Developing a role for *Rhizopus oryzae* in the biobased economy by aiming at ethanol and cyanophycin coproduction

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Bas Johannes Meussen

Thesis

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Contents

Chapter 1.
General Introduction

1.1. The transition to a biobased economy

Fossil fuels, including oil, natural gas, and coal are primary energy sources and account for 86% of the world's energy consumption [1]. These fossil fuels are also used as a feedstock for the production of chemicals and materials. It took millions of years to form these fuels from biomass and their consumption is therefore considered non-renewable. At a certain moment the reserves of these fossil resources will be depleted and prior to that moment mining for bulk use will be economically unfeasible. Hubbert formulated the theory of 'peak oil', which predicts the moment at which the maximal daily extraction of oil from oilfields is reached and the production decreases [2]. In the 'peak oil' model the rate of extraction will first exponentially increase, reach a breakeven point, and subsequently decline. After this breakeven point the net reserves of oil will start to deplete. For some countries like the United States of America the peak has already passed around 1970 [2]. Hubbert predicted the peak oil moment for the world to occur in 2000. This prediction did not come true since it did not account for the developments in recovery from existing fields and discoveries of new reserves, e.g. deep water and arctic oilfields. It also did not predict the use of unconventional sources such shale oil and gas, tar sand or extra heavy oils. The new extraction methods and sources pushes the time point of "peak oil" further forward in time and the debate is ongoing when this moment will occur. Figure 1 gives an overview of the peak oil moment using the proven oil reserves versus the expected discoveries.

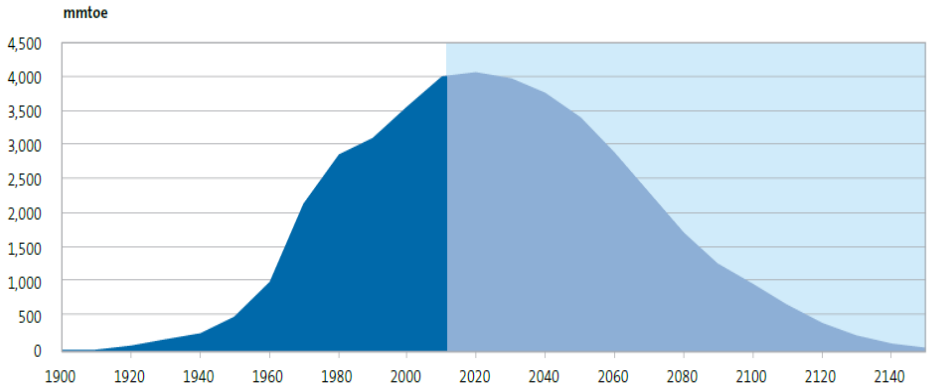

Figure 1. World “peak oil” moment in time and production volume in million tonnes of oil-equivalent. The dark blue area indicates our consumed oil reserves and the light blue indicate predicted oil reserves [3].

Besides the fact that fossil fuels will eventually run out for bulk use, there are many negative impacts on the environment. These include the pollution of the environment and global warming. It is generally considered that global warming is caused by the release of greenhouse gasses, such as CH_4, NO_x, water vapour and CO_2 into the atmosphere [4]. According to the Intergovernmental Panel on Climate Change the incineration of fossil fuel releases large amounts of anthropogenic CO_2 into the atmosphere [5]. Global warming will result in the rise of sea levels due to thermal expansion of water and melting of ice stored on landmasses. This can have considerable effects on the world population since 40% lives within 100 km of the coast [6]. In addition, it is expected that global weather patterns will change, which will have global effects. These threads have been recognized by the global community and measures to reduce global warming have been agreed upon as described in the Paris treaty. These measures include the use of geothermal, water, wind, and solar energy, as viable alternatives for the generation of heat and power. However, these resources do not form an alternative for the production of chemicals and materials since this requires (chemical) building blocks. Biomass can form a renewable and viable alternative since the building blocks are formed using energy from sunlight [7].

1.2. Biomass as resource for production of chemicals and fuels

In the past mankind used renewable energy resources such as wind and water for a wide range of applications e.g. sailing, water wheels and windmills. These resources are sometimes not readily available hampering robust operation. As an alternative biomass-derived energy in the form of firewood was used. Eventually, biomass-derived energy lost in favour over fossil fuels due to the higher availability and energy density of the latter in order to support an ever increasing population size. Due to negative properties of fossil fuels renewable resources such as biomass obtained a renewed interest in the last decades.

1.3. Food versus Fuel

Based on the method of biomass conversion a division can be made in two generations. In the first generation processes, carbohydrates, vegetable oils or protein are directly converted into the end product by biological or chemical conversion processes. An example is the production of bioethanol from corn starch in the United States of America. Since starch is produced in plants which are historically grown as dedicated food crops there is the risk of diverting farmland or crops to the production of fuels. The debate regarding the detriment of the food supply is called “food versus fuel” and should be taken into consideration when using food grade biomass if a shortage of food is the reason for hunger. In order to protect the food supply dedicated food and non-food crops can be co-cultured on the same land. Alternatively, the inedible parts of the food crops can be used as feedstock in so called second generation production processes.

In second generation processes the production potential of a land area is increased by using (specially grown) lignocellulosic biomass or agricultural residues.

1.4. Lignocellulose

Lignocellulosic biomass is rich in polysaccharides such as cellulose (35-50%) and hemicellulose (5-20%) in a matrix of lignin [8] (Figure 2). Lignin is comprised of cross-linked phenolic polymers and as a result is very resistant to degradation. Cellulose is comprised of β-1,4-D-glucose molecules in long linear chains linked to each other by hydrogen bonds to form a stable crystalline lattice [9]. Hemicellulose is more complex and is comprised of various sugars. It is classified according to the main sugar in the backbone of the polysaccharide. Various forms are: mannan (β-1,4-linked D-mannose), xyloglucan (β-1,4-linked D-glucose) and xylan (β-1,4-linked D-xylose). The backbone can be decorated with side groups such as D-galactose, D-xylose, L-arabinose, and D-glucuronic acid. In plant cell wall material xylan is generally the most abundant hemicellulosic polysaccharide. The precise composition of hemicellulose is dependent on the plant species and tissue and is extensively addressed in a review by Dodd and Cann [10].

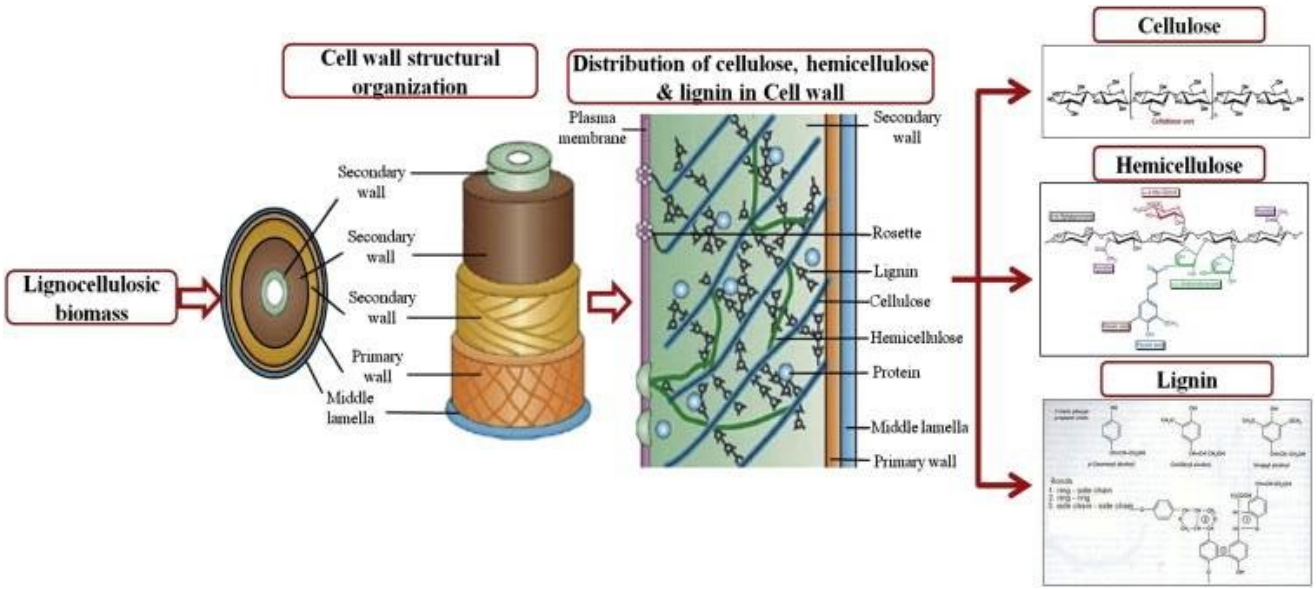

Figure 2. The organization and structure of plant biomass ranging from de cell wall to individual biomass constituents indicating the complexity of biomass [11].

Biomass in its pure form is complex and can in most instances not directly be used for the production of renewable compounds. Just like fossil fuels it should be processed or biorefined to obtain building blocks for chemicals and materials. In the biorefining process all the non-food fractions such as the oils, gums, carbohydrates, proteins, and lignin should be used for their most energetically favourable applications.

1.5. Microbial conversion of biomass into chemicals

Microorganisms convert biomass into a wide range of commercial compounds which cannot be cost effectively produced from chemicals. In general, these compounds follow the rule of low volume with high value or low value with large volume (Figure 3). High value products can be growth hormones or insulin produced by genetically engineered microorganisms. The global production volume is relatively small and measured in kilograms with a value as high as 60 euro per milligram [12]. The reactor volumes are small and the costs of the nutrients and utilities are a minor factor in the overall feasibility and profitability. On the other side of the spectrum are the low value and high volume compounds such as transportation fuel. For ethanol the average value is 0,42 euro per litre and feedstock price is important for a profitable process [13].

