of advantage for PGA production. At the same time, the previously used *Ec*PGA strain expressed PGA under the control of the p*AOX1* promoter as opposed to the formate dehydrogenase promoter p*FMD* used in this study (Weinhandl *et al.*, 2014). The studies of Camara *et al*. (Camara *et al.*, 2017, Camara *et al.*, 2019) showed that p*AOX1* faces a transcriptional limitation when heterologous genes are expressed under the control of this promoter. This results in a limitation of the methanol assimilation capacity of such strains due to low availability of methanol expression regulator 1, a trans-acting factor essential for significant levels of methanol pathway (Lin-Cereghino *et al.*, 2006).

The strain pPpB1_*pFMD*_Da-PGA was used in the consequent experiments where so-called "helper" proteins were co-produced along with PGA. The major mechanisms related to protein production in *P. pastoris* can be divided into several modules: translocation to ER, protein folding, vesicular trafficking, ERAD, UPR, and exocytosis (Fig. 3). 9 potential genes from the individual modules, which were previously described to increase heterologous protein production, were selected and co-produced with PGA (Grote *et al.*, 2000, Boer *et al.*, 2003, Wilkinson & Gilbert, 2004, Joo *et al.*, 2011, Gu *et al.*, 2015, Wu & Guo, 2015, Preston & Brodsky, 2017, Saito *et al.*, 2017, Han *et al.*, 2020). Using this approach, we were able to further significantly increase the PGA yields when compared to strain *Ec*PGA cultivated under the same cultivation conditions. The helpers having the main impact on PGA production and secretion were those connected to the vesicular trafficking and exocytosis.

Co-production of WSC4, which plays an important role in the translocation of proteins in ER and also interacts with the regulators of UPR pathway (Mamoun *et al.*, 1997, Zu *et al.*, 2001), increased the PGA yields 5.4-fold, marking protein trafficking as an important bottleneck of efficient PGA secretion. Nevertheless, the best results were observed when SSO2 was co-produced – up to 7-fold

increase in PGA yields was observed. SSO2 is a plasma membrane target of SNAP receptors (tSNAREs, the syntaxin homologues), that helps with the fusion of secretory vesicles at the plasma membrane (Gasser *et al.*, 2007). This indicated that exocytosis may represent another of the major bottlenecks in PGA-*P. pastoris* system. Interestingly, for both those proteins, the effect was only observable while they were produced under the control of constitutive promoters. That indicates that the respective pathways were boosted prior the production of PGA, which only started after the methanol induction. When produced under inducible promoter, i.e. when the production of the helper was started at the same time as PGA production, the effect of the helper was significantly lower. From this we can hypothesize that initial "prevention" of the bottleneck plays an important role in PGA secretion. Similar results were also observed in the work of Gu *et al.* (2015) or Gasser *et al.* (2007).

However, it is not only the two mentioned helpers that had a significant impact on PGA yields. Improvement was observable while co-producing "helpers" from each described module; PGA yields were also increased while co-producing helpers involved in activation of general stress response of the cells, ER chaperones, and Hac1p activator of the UPR pathway. This indicates that further steps in preparation of the PGA-*P. pastoris* platform should employ a combination strategy, where helpers from different modules would be co-expressed together to further improve the yields of PGA and push its secretion beyond existing benchmarks.

The current study presents the first systems biotechnology-based strategy for engineering *P. pastoris* strain producing PGA. The study proved the great potential of *P.* pastoris for producing PGA. Obtained knowledge should therefore facilitate the development of tailored process strategies to design an optimum PGA-secreting strain and, consequently, to allow for the production of PGA in ways that are feasible for large-scale manufacturing.

7. Summary and Outlook

In conclusion, the first "discovery" part of this thesis offered new insights into the changes in microbial communities of soils exposed to anthropogenic activity as well as into indications that those soils may represent a hotspot for biotechnologically interesting targets. The analyses of the second "upstream" part represent a significant contribution to the understanding of the behaviour and physiology of PGA-*P. pastoris* system and, as such, represents a valuable input for further studying and optimization of *P. pastoris* production platform in order to generate opportunities for expanding its biotechnological application potential.

The main messages of the "discovery" project "Biotechnological potential of soil microbiomes affected by industrial production of antibiotics" can be summarized as follows:

- Long-term selection pressure caused by xenobiotic pollutants formed at a penicillin G manufacturing site causes significant changes in microbial consortia of the affected soils, leading to a significant change of composition on both phyla and genera level that potentially results in decreased functionality of the soil. On the other hand, a significant increase in diversity and richness of the impacted consortia was observed.
- Metagenome of the anthropogenic soil represents a promising reservoir for discovering enzymes involved in the bio-transformation of beta-lactam antibiotics that exhibit novel functions and abilities.

The main messages of the "upstream" project "Relationship between yeast cell physiology, molecular design of expression system, and secretion of heterologous penicillin G acylases" can be summarized as follows:

- Production of Penicillin G acylase in *Pichia pastoris* is hindered at the level of efficient secretion of the produced enzyme. Limitation in the secretory machinery can be attributed to the cellular stress caused by accumulation of the (unfolded) protein in the endoplasmic reticulum.
- Specific product formation rate (q_P) exhibits temporal change during the production phase of *P. pastoris* fed-batch cultivation. Moreover, initial product formation is related to growth (μ) in a bell-shaped manner, while after the transition period *the $q_P(\mu)$* relationship shifts towards a linear production kinetics, which is usually found for constitutive promoters. Also, after the described shift, the specific secretion rate of the cell machinery is up to three times higher.

- The limitations of PGA production and secretion by *P. pastoris* could be overcome by rational design of the production strain and suitable cultivation strategy.
- Overall, the obtained results strongly indicate that PGA-*Pichia* production platform could find its place in the industrial biotechnology, though further development steps need to be performed.

The presented thesis also gave rise to the consequent studies that should follow:

1. Detailed analysis and isolation of the *pga* genes from Paper I
2. Further development of the *P. pastoris*-PGA production platform based on Pilot study VII using combinatorial strategy and further scale-up and development of the most suitable cultivation strategy for bioreactor cultivations.

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9. Publications

PAPERS I-VI and PILOT STUDY VII are annexed to this thesis.