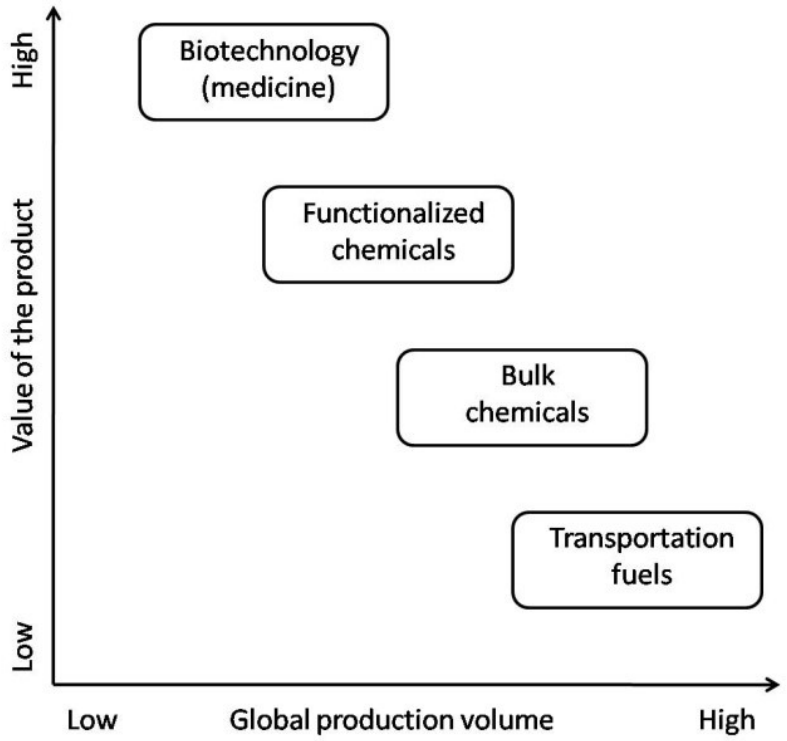

Figure 3. Relation between production volume and value of various products. Demonstrating that functionalized chemicals have reasonable high value and low volumes, adapted from Sanders and co-workers [14].

The U.S. department of Energy identified the top twelve building block chemicals that can be produced from carbohydrates via biological or chemical conversions. These primarily include 1,4-diacids (succinic, fumaric and malic) and mono-acids due to the reactivity of the carboxyl groups [15]. With regard to the feedstock a pure carbon source results in simpler production process compared to hydrolysates or untreated biomass.

However, the use of pure sugars as carbon source could significantly increase the production costs of the desired product. As a result, various cheap nutrient rich sugary and starchy materials are investigated such as molasses, corn starch, Jatropha seed cake and various flours [16]. Despite their low costs, these materials require pretreatment and hydrolysis using various chemical, acid, alkali or enzymatic methods in order to liberate the constituent monosaccharides [17]. These pretreatment methods result in additional costs and can produce toxic by-products which hamper the fermentation process. Ideally, the microorganisms should therefore be able to produce enzymes that hydrolyse both the cellulose and hemicellulose fraction and are able to utilize all constituent monosaccharides.

1.6. Conversion of protein into N-containing chemicals

Another important fraction of biomass is protein. Protein or the amino acids it consists of are especially interesting for the production of nitrogen containing chemicals. When nitrogen containing functionalized chemicals are produced from naphtha the end product has reduced energy content. The nitrogen it contains is derived from atmospheric nitrogen and this nitrogen fixation step is very energy intensive. In the biorefinery approach on the other hand, nitrogen containing amino acids can be converted to nitrogen containing chemicals, and the energy intensive nitrogen fixation is not required. This concept is depicted in Figure 4, the rise and fall of the dotted line representing an overall loss in energy [18]. The nitrogen containing chemicals that can be produced from an amino acid depend on the presence of carboxyl, hydroxyl, amine, indol, imidazole and phenyl groups.

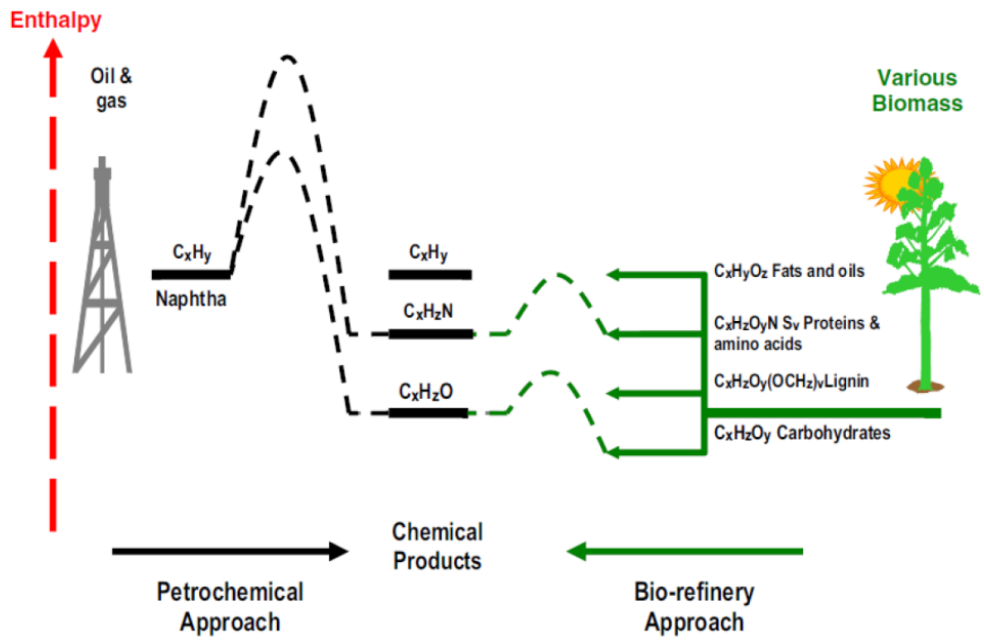

Figure 4. The biorefinery approach in comparison to the traditional petrochemical approach in respect to enthalpy indicating the larges loss in energy using the petrochemical approach [18].

Proof of this principle is given by the conversion of the amino acids glutamic acid and arginine to bulk chemicals [19, 20]. Additionally these processes can become economically interesting since 40% of the integral cost price of nitrogen containing chemicals from fossil resources is formed by the energy and process costs [18]. Using amino acids, the capital costs can also be significantly reduced.

Traditionally, commercially available amino acids are obtained through microbial fermentations. These production volumes are generally low and the price is too high for bulk use. Therefore, alternative sources need to be investigated which can supply large volumes of amino acids at low cost. Residual protein streams from food, agro and biofuel industry can form a suitable source of amino acids since the cost is low. The main waste streams of bio-ethanol and bio-diesel production is dried distillers grains and soluble (DDGS) (30% protein by dry weight) and oilseed cake (40% protein by dry weight), respectively [21]. The volume of waste streams from the biofuel industry are expected to increase since the goal is to replace 10% of our transportation fuels with biofuels by 2020. This results in an expected increase of

100 million tons of total protein, thus on average 5 million tons of each individual amino acid [18]. Some waste streams such as Protamylasse already contain free amino acids. Protamylasse is a waste stream from the potato starch industry and has an annual volume of 100.000 tons and contains high amounts of free amino acids [18].

1.7. Cyanophycin as intermediate for the production of nitrogen containing chemicals

A major hurdle in the biobased economy and the subsequent refinery processes is formed by the diluted feedstock. Biomass comprises of relatively small amounts of proteins and these proteins are build-up from various amino acids. Depending on the extraction or hydrolysis method the (proteinogenic) amino acids can be separated using various methods e.g. ion-exchange chromatography, electrodialysis or reactive extraction. Additionally, amino acids can be extracted from a feed stream by the production of non-ribosomal peptides. This offers an advantage in the downstream processing since only the incorporated amino acids are present in the end product. Ideally, the concentrated fractions contain as little compounds as possible. An example is the production of cyanophycin in transgenic microorganisms using Protamylasse as substrate [22]. Non-ribosomal proteins have an additional advantage in comparison to ribosomal proteins: every elongation of the protein chain with one amino acid only requires the input of 1 ATP, instead of 7 ATPs. Consequently, production of non-ribosomal proteins is energetically more efficient.

Cyanophycin or cyanophycin granule peptide (CGP) consists of L-arginine and L-aspartic acid and is naturally produced in all groups of the cyanobacteria [23]. It consists of an α-amino-α-carboxy linked aspartic acid backbone with L-arginine side change linked to the β-carboxyl group of each aspartic acid (Figure 5) [24]. In the bacteria it serves as a temporary nitrogen- and carbon-storage compound. CGP is a polypeptide that results from non-ribosomal protein synthesis by cyanophycin synthetase [25].

As a result less energy is required for the synthesis in comparison to ribosomal synthetized proteins thereby reducing the metabolic burden on the cell.

Generally the molar ratio of the two amino acids is 1:1, although variants are known that have different ratios as a result from amino acid substitution of the arginine [26]. Since the synthesis is non-ribosomal the size distribution varies. In addition, the molecular mass depends on the CGP producing organism [27]; in cyanobacteria the molecular mass ranges from 25-100 kDa [28-31]. At neutral pH and under physiological ionic strength CGP is in general water-insoluble, although CGP forms are described that are water-soluble at neutral pH [32]. The water-insoluble CGP can become water-soluble at extreme pH values (below 3 or above 9) or under high ionic strength [30, 33]. The insolubility, composition and low energy requiment to synthesize make the formation and subsequent isolation of CGP a suitable method to selectively isolate L-aspartic acid and L-arginine from diluted waste streams [34].

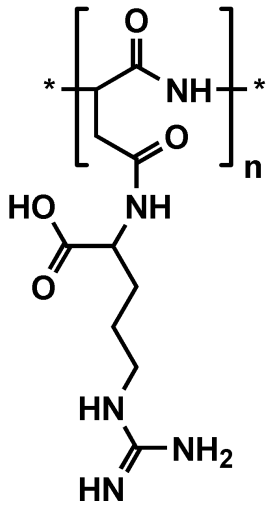

Figure 5. Schematic representation of a CGP molecule with an L-aspartic acid backbone and L-arginine side chains [24].

1.8. The filamentous fungus *Rhizopus oryzae*

Compared to bacteria, yeasts, and other moulds, filamentous fungi stand out with a larger repertoire of products such as enzymes, organic acids, alcohols, antibiotics and bioactive compounds with numerous high-value biotechnological applications.

In most cases microorganisms display undesired traits for a production process e.g. the carbon flow is not fully directed towards the formation of the desired product, the morphology is unfavourable or completely new pathways should be introduced. It is therefore desired that the producing organism is genetically accessible in order reduce the undesired traits.

The filamentous fungus *R. oryzae* (and close relatives *R. arrhizus* and *R. delemar*) is a ubiquitous filamentous fungus found on decaying organic material. It is classified in the order of Mucorales in the phylum Zygomycota. The Zygomycota are considered a primitive and early diverging group of fungi which are quite distinct from the Ascomycota and Basidiomycota. Zygomycota are characterized by the formation of haploid coenocytic mycelia, meaning that multiple nuclei are present in the hyphae and septa are lacking. Classically, *R. oryzae* strains are used in Southeast Asia, China and Japan in the production of alcoholic beverages, ragi and tempeh [35]. *R. oryzae* is considered GRAS (Generally Recognized As Safe) by the U.S Food and Drug Administration (FDA) and thus, can be used for human consumption within the U.S. Nowadays, fermented food products are a new trend since they are believed to be more nutritious and result in various positive health effects. Fermentation with *R. oryzae* can be used to alter flavours, tastes and generate a broad number of new textures [36, 37]. Cantabrana and co-workers presented a number of workflow diagrams to produce fermented food products using *R. oryzae* under household kitchen conditions.

Typical for *Rhizopus* species morphology are the rhizoids, a root like tissue, attached to the stolon. Opposite of the rhizoids on the stolon are the sporangiophores which have a length up to 1500 µm and a width of 18 µm.

Sporangia are globular in shape containing the rough, ellipsoidal to subglobular sporangiophores (Figure 6). On minimal medium containing D-glucose colonies are fast growing at 30 °C. Upon a temperature increase to 40°C the fastidious growth is still observed yet an increase in temperature to 45°C eliminates growth. In addition, Zygomycetes have a different cell wall composition from most other fungi. The characteristic difference is formed by the presence of the structural polysaccharide chitosan which is the deacytylated homopolymer of the more common chitin.

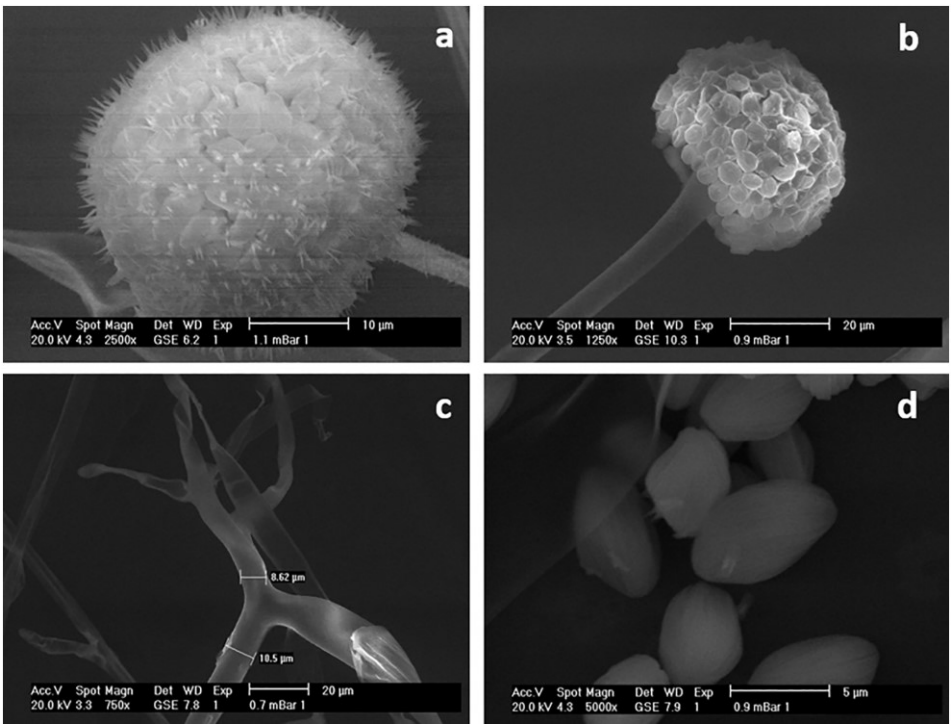

Figure 6. Low Vacuum SEM Photomicrographs of *R. oryzae* (MUCL 28168): (a and b) sporangium, (c) hyphae, and (d) sporangiospores [38].

R. oryzae strains are capable of producing ethanol, fumaric and L-lactic acid from various carbon sources. In the central carbon metabolism all fermentable substrates are first metabolized into pyruvate. Under (micro) anaerobic conditions the pyruvate pool is channelled mostly to the formation of ethanol whereas under aerobic conditions the organic acids are formed [39]. During (micro) anaerobic fermentation of D-glucose ethanol is formed with a yield close to the theoretical yield [40, 41]. The current benchmark microorganism for ethanol production on D-glucose

is *S. cerevisiae*. The main disadvantage of wild type *S. cerevisiae* for ethanol production is the inability to utilize pentose sugars, which are present in hemicellulosic hydrolysates. Using *R. oryzae* with optimal conditions the maximal yields for L-(+)-lactic and fumaric acid are in excess of 85% and 65% of the theoretical yields, respectively [42, 43]. *R. oryzae* can efficiently produce the various fermentation products and it is presumed that the production of organic acids is an overflow mechanism resulting from unnatural high sugar concentrations in the environment [44]. The ethanol fermentative pathway is presumed to be present to allow growth for short periods of time in the absence of oxygen [45, 46]. The availability of oxygen is the key for the flow of pyruvate and thus the product formation. The formation of multiple products is therefore common if the oxygen availability or the mycelium formation is not closely controlled.

The species *R. oryzae* are classically identified by sporangiophore morphology and growth temperature. Based on the production of lactic or fumaric acid and phylogenetic analysis it was proposed to reclassify the fumaric acid producers to *R. delemar* [47]. Since this reclassification is not universally adopted both species will still be addressed as *R. oryzae* in this thesis.

The mycelium can have various forms in submerged culture but three main variants can be found; tight pellets, flocks and filaments. The optimal morphological form is small pellets with a diameter of less than one millimetre. This is presumably due to limitations in mass transfer regarding oxygen, substrate and product [48]. In addition, the viscosity increases when flocks or filaments are present. A rise in viscosity results in a reduction in oxygen transfer into the medium. This in turn led to an increased production of ethanol. Furthermore, the pH of the fermentation broth should be controlled. Preferably, $CaCO_3$ has been used as a neutralizing agent. $CaCO_3$ and Ca salts have a low solubility facilitating the recovery of products. Although it should be considered that undissolved particles in the fermentation broth are generally encased in the biomass leading to large lumps or pellets. Next to the shifts in product formation due to low dissolved oxygen tensions the medium composition itself influences the biomass formation and thereby the efficiency of the fermentation.

The formation of mycelium can be reduced by limiting the nitrogen content. In a review by Roa Engel and co-workers various methods to increase the formation of fumaric acid are discussed [49]. Some of these methods can also be used to increase the production of lactic acid since both products are produced under aerobic conditions.

Earlier it was described that microorganisms capable of simultaneous saccharification and fermentation are favoured. *R. oryzae* strains are capable of a wide range of enzymatic activities such as amylolytic [50], xylanolytic [51], pectinolytic [52] and cellulolytic [53-55] capabilities, thereby enabling the conversion of polymeric agricultural residues to monomeric carbohydrates. It should be noted that the enzymatic capacities varies strongly between strains. Currently, enzymes from *R. oryzae* are already used in various industrial processes demonstrating that this organism is a suitable organism for production of cyanophycin using agro-waste streams. An example of the enzymatic capacities are formed by the starch degrading enzymes which are currently used in the starch industry due to the high activity and stability [56]. Besides ethanol biodiesel is also used as transportation fuel. Biodiesel is produced by the esterification of plant or animal oils using methanol and a base catalyst with a yield of 90%. Compared to the enzymatic conversion with lipases the chemical reaction can easily be influenced by the presence of free fatty acids and other contaminants [57]. Non-edible vegetable oils contain high levels of free fatty acids and esterification using lipases forms an interesting alternative to the current chemical conversion. The lipases of *Rhizopus* species are versatile enzymes that can be used in fat and oil modification due to their strong 1,3-regiospecificity. Reactions with immobilized *R. oryzae* lipase and mixtures with *Candida rugosa* resulted in reactions with a yield of 90% and >99% [58, 59]. Additionally, these reactions require less process steps resulting in a drastically reduction in wastewater and improve product separation [60]. An overview of possible enzyme that can be produced with *R. oryzae* and substrates are provided in a review by Gosh and Ray [37] and Battaglia and co-workers [61].

Transformation and subsequent expression of new genes in *R. oryzae* is problematic. Integration of plasmid DNA into the genome is very rare. The plasmids

reproduce extrachromosomally in high molecular weight concatenated structures [62]. As a result, the transformants usually display a mitotically unstable phenotype. Currently, there are three transformation systems which were used for the expression of genes or show promise to increase the integration into the genome. The system developed by Skory and Ibrahim [39] to increase fumaric [63] and lactic acid production [39]. The system by Yuzbashev and co-workers is based on a similar plasmid and transformation method but contained various genomic repeats which facilitated integration into the genome [64]. The plasmid containing a genomic repeating sequence named H (rptH) resulted in integration into the genome in multicopy fashion with up to 25 copies arranged in tandem. Yet this system was not used for the expression of additional genes. The last system was based on electroporation of germinated spores [65]. With this system EGFP was expressed, according to the authors this method had high efficiency, short handling time, and reliable reproducibility.

1.9. The N-ergy project funded by SenterNovem

The production of bioethanol from waste streams coupled to the concentration and subsequent conversion of amino acids culminated into the N-ergy project (Figure 7). This project was supported by a grant (EOSLT02034) provided by SenterNovem (Utrecht, the Netherlands). The group at Fungal Genomics (Wageningen UR) was working on the production of cyanophycin using *R. oryzae*. In Germany, the Institute for Molecular Microbiology and Biotechnology of the Westphalian Wilhelms University in Münster was involved, working on the production of this polymer in *S. cerevisiae*. The other two partners were working on converting cyanophycin into nitrogen-rich bulk chemicals. These partners are the Valorisation of Plant Production Chains chair group and the Organic Chemistry Laboratory at Wageningen UR. The Netherlands Energy Research Centre (ECN) was helping to shape the industrial process, and Easthouse Business Solutions BV was marketing the process. The Agrotechnology & Food Sciences group was involved in testing the possibility of converting residual agricultural flows into cyanophycin and ethanol.

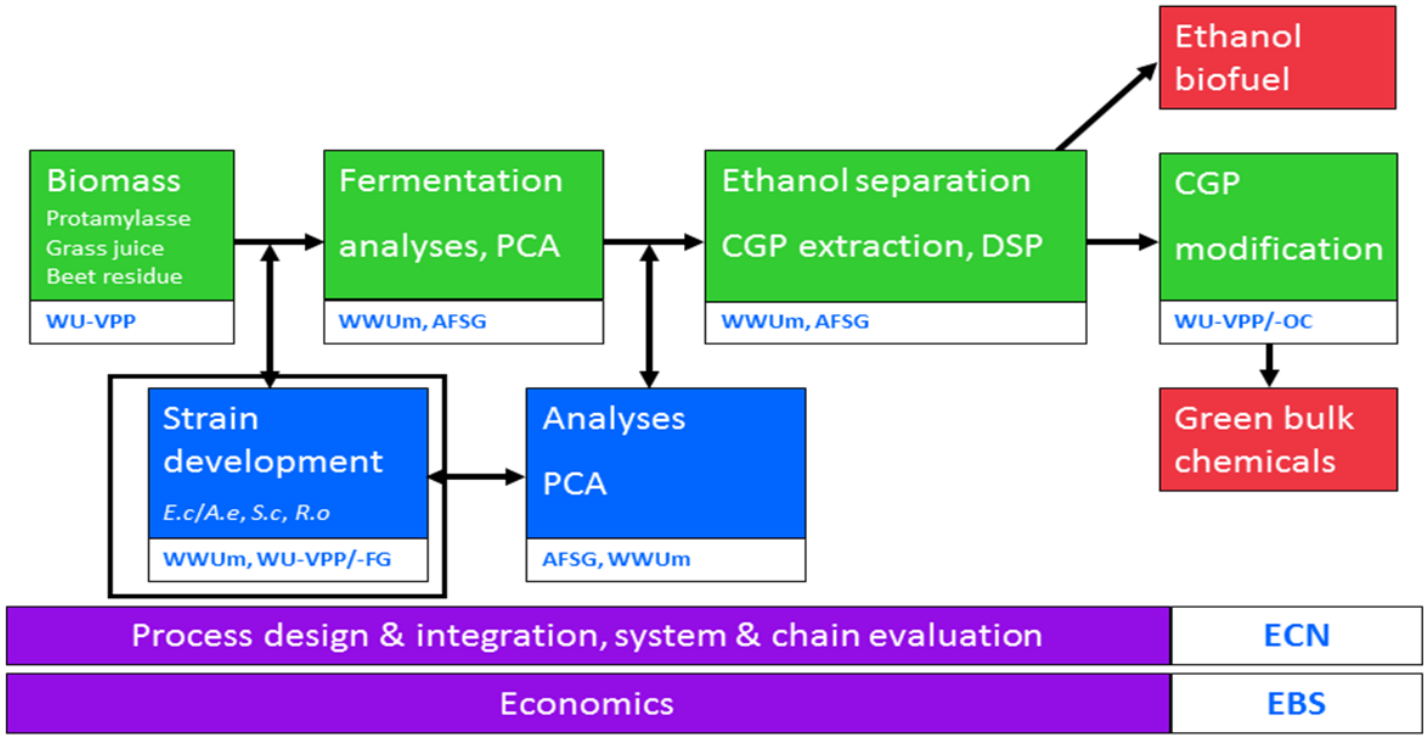

Figure 7. Organizational chart of the N-ergy project.

WU-VPP: Wageningen University Valorisation of Plant Production Chains, FG; Fungal Genomics, OC Organic Chemistry AFSG;Agrotechnology and Food Science Group, Westfälische Wilhelms-Universität Münster, ECN; Energy Research Centre, EBS; Easthouse Business Solutions.

1.10. Outline of this thesis

The research described in this thesis is part of the N-ergy project funded by SenterNovem, which on behalf of the Dutch Ministry of Economic Affairs supported us with a grant of the Program Energie Onderzoeksstrategie Lange Termijn, project EOSLT02034. The aim of the research described in this thesis was to confirm the suitability of *R. oryzae* as a cell factory for the production of platform chemicals from lignocellulosic side streams, and to develop genetic tools to enable cyanophycin production by this microorganism and enhance its saccharolytic potential.

Chapter 2 is a literature study on the applications of *R. oryzae* as a production organism for platform chemicals with an emphasis on the molecular biology and genetic modification. The common platform chemicals that can be produced with *R. oryzae* are ethanol, L-(+)-lactic acid, and fumaric acid. Currently, ethanol is used in bulk as a fuel additive to gasoline. The organic acids are of interest due to the functional groups which make them suitable for the production of renewable chemicals. *R. oryzae* strains also produce a wide range of hydrolysing enzymes making this organism interesting for simultaneous saccharification and fermentation processes. At the start of this project no functional transformation systems were available.

Chapter 3 describes the formation of transformants of *R. oryzae* 99-880 in order to produce cyanophycin. To this end, cyanophycin synthetase genes from *Synechocystis* sp. strain PCC6803, *Anabaena* sp. strain PCC7120, and a codon optimized version of latter gene were introduced. The generated transformants were screened for the expression the cyanophycin synthetase genes using qPCR, specific enzyme activity using scintillation assay and the accumulation of cyanophycin.

The conventional analysis methods of proteinogenic amino acids are costly and time consuming. **Chapter 4** state the results of the development of a fast and low cost UPLC method to determine the proteinogenic amino acid composition based on acid hydrolysis and pre-column derivatisation using o-phthalaldehyde/ethanethiol reagent in combination with 9-fluorenylmethyl chloroformate.

R. oryzae strain 99-880 produces various organic acids and ethanol but few hydrolysing enzymes. **Chapter 5** presents the results of the introduction of a xylanase encoding gene of *Aspergillus niger* in order to increase the hydrolytic capacities towards hemicellulosic biomass.

Lastly, **chapter 6** is a general discussion chapter which will summarize the findings of the previous chapters, and contemplate the use of *R. oryzae* as an organism in the biobased economy. In addition it summarises the general conclusions from this thesis.

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Chapter 2.
Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals

2.1. Abstract

Rhizopus oryzae is a filamentous fungus belonging to the Zygomycetes. It is among others, known for its ability to produce the sustainable platform chemicals L-(+)-lactic acid, fumaric acid and ethanol. During glycolysis all fermentable carbon sources are metabolized to pyruvate and subsequently distributed over the pathways leading to the formation of these products. These platform chemicals are produced in high yields on a wide range of carbon sources. The yields are in excess of 85% of the theoretical yield for L-(+)-lactic acid and ethanol and over 65% for fumaric acid. The study and optimization of the metabolic pathways involved in the production of these compounds requires well-developed metabolic engineering tools and knowledge of the genetic makeup of this organism. This review focuses on the current metabolic engineering techniques available for *R. oryzae* and their application on the metabolic pathways of the main fermentation products.

2.2. Introduction

Fossil resources such as coal, oil and gas are estimated to become exhausted or unattractive to mine for bulk use within the coming decades. Added to this foreseen unavailability are the current ever-increasing costs, geo-political instability of oil-producing regions and sustainability issues. This formed a strong incentive for the development of alternative sources of feedstock to replace fossil resources. For energy generation alternatives are already available in the form of geothermal, water, wind, solar and nuclear energy. For the generation of transportation fuels and platform chemicals the only feasible alternatives are biomass-derived products [1]. Biomass can be converted into fuels and platform chemicals using fermentation processes with the aid of microorganisms. To be able to compete with processes based on a petrochemical feedstock, these microorganisms should exhibit high product yields (g/g), productivities (g/l/h) and product titers (g/l). Other prerequisites are the ability to use many carbon sources, resistance against fermentation inhibitors that are released during biomass pretreatment, the ability to grow in absence of complex growth factors, etc.

R. oryzae is a fungus able to produce ethanol, L-(+)-lactic and fumaric acid in high quantities using sugars derived from biomass [2-5]. The market for these fermentation products is large, indicating the potential of this microorganism for the production of platform chemicals. In 2000 the global production of ethanol was 17.25 billion liters [6] and increased to a volume over 74 billion liters in 2009 [7]. This market is expected to increase and exceed 125 billion liters by 2020 [8]. The global market for L-(+)-lactic acid in 2000 exceeded 100,000 tons [9] and is expected to increase to 259,000 tons in 2012 [10]. L-(+)-lactic acid is currently primarily used as food or feed acidulant, but it can also be used in the fast expanding market of renewable plastics, solvents or oxygenated chemicals [11, 12] and animal feed. The market for fumaric acid is smaller but still considerable with an annual estimated volume of 90,000 tons in 2007 [13]. This market is also expected to increase in the coming years. Fumaric acid is currently used in the food industry directly as a pH adjuster, preservative or flavor enhancer. Due to its structure it can be used for the production of polyester and alkyd resins [14]. In addition to these platform chemicals *R. oryzae* is also used for the production of a wide range of commercially relevant

enzymes. The application of *R. oryzae* in biotechnological processes has recently been reviewed by Ghosh and Ray [15].

In order to optimize product formation in *R. oryzae*, metabolic engineering techniques should be available, enabling the production of chemicals by the introduction of heterologous genes, overexpression of homologous genes or knocking out existing competing pathways. This review describes the current state-of-art molecular techniques available for pathway engineering in *R. oryzae* and how they have been employed to study and enhance the production of its main fermentation products.

2.3. The organism *Rhizopus oryzae*

R. oryzae is a filamentous fungus that is classified in the order of Mucorales in the phylum Zygomycota. The genus *Rhizopus* was first established in 1820 by the description of *R. nigricans* [16] and is known for the formation of fermentation products like ethanol, L-(+)-lactic acid, fumaric and to a lesser extent L-(+)-malic acid. The ability to produce fumaric acid is what sets this genus apart from *Aspergillus*, *Fusarium* and *Penicillium* [17]. *R. oryzae* strains are often used in Asia for food fermentation to manufacture alcoholic beverages, ragi or tempeh and the strains are generally regarded as safe. Nevertheless, *R. oryzae* is also known as an opportunistic human pathogen and has a high prevalence under mucormycosis infections [18]. Most mucormycosis cases have an underlying illness such as an elevated serum iron level, trauma or a weakened immune system [19].

R. oryzae is ubiquitous in nature and found on decaying organic material. It is able to grow on a wide range of carbon sources e.g. glycerol, ethanol, lactic acid, glucose, mannose, fructose, sucrose, xylose, cellobiose, fatty acids and oils [20-24]. All mentioned sugars have been shown to be a substrate for L-(+)-lactic or fumaric acid production. Moreover, *R. oryzae* has amylolytic [25], xylanolytic [26], pectinolytic [27] and cellulolytic [28-30] capabilities, enabling the conversion of polymeric agricultural residues. It is able to grow well at a wide temperature range (up to 40 °C) and pH range (from 4 to 9), indicating a robust behavior and widely applicable potential.

2.4. Production of chemicals by *Rhizopus oryzae*

The first references on the ability of *Rhizopus* species to produce organic acids appeared in 1911: Saito described lactic acid production by *R. chinensis* [31] and Ehrlich reported the production of primarily fumaric acid, together with lactic acid, succinic acid and malic acid by *R. nigricans* species [32]. Studies by Takahashi and co-workers [33] indicated that also other products were formed, including ethanol. Ward and co-workers [34] described the production of L-(+)-lactic acid from glucose by *R. oryzae* strains, culminating to a yield of 0.62 g/g. Since then *R. oryzae* has been extensively studied for the production of organic acids, ethanol, enzymes and other commercially interesting products. These studies have been reviewed recently by Ghosh and Ray [15].

As mentioned before *R. oryzae* can efficiently produce the fermentation end products. The maximal theoretical yield for L-(+)-lactic acid production by aerobic respiration is 2 mol of L-(+)-lactic acid per mol of D-glucose, this equals 1.0 g L-(+)-lactic acid per gram D-glucose. For fumaric acid the maximal theoretical yield is 2 mol fumaric acid per mol D-glucose consumed, which correlates to 1.3 g fumaric acid per g D-glucose. The highest reported yield for L-(+)-lactic acid was 0.88 g/g, and 0.86 g/g for fumaric acid with ethanol as main byproduct [35, 36] (Table 1). These results also demonstrate the ability of *R. oryzae* to withstand high product and D-glucose concentrations in excess of 100 g/L. The theoretical yield is two moles of ethanol per mole of D-glucose (0.51 g/g D-glucose). When grown on D-glucose the yields obtained with *R. oryzae* are close to this maximal yield. Table.1 lists some experimental data on the production of ethanol by *R. oryzae.* The current benchmark microorganism for ethanol production on D-glucose is *S. cerevisiae*. The main disadvantage of wild type *S. cerevisiae* for ethanol production is the inability to utilize pentose sugars, which are present in hemicelluloses hydrolysates. *R. oryzae* can grow on many carbon sources including C5 sugars and has low growth requirements. Furthermore, it is able to tolerate the inhibitors present in acid hydrolysates of lignocellulosic biomass [37, 38], and is able to –although at slow rate–utilize cellulose and hemicellulose directly [39].

Table 1. Literature data on the main fermentation end product production by *R. oryzae* strains.

Product	*R. oryzae* strain	Reactor type	Sugar consumed (g/L)	Final product titer (g/L)	Yield (g/g)	Reference
L-(+)-lactic acid	NRRL 395	ALB	120	105	0.87	[93]
	ATCC 52311	ALB	94	83	0.88	[36]
	GY 18	SF	160	115	0.81	[4]
Fumaric acid	*R. arrhizus* 2582	STR	130	103	0.79	[94]
	ATCC 20344	RBC	108	93	0.86	[35]
	R. arrhizus NRRL1526	SF	120	98	0.82	[95]
Ethanol	CCUG 28958	SF	50	21	0.42	[38]
	CCUG 22420	SF	50	22	0.44	[38]
	CCUG 18663	SF	50	19	0.38	[38]
	NRRL1501	SF	50	25	0.50	[39]
	NRRL2625	SF	50	25	0.50	[39]
	NRRL395	SF	50	19	0.38	[39]

The *R. arrhizus* strains are currently classified as *R. oryzae* strains. ALB = air lift bioreactor, SF = shake flask, STR = stirred tank reactor, RBC = rotary bed contactor

2.5. Genetic diversification of *Rhizopus oryzae* strains

R. oryzae strains can be divided into two types based on the primary organic acid produced when grown on D-glucose [40]. One group produces primarily L-(+)-lactic acid while fumaric and L-(+)-malic acid are the main fermentation products of the other group. To obtain information on this division an analysis of the lactate dehydrogenase (LDH) encoding genes and proteins was performed. It was determined that *R. oryzae* NRRL 395 has two NAD-dependent isoenzymes (LDHA and LDHB) [41].

The LDHA-encoding gene was expressed during growth in the presence of fermentable carbon sources such as D-glucose, D-xylose or trehalose. In contrast, the LDHB-encoding gene was expressed on non-fermentable carbon sources such as ethanol, glycerol and lactate [41]. A relationship between the L-(+)-lactic acid production and the LDH encoding-genes was found by Saito and co-workers [42]. Strains that produced L-(+)-lactic acid contained both *ldhA* and *ldhB* and were classified as type I strains. The fumaric and L-(+)-malic acid producing strains contain only *ldhB* and were classified as type II strains. After sequence analysis of the various genes and markers from the two different types it was determined that they were phylogenetically distinct. On the basis of these results it was proposed to reclassify the strains which produce predominantly fumaric and L-(+)-malic acid as *R. delemar* [43], since this was the first name given to a *Rhizopus* strain belonging to the type II strains [44]. The proposed reclassification of *R. delemar* for type II strains is not widely used in literature. As a result of this the *R. delemar* strains will still be addressed as *R. oryzae* in the review.

2.6. Genome analysis

In 2004 the genome of *R. oryzae* strain 99-880 (a type II strain) was published. This formed a great contribution to this research field and gave new insights for molecular techniques. This strain was the first organism to be sequenced in the polyphyletic basal lineage described as the Zygomycetes. It has an unusual high degree of gene duplication, which was analyzed by Ma and co-workers [45]. The genome of *R. oryzae* 99-880 is 45.3 Mbp in size and 20% comprises of transposable elements. In total 13,895 protein coding genes were predicted which did not overlap with transposable elements. After analysis of the duplicated gene pairs and their common phylogenetic order, the conclusion was drawn that an ancestral whole-genome duplication event occurred. This event - in combination with recent gene duplications - resulted in a two to tenfold increase in gene families related to pathogen virulence, fungal-specific cell wall synthesis and signal transduction. This whole-genome duplication allows for growth under a wide range of adverse conditions. This can include host immune defense response and can explain the high prevalence of *R. oryzae* strains in mucormycosis infections [18]. As a result of

the whole-genome duplication considerable difficulties are encountered in pathway modifications by gene knockout or silencing strategies.

2.7. Transformation of fungi belonging to the Mucorales

Currently, there are several transformation systems developed for organisms in the order of Mucorales. These include: *Absidia glauca* [46], *Mucor circinelloides* [47], *M. miehei* [48], *Parasitella simplex* [49], *Phycomyces blakesleeanus* [50], *R. niveus* [51] and *Rhizomucor pusillu*s [52]. In general, the main bottleneck in heterologous gene expression for Mucorales is formed by the recombination and replication mechanisms affecting the introduced DNA. The DNA introduced by transformation will remain extra chromosomal and replicate autonomously since it does not require a defined origin of replication [50, 53]. As a result, transformants usually display a mitotically unstable phenotype. In addition to autonomous replication, these plasmids will form high molecular weight concatenated structures. These structures will co-migrate with genomic DNA, resulting in incorrect conclusions regarding integration [54, 55]. To increase the likelihood of integration in *R. oryzae*, a double-strand break (DSB) was introduced in the homologous region of the plasmid used for the transformation. This increased occurrence of integration to 20% with the remainder of the transformants still containing concatenated plasmids [55]. It was hypothesized that the concatenated plasmids were a result of the re-ligation prior to integration into the genome. This re-ligation can be the result of repair mechanisms known as non-homologous end joining (NHEJ) [56]. For the NHEJ to occur only a few homologous base pairs are required in the break. When the homology on both ends is larger, single strand annealing (SSA) is the dominant method of repair. For in-depth review on DNA repair we advise the review of Pardo and co-workers [57]. Selection against NHEJ has been achieved by a frame-shift mutation in the selection marker on the vector, combined with a recipient strain that already contains a mutation in the genomic selection marker [58]. Growth can only be restored when the plasmid integrates by a single homologous crossover into the genome, which results in a functional selection marker and a copy containing both mutations.

After transformation with this vector only 8% of the transformants tested displayed the prototrophic phenotype. The remainder of the transformants still contained concatenated plasmids [58]. When the sequences of the genes involved were analyzed, it was determined that the non-functional copy on the vector was repaired instead of the genomic copy. It was hypothesized that this was due to non-crossover mechanisms like break-induce replication or by synthesis-dependent strand annealing. Another study with the goal to increase the likelihood of double strand break repair examined the effect of the DNA break on NHEJ [59]. In this experiment, a vector digested at a single site with various overhangs was added to cell free extract. After 30 minutes of incubation dimers, trimers and degradation products of the vector were observed. An extension of the incubation time to 1.5 hours resulted in a reduction of dimers and trimers and the appearance of high molecular weight structures. Upon transformation of spores with vectors containing either a 5' or 3' overhang no difference was observed in the restoration of prototrophic growth. In the same study a vector was designed which selected for integration into the genome. This vector contained a truncated *pyrG* selection marker containing the 5' half of the gene. The recipient strain already contained a point mutation in the 3' half of the genomic copy of the selection marker. Viable transformants could only be obtained when integration into the genome occurred. All of the transformants generated with the truncated selection marker that were tested contained integrated vectors, and multicopy inserts were frequently found. The efficiency of transformation with the truncated vector was 20 fold lower in comparison to a non-truncated vector, this was likely caused by the selection for a single integration event [59].

2.8. Metabolic engineering tools for *Rhizopus oryzae*

Several methods have been applied to alter the genome of *R. oryzae* and its transcription. These methods were based on the introduction of foreign DNA or random mutagenesis.

2.8.1. Random mutagenesis of Rhizopus oryzae

Random mutagenesis is a powerful tool to disrupt gene functionality or to increase the productivity of metabolic processes. This has been accomplished in

R. oryzae by chemical mutagenesis, for example with N-methyl-N'-nitro-N-nitrosoguanidine to generate auxotrophic mutants [60] or with diethyl sulfate to increase L-(+)-lactic acid production [61]. Random mutagenesis has also been performed by radiation with UV light, gamma radiation with ^{60}Co [61] or by low energy ion implantation [62]. The downside of this technique is formed by the risk of generating multiple mutations. Also considerable amount of time is required for screening and subsequent selection rounds. To screen the mutants for desired traits, efficient screening methods are required, an example of such a method is the screening method developed by Huang and co-workers to screen for mutants with a higher acid production [63].In this study *R. oryzae* spores were mutagenized with UV radiation and an increase in the acidification was screened on agar plates by the color change of a pH indicator.

2.8.2. Transformation with heterologous DNA

Next to the previously described random mutagenesis another method to alter the genome is formed by the introduction of heterologous DNA. In recent years the knowledge regarding (heterologous) gene expression in *R. oryzae* increased tremendously and 3 transformation systems were described. One of the systems was based on DNA transfer by the microorganism *Agrobacterium tumefaciens* [64]. *A. tumefaciens* has the ability to transfer a part of its DNA called transfer DNA (T-DNA) to a broad range of hosts. For heterologous expression this T-DNA is altered to contain the gene of interest. Upon transformation of biomass this T-DNA integrates into the chromosome. In a proof of principle study for genetic modification by Michielse and co-workers, an auxotrophic strain of *R. oryzae* (COM1291) was used as target organism [64]. The *R. oryzae* strain contained a mutation in orotidine-5'-monophosphate decarboxylase (*pyrG*) gene which was complemented by the introduction of the T-DNA. Transformants could be generated using protoplasts as starting material but not with spores or germlings. In total eight transformants were isolated that were all mitotically stable under non-selective conditions. After further analysis it was concluded that in two transformants a gene conversion occurred. This conversion resulted in the restoration of the function of the genomic *pyrG* copy without the introduction of additional DNA. In the remaining six transformants an extra *pyrG* copy integrated in the genome.

Interestingly, the DNA introduced in the six transformants was integrated at the same locus indicative for a hotspot for integration. No vector DNA outside the T-DNA borders or of the second gene inside these borders was detected in the genome. With this system it was not possible to express a gene of interest and therefore it is not suitable for heterologous gene expression although integration into the genome was achieved. It was hypothesized that the integration is a result of the virulence factor proteins that coat the DNA and protect it from modification events.

In the same study describing the *Agrobacterium* system a second method to transfer DNA was described. For this method protoplasts were generated from mycelium and transformed with vector DNA by the $CaCl_2$/PEG method [64]. The vector DNA used in this study was circular or linear in order to determine if linear material increased the likelihood of integration. In contrast to the *Agrobacterium* system none of the generated transformants had stable phenotypes and the vector DNA remained extra-chromosomal and replicated autonomous.

The third system to introduce DNA is formed by a particle bombardment DNA delivery system. The particle bombardment system was first developed to introduce DNA in plant cells [65]. It is currently the only system successfully used for heterologous gene expression in *R. oryzae*. With this system spores are transformed using tungsten particles coated with vector DNA. For *R. oryzae* it was first used to determine the fate of introduced DNA in a uracil auxotrophic strain derived from *R. oryzae* NRRL 395 [55]. The uracil auxotrophy was complemented by the introduction of a vector with the functional *pyrG* copy from the same strain. Over the years this system was further improved to express homologous genes such as *ldhA* [66]. This was followed by the expression of the green fluorescence protein (GFP) as proof of principle for heterologous gene expression [67]. In 2007 a uracil auxotrophic strain derived from *R. oryzae* 99-880, a type II strain, was transformed with the *ldhA* gene from a type I strain [68]. Functional heterologous gene expression was achieved in this strain with a cyanophycin synthetase-encoding gene (*cphA*) from a cyanobacterium with the goal to produce cyanophycin in *R. oryzae* [69]. Cyanophycin has a unique structure and can be used for the production

of green-chemicals. Also a xylanase-encoding gene (*xynB*) from *Aspergillus niger* was expressed in *R. oryzae* (Meussen et al. in preparation).

The vectors developed for the GFP expression contained three different promoter elements originating from the phosphoglycerate kinase 1 (*pgk1*), pyruvate decarboxylase A (*pdcA*) or glucoamylase A (*amyA*). Of these promoter elements the *pdcA* promoter gave the strongest signal [67] and was selected as the promoter element for all the expression constructs described up to date.

Upon analysis of the GFP-expressing transformants it was discovered when the fluorescent signal was present in a hyphen it was not localized in a particular organelle or at the hyphal tip but evenly distributed. After transcript analysis in these transformants it was concluded that there was a clear correlation between transcript level and GFP accumulation. Interestingly, the copy number of the genes did not significantly influence the accumulation of protein [67].

2.8.3. Gene knockout

Next to introducing genes for the production of new enzymes, DNA has also been introduced to create gene knockouts. With a double cross-over event genes are knocked out and as a result the specific loss of gene function or its effect on metabolic pathway can be studied. Currently, the only example of a successful double cross-over event described in detail for *R. oryzae* was for a high-affinity iron permease-encoding gene (*ftr1*). This gene is strongly expressed in *R. oryzae* during a host infection, suggesting a role of FTR1 in the pathogenicity of *R. oryzae*. To investigate the role of FTR1, a double cross-over homologous recombination gene knockout was successfully generated in an auxotrophic mutant derived from *R. oryzae* 99-880 [70]. In the generated transformants the FTR1-encoding gene was not detected under non-selective conditions. Yet, under selective pressure the phenotypic effect in putative *ftr1* null mutants was lost and the FTR1-encoding gene was once again detected. Under the selective condition the polynucleated nature of *R. oryzae* overcame the *ftr1* null mutant's phenotype. After 14 consecutive sporulation events and single colony inoculations under non-selective condition the gene was not detected by PCR analysis.

Nevertheless, within 48 hours of growth in selective medium the gene was again amplified by PCR [70]. It was speculated that the *ftr1*-encoding gene might be essential for the organism in iron depleted media and therefore it was impossible to obtain a null mutant.

In addition, in this paper it was claimed that it was possible to obtain an imidazoleglycerol-phosphate dehydratase (*his3*) null mutant in this strain although these data were not published.

2.8.4. RNA interference

Another elegant method to down regulate the expression of genes is formed by RNA interference (RNAi). RNAi is a recently discovered mechanism where double stranded RNA triggers the degradation of a homologous sequence of messenger RNA (mRNA). As a result, translation of the corresponding protein is diminished or abolished [71]. For the RNAi machinery several proteins are required: a dicer, the Argonaut and RNA-dependant RNA polymerase (RdRP). The dicer cuts dsRNA to double stranded short interference RNA (siRNA). Argonaut subsequently binds to the siRNA fragments and retains single stranded RNA. The Argonaut complex recognizes the homologous sequence of the mRNA and cleaves the strand, thereby rendering the mRNA unfit for protein translation. The cleaved mRNA strands are recognized by the RdRP, which generates more siRNA, thereby increasing the severity response. For fungi an efficient and stable method for RNAi is formed by hairpin RNA (hpRNA) expressing plasmids [72]. In addition, synthetic siRNA's have been introduced which trigger the RNAi response. Based on the genome sequence of *R. oryzae* strain 99-880 it was predicted that this strain contains two Argonaut copies, one dicer and five RdRP-encoding genes [73]. In the same study, which investigated the role of the high-affinity iron permease-encoding gene (*ftr1*), RNAi was used to silence the gene and study its effect. A construct was generated with a sense and antisense (both 450 bp) of the *ftr1*gene held apart by a spacer element [70]. After transcription of the construct a hairpin structure was formed (hpRNA) which initiated the RNAi process. This method was successful in silencing the gene, and the iron uptake of the transformants generated with this construct was reduced by roughly 50%. In addition the pathogenicity towards mice was greatly reduced.

The transformants were used to infect mice and re-isolated from the mice that succumbed to the infection and healthy mice. It was discovered that the transformants from mice that succumbed to the infection had lost the RNAi vector and in the healthy population the vector was still present.

Next to the hpRNA interference, siRNA's were successfully employed to silence the *ldhA* and *ldhB* genes [74]. These genes were silenced in an effort to reduce the pyruvate flow towards L-(+)-lactic acid and thereby increasing the ethanol formation. To this end synthetic siRNA's were designed for a region in the LDHA-encoding gene, which had the highest sequence similarity to *ldhB*. The siRNA's were 25 nucleotides long and used to transform protoplasts generated from of *R. oryzae* CCUG 28958. In total six knockdown transformants were isolated and grown in medium containing 30 g/L D-glucose. The average L-(+)-lactic acid yield for the knockdown transformants was 0.01 g/g D-glucose, this represented a decrease of 86% in comparison to the parent strain which had a yield of 0.07 g/g. Ethanol production increased with an average of 15% from 0.39 g/g to 0.45 g/g. After the knockdown the yield of glycerol, succinate and pyruvate increased coupled to a decrease in biomass production. The effect of gene silencing with siRNA is transient and therefore not suitable for an industrial process. The results of both studies demonstrated that RNAi can effectively be used for down regulation of gene expression.

In conclusion, it is possible to alter the genome of *R. oryzae* strains by random mutagenesis and genetic modification. The main bottleneck for heterologous gene expression is formed by the difficulties to obtain genomic integration of the vector DNA. In addition there is a lack of dominant selection markers and the absence of multiple auxotrophic markers. Therefore more research is required to further exploit the potential of *R. oryzae* for the production of platform chemicals.

2.9. Pathways of the main fermentation products

In *R. oryzae* the pathways for the main fermentation products are linked to each other by the availability of pyruvate. In *R. oryzae* all fermentable carbon sources are metabolized to pyruvate. The pyruvate is subsequently channeled to a number of pathways, including the pathways responsible for the formation of fermentation end products. This junction is named the pyruvate branch point (Figure 1). The dissolved oxygen in the medium influences the flow of pyruvate from the branch point. Under (micro) anaerobic conditions the carbon flow is directed towards the formation of ethanol, while under aerobic conditions, with excess of carbon substrate, the flow is directed towards organic acid production. This effect was clearly demonstrated in a study using a rotating fibrous bed reactor containing 70 g/L D-glucose [75]. The highest ethanol yield (37% of the theoretical yield) was obtained with 20% dissolved oxygen (DO). Upon an increase to 25 and 50% the ethanol yield decreased to 26 and 15% of the theoretical yield respectively. These results were also demonstrated using stirred tank bioreactors containing 70 g/L D-glucose [76]. The highest ethanol yield was in excess of 50% of the theoretical yield and was obtained at a low rpm. The yield decreased upon an increase in agitation or aeration rate.

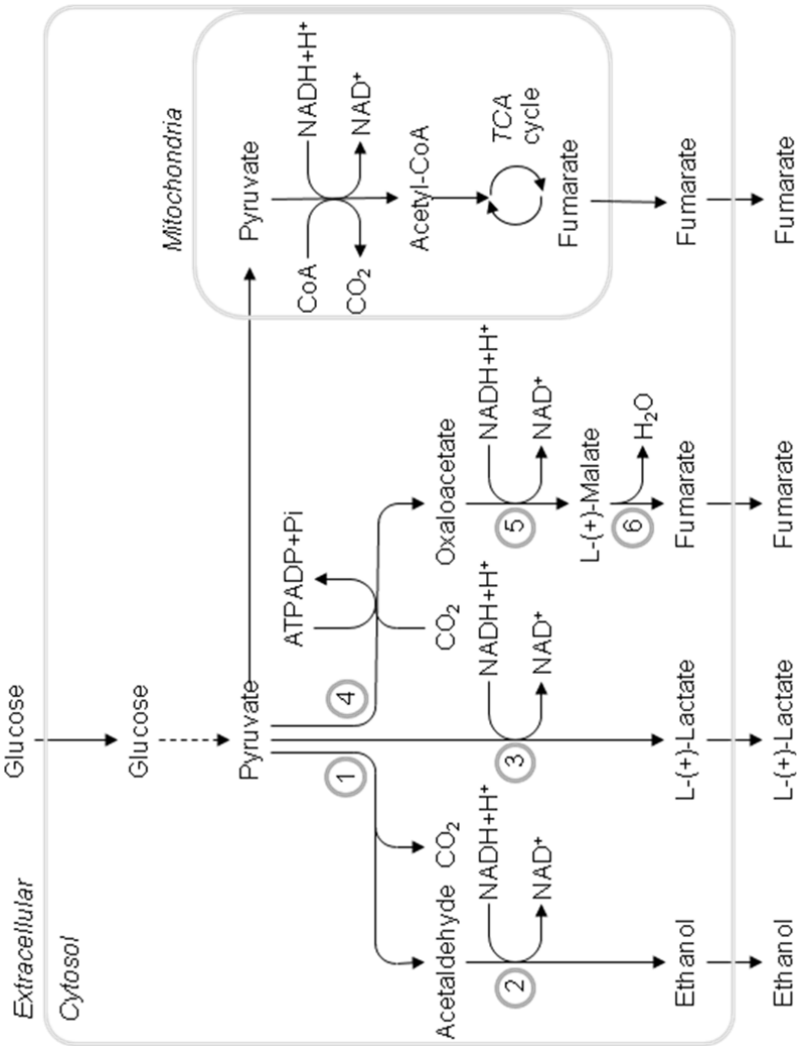

Figure 1. simplified overview of the main fermentation routes with glucose as carbon source adapted from [68]. The numbers indicate key enzymes in this pathway. 1: pyruvate decarboxylase (PDC), 2: alcohol dehydrogenase (ADH), 3: lactate dehydrogenase (LDH), 4: pyruvate carboxylase (PYC), 5: malate dehydrogenase (MDH), 6: fumarase (FUM).

The ethanol fermentative pathway is presumed to be present to allow growth for short periods of time in the absence of oxygen [77, 78]. The production of organic acids under high sugar concentrations is presumed the result of an overflow mechanism, since cells will not encounter these high concentrations in environmental samples [79].

2.10. Ethanol producing pathway

The production of ethanol from pyruvate is catalyzed through the combined action of the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). In *R. oryzae* NRRL 395 two PDC-encoding genes were detected which were not expressed in the presence of the non-fermentable carbon source glycerol, but were readily expressed after the addition of D-glucose [80]. In addition, the absence of oxygen increased the transcript levels of these genes. When the PDC activity was compared between anaerobic and aerobic conditions the enzyme activity was roughly three times higher under anaerobic conditions [80]. While some knowledge is available on the PDC activity and the encoding genes, little work is performed on the characterization of ADH enzymes and encoding genes. When *R. oryzae* NRRL 395 was grow under anaerobic conditions the ADH activity was around seven times higher in comparison to aerobic conditions [60].

In addition it appears that that type II strains produced more ethanol in comparison to type I strains [43].This can be an effect of the absence of the L-(+)-lactic acid producing pathway. During fermentation the carbon sources are metabolized to pyruvate. The available pool of pyruvate is distributed over a number of pathways. When one pathway is absent it can be assumed that more pyruvate is available for the remaining pathways, thereby increasing the amount of ethanol formed in a carbon rich environment.

2.11. L-(+)-Lactic acid pathway

Under aerobic conditions the main fermentation products are organic acids, presumable due to the down regulation of the PDC and ADH gene expression. L-(+)-lactic acid is produced in a single enzymatic step by a NAD^+-dependent L-lactate

dehydrogenase using pyruvate. With the aid of the particle bombardment transformation system it was possible to gain further insight in the molecular mechanisms behind the L-(+)-lactic acid production. The LDHA-encoding gene from the type I strain *R. oryzae* NRRL 395 was introduced in an auxotrophic mutant derived of the type II strain *R. oryzae* 99-880 [68]. The generated transformants converted more than 25% of the starting D-glucose to L-(+)-lactic acid, whereas the recipient strain did not produce any L-(+)-lactic acid. This increase in L-(+)-lactic acid production was coupled to a reduction in the formation of biomass, fumaric acid and ethanol, thereby demonstrating a redirection of the pyruvate flow from the pyruvate branch point away from other products towards L-(+)-lactic acid. To further study the molecular mechanics of L-(+)-lactic production the LDHB-encoding genes from the donor and recipient strains were expressed in *Escherichia coli* [68]. Enzymatic analysis of both purified LDHB proteins demonstrated the ability to convert pyruvate to L-(+)-lactic acid using NADH as cofactor. Yet the untransformed type II strain was unable to produce any L-(+)-lactic acid. It was hypothesized that the lack of L-(+)-lactic acid production was caused by tight transcriptional regulation. With Northern blot analysis no *ldhB* transcript was detected and a gene transcript was only detected with the more sensitive RT-PCR. The presence of very low amounts of transcript could be the cause why some type II strains are able to produce small amounts of L-(+)-lactic acid [42].

2.12. Fumaric and L-(+)-malic acid producing pathway

Other fermentation products originating from the pyruvate branch point are fumaric and L-(+)-malic acid. These organic acids are produced mainly via the reductive tricarboxylic acid (TCA) branch that is located in the cytoplasm. The first enzyme in this pathway is pyruvate carboxylase (PYC) located in the cytoplasm [81]. In eukaryotic organisms this enzyme is normally only present in mitochondria [82]. PYC is a biotin dependent enzyme and carboxylates pyruvate to oxaloacetate. In order to fully convert pyruvate to L-(+)-malic and fumaric acid two other enzymes are required which are also located in the cytoplasm.

Malate dehydrogenase (MDH) converts oxaloacetate to L-(+)-malic acid, which is hydrated (reversibly) to fumaric acid by the enzyme fumarase (FUM). The fumaric acid is subsequently transported across the cell membrane.

Of the enzymes involved in the production of fumaric acid, FUM is the best studied in *R. oryzae*. It was hypothesized by Friedberg and co-workers on the basis of Northern blot and primer extension analysis that *R. oryzae* has one gene (*fumR* GenBank GU013473) encoding for the enzyme FUM [83]. But the kinetics of the cytoplasmically and mitochondrially located enzymes are different. The different enzyme kinetics can be a result of specific conditions in the compartments or due to posttranslational modification events. It is common for eukaryotic organisms, such as rat, that both the cytosolic and mitochondrial enzymes originate from the same gene [84]. However, Goldberg and co-workers suggested that there are two genes coding for the FUM enzymes present in the cytoplasm and the mitochondria [12]. In order to determine which of the hypothesis is correct a fumarase (*fumR* GenBank GU013473) was cloned from genomic DNA of *R. oryzae* NRRL 1526 which produced a single transcript [83]. Antiserum produced against the *S. cerevisiae* FUM partially neutralized the enzyme activity in *R. oryzae* cell free extracts (unpublished data from Battat, Pines and Goldberg cited by [12]). In order to determine whether different fumarases were present at various growth stages the enzyme activity was determined in cell free extract from mycelium in a growth stage and in the acid producing stage. The K_m of the FUM from the growth stage mycelium was 0.78 mmol L^{-1} for fumarate and 2.9 mmol L^{-1} for L-(+)-malic acid. The fumarase activity was not inhibited by the presence of fumaric acid. The FUM activity from the acid producing mycelium however was completely blocked by the presence of 2 mmol L^{-1} fumaric acid. According to the authors it was possible that a unique FUM was induced under acid-producing conditions. This enzyme would produce fumaric acid and the reverse reaction was completely blocked by increased amounts of fumaric acid. Attempts were made by the authors to obtain this unique FUM which had a half-life of two hours. The gene cloned by Friedberg and co-workers was not further characterized [83]. When fresh medium is inoculated with pre-grown biomass a delay in acid fumaric acid production is observed, suggesting that two different genes are present [85, 86]. To further elucidate the hypothesis of the fumarase

enzymes, Song and co-workers cloned a FUM-encoding gene (GenBank X78576) from *R. oryzae* ATCC 20344. This gene was expressed in *E. coli* and the corresponding enzyme was analyzed [87]. The conclusion was drawn that this FUM-encoding gene was responsible for the production of fumaric acid. The K_m for L-(+)-malic acid was 0.46 mM and the reaction from fumaric acid to L-(+)-malic acid was blocked when the fumaric acid concentration exceeded 2 mM. These were the same observations as the unpublished results from Battat, Pines and Goldberg cited by [12]. Song and co-workers then compared both the *fumR* sequence and determined that they were identical with the exception of a fifteen amino acid sequence at the N-terminal end [87]. The presence of this amino acid sequence may be responsible for the localization and activity of the enzyme. Nevertheless, the authors suggested that additional tests were required to determine if this hypothesis is correct.

It was suggested by Goldberg and Song [12, 87] that the exceptional fumarate production by *R. oryzae* is caused by the irreversibility of the reaction catalyzed by FUM at higher fumarate concentrations. However, the ΔG_0'of the conversion of L-(-)-malic acid to fumaric acid is 3.6 kJ per mol [88], indicating that at equilibrium the L-(+)-malic acid concentration is higher than the fumaric acid concentration. This suggests that a dicarboxylic acid transporter with a high selectivity for fumaric acid also plays an important role in fumaric acid production in *R. oryzae*.

When *R. oryzae* is grown in a fermenter under low pH values (3.0) the cell specific fumarate production rate is lower in comparison to a higher pH value (5.0). This can be explained by an increase in the energy requirement to maintain the internal pH, which results in the conversion of more glucose to CO_2 and less to fumarate [85].

2.13. Conclusions and future prospects

With the ever-growing demand for platform chemicals, the increased costs of raw materials and sustainability requirements, efficient biocatalysts are required. One of these biocatalysts is formed by the filamentous fungus *R. oryzae*. This organism is able to produce ethanol, L-(+)-lactic acid and fumaric acid and the ability to produce fumaric acid is what sets this genus apart from other fungi.

These platform chemicals are produced in high yield on a wide range of carbon sources, in excess of 85% of the theoretical yield for L-(+)-lactic acid and ethanol and 65% for fumaric acid. In the cell, all the metabolic end products are formed from a common pyruvate pool and the pathways resulting to the formation of these products are known. Recently, metabolic engineering tools have become available with which it is possible to further increase the yield. These tools consist of RNAi, random mutagenesis and gene knockout strategies. Furthermore, it is currently possible to introduce heterologous genes, resulting in new product formation and eventually the introduction of entire pathways. To achieve the introduction of multiple genes, dominant and multiple auxotrophic selection markers should be developed. This should be coupled to additional research to gain further understanding of the fate of the DNA introduced in the cells as this rarely integrates into the genome.

While it is possible to alter the genome and to introduce heterologous DNA, the genome of *R. oryzae* itself also forms a source for interesting enzymes. Examples are the introduction of the FUMR encoding gene in *S. cerevisiae* for the production of fumarate [89], the expression of *ldhA* in *S. cerevisiae* for lactic acid production [90] and the expression of a *R. oryzae* lipase in *Pichia pastoris* [91] and *S. cerevisiae* [92]. The organism *R. oryzae* is a versatile organism that is already used for a wide range of applications. It is expected that this will increase in the future due to the afore mentioned reasons.

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Chapter 3.
Production of cyanophycin in *Rhizopus oryzae* through the expression of a cyanophycin synthetase encoding gene

3.1. Abstract

Cyanophycin or cyanophycin granule peptide is a protein that results from non-ribosomal protein synthesis in microorganisms such as cyanobacteria. The amino acids in cyanophycin can be used as a feedstock in the production of a wide range of chemicals such as acrylonitrile, polyacrylic acid, 1,4-butanediamine, and urea. In this study, an auxotrophic mutant (*Rhizopus oryzae* M16) of the filamentous fungus *R. oryzae* 99-880 was selected to express cyanophycin synthetase encoding genes. These genes originated from *Synechocystis* sp. strain PCC6803, *Anabaena* sp. strain PCC7120, and a codon optimized version of latter gene. The genes were under control of the pyruvate decarboxylase promoter and terminator elements of *R. oryzae*. Transformants were generated by the biolistic transformation method. In only two transformants both expressing the cyanophycin synthetase encoding gene from *Synechocystis* sp. strain PCC6803 was a specific enzyme activity detected of 1.5 mU/mg protein. In one of these transformants was both water-soluble and insoluble cyanophycin detected. The water-soluble fraction formed the major fraction and accounted for 0.5% of the dry weight. The water-insoluble CGP was produced in trace amounts. The amino acid composition of the water-soluble form was determined and constitutes of equimolar amounts of arginine and aspartic acid.

3.2. Introduction

Cyanophycin or cyanophycin granule peptide (CGP) was first discovered in 1887 by Borzi while examining cyanobacteria with a microscope [1]. Later, it was discovered that all groups of cyanobacteria produce CGP [2] as well as many heterotrophic bacteria [3]. CGP is a polypeptide that results from non-ribosomal protein synthesis by a single enzyme, cyanophycin synthetase (CphA) [4]. The molecule consists of an α-amino-α-carboxy linked aspartic acid backbone to which arginine residues are linked to the β-carboxyl group of each aspartic acid [5] (Figure 1). The non-ribosomal synthesis results in a polydisperse size distribution. In addition, the molecular mass depends on the CGP producing organism [3]; in cyanobacteria, the molecular mass ranges from 25 to 100 kDa [6-9]. At neutral pH and under physiological ionic strength, CGP is in general water insoluble, although a CGP form was described that is water soluble at neutral pH [10]. The water-insoluble CGP can become water soluble at extreme pH values (below 3 or above 9) or under high ionic strength [6, 11].

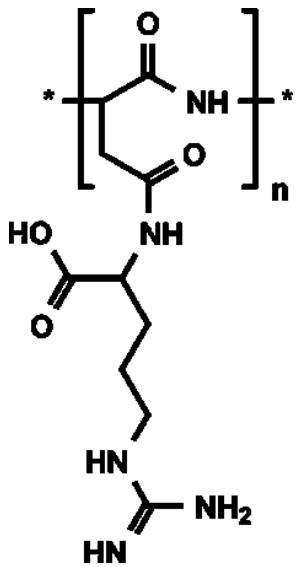

Figure 1. Schematic overview of a CGP molecule. Schematic representation of a CGP molecule with an aspartic acid backbone and arginine side chains [5].

CGP has potentially many industrial applications, it can be used for the production of poly(L-aspartic acid) (PAA) generated by β-cleavage of the side chains. PAA can be used as substitute for non-degradable poly(anionic) molecules such as polyacrylic acid [12]. CGP can also be applied for the production of anti-scalants, dispersing agents, or bulk chemicals such as acrylonitrile [13], urea, and 1,4-butanediamine [14-16].

Currently, the application of CGP is hampered by the lack of a cost-effective production process in cyanobacteria as a result of the low yield and strict growth requirements such as light and complex media. In order to increase the production efficiency, *cphA*-encoding genes from various biological sources were expressed in *Escherichia coli* [3, 4, 9, 10, 17-19] and other commercially relevant bacteria such as *Corynebacterium glutamicum*, *Ralstonia eutropha*, and *Pseudomonas putida* [20-22]. The maximal yield in these bacteria varied strongly with a maximum yield of 35% (*w*/*w*). In eukaryotic microorganisms like *Saccharomyces cerevisiae* [23] and *Pichia pastoris* [24], the maximal CGP yield dropped to 15% (*w*/*w*).

Filamentous fungi of *R. oryzae* spp. have great potential in biotechnological applications. This is due to their ability to utilize a range of simple carbon substrates such as D-glucose, D-xylose, sucrose, and lactose [25]. Next to simple carbohydrates, *R. oryzae* spp. can also grow on agricultural waste streams [26-32]. Using these carbon sources, it can produce ethanol and organic acids like L-(+)-lactic, fumaric and L-(+)-malic acid [33, 34]. These organic acids have wide applications in the food and feed industry. In addition, these compounds can be applied as feedstock in order to produce renewable resources like plastics, fibers, solvents, and oxygenated chemicals [35-38]. The biotechnological potential of *R. oryzae* spp. further increased by the publication of the genome sequence of strain 99-880 in 2004 and with the development of transformation systems based on uracil auxotrophy [39].

To investigate the potential for the production of CGP in a fungal expression system, we have expressed the *cphA*-encoding genes from *Synechocystis* sp. strain PCC6803, *Anabaena* sp. strain PCC7120 and its codon-optimized version in the auxotrophic mutant *R. oryzae* M16 derived from *R. oryzae* 99-880.

3.3. Materials and methods

3.3.1. Strains, media, growth conditions, and methods

One Shot® Mach1™ T1 Phage-Resistant *E. coli* (Invitrogen Carlsbad, CA) was used for plasmid maintenance and propagation. The cells were grown in Luria-Bertani (LB) medium containing 50 µg/ml ampicillin or kanamycin at 37°C with agitation at 250 rpm. In this study, *R. oryzae* 99-880 (Fungal Genetics Stock Center FGSC 9543) for which the genome sequence is known and the from this strain-derived orotate phosphoribosyltransferase (*pyrF*) auxotrophic mutant M16 [39] were used. The auxotrophic mutant M16 was a kind gift of Dr. C. D. Skory of the USDA, Peoria, IL. Spores were obtained by cultivation on synthetic *Rhizopus* (RZ) medium [40] containing 1.5% (*w*/*v*) agar and 2% D-glucose. For growth of *R. oryzae* M16, the medium was supplemented with 0.5 mg/ml uracil. The plates were incubated for 4 days at 30°C. The spores were harvested using a saline Tween-80 solution [0.9% (*w*/*v*) NaCl, 0.005% (*v*/*v*) Tween-80]. Details for the strains and plasmids are listed in Table 1. All experiments were performed with biomass obtained from liquid cultures unless stated otherwise. Biomass of *R. oryzae* transformants and wild-type was generated by cultivation in shake flasks containing 100 ml RZ medium using 100 g/l of D-glucose as a carbon source, inoculated with 10^6 spores per milliliter. The cultures were incubated for 72 h at 30°C with constant agitation in an orbital shaker at 200 rpm. To maintain a stable pH, 10 g/l $CaCO_3$ was added after 18 h. When the $CaCO_3$ was almost dissipated, fresh $CaCO_3$ was added.

Table 1. Strains and plasmids used in this study.

Ap^r, ampicillin resistance, Km^r, kanamycin resistance and Cm^r, Chloramphenicol resistance

Strain or plasmid	Description	Reference or source
E. coli One Shot® Mach1™	F-φ80 (lacZ) Δ*M15* Δ*lacX74* hsdR (rK-mK+) Δ*recA1398 endA1 tonA*	(Invitrogen Carlsbad, CA)
R. oryzae 99-880	Sequenced strain	Fungal Genetics Stock Center FGSC 9543)
R. oryzae M16	Auxotrophic mutant derived from *R. oryzae* 99-880 Δ*pyrF*	[39] Gift of Dr. C. D. Skory
pBBR1MCS-2::$cphA_{7120}$	Km^r, broad host range vector, lacPOZ' harbouring *cphA* from *Anabaena* sp. strain PCC7120 collinear to *lacPOZ'*	[21] Gift of Prof. Dr. A. Steinbüchel
pJet::$cphA_{7120}$	Ap^r harbouring *cphA* from *Anabaena* sp. strain PCC7120	This study
pMa/c5-914::$cphA_{6803}$	Ap^r Cm^r c1857ts _ PL/PR, translational initiation region harbouring a 2.6-kb PCR product from *Synechocystis* sp. strain PCC6803	[19] Gift of Prof. Dr. A. Steinbüchel
pJet::$cphA_{6803}$	Ap^r harbouring a 2.6-kb PCR product from *Synechocystis* sp. strain PCC6803	This study
pPdcExPyrF	Ap^r *pdcA* promoter and terminator	Gift of Dr. C. D. Skory
pPdcExPyrF::$cphA_{6803}$	pPdcExPyrF harbouring a 2.6-kb PCR product from *Synechocystis* sp. strain PCC6803 cloned with *Sph*I and *Pac*I	This study
pPdcExPyrF::$cphA_{7120}$	pPdcExPyrF harbouring *cphA* from *Anabaena* sp. strain PCC7120 cloned with *Sph*I and *Pac*I	This study
$cphA_{7120}$ codon optimized	Codon optimized *cphA* from *Anabaena* sp. strain PCC7120	DNA2.0, Menlo Park, CA
pPdcExPyrF::$cphA_{7120}$ codon optimized	pPdcExPyrF harbouring a codon optimized *cphA* from *Anabaena* sp. strain PCC7120 cloned with *Sph*I and *Pac*I	This study

3.3.2. DNA techniques

The *cphA*$_{7120}$ gene originating from *Anabaena* sp. strain PCC7120 was cloned from plasmid pBBR1MCS-2::*cphA*$_{7120}$ [21]. The *cphA*$_{6803}$ gene originating from *Synechocystis* sp. strain PCC6803 was cloned from plasmid pMa/c5-914::*cphA*$_{6803}$ [19]. Both plasmids were a kind gift from Prof. Dr. A. Steinbüchel of the WWU Münster, Germany. The genes were cloned by PCR amplification using Phusion DNA polymerase (Finnzymes, Espoo, Finland). The oligonucleotide primers used for PCR amplification were 7120FS and 7120RS for *cphA*$_{7120}$ and 6803FS and 6803RP for *cphA*$_{6803}$ (Table 2).

Table 2. Oligonucleotide primers used in this study.
Characters in bold indicate the restriction sites that were introduced. The underlined character indicates the location of a silent mutation in order to remove an existing restriction site. Primer 7120RP is designed to have an overlap with the donor plasmid to facilitate cloning.

Oligonucleotide primer	Oligonucleotide primer sequence 5' to 3' orientation
7120FS	**GCATGC**GAATCCTCAAGATCCAGACC
7120RP	GGGAATCACCACATCTCTACTA**TTAATTAA**AGCAAAGTA
7120qF	CTGGATGAAACCCAAGCAAT
7120qR	CGGTTGTCGAGGAATTTTGT
6803FS	**GCATGC**AAATTCTTAAAACCCTTACCCTCCGC
6803RP	**TTAATTAA**CCAATGGGTTTACGGGCTTTGATTAAC
6803qF	TCAATCTGGGTCGGTACCAT
6803qR	GGCCCCGTTTATCATCATCT
kanqF	AGCATTACGCTGACTTGACG
kanqR	AGGTGGACCAGTTGGTGATT
7120coqF	TTAAACCTGATGCCCGATATG
7120coqR	TGACCAAGCCTCTCAGTTTG
PDCqF	ACAGCCGAATTTGCTTCACT
PDCqR	GATAGCGGCCCTACAGAGG

The correct size of the DNA fragments was verified by agarose gel electrophoresis using 0.8% agarose with 0.5 times Tris–acetate–EDTA (TAE) buffer. The amplicons were ligated into the plasmid pJET1.2/blunt using the CloneJET™ PCR Cloning Kit (Fermentas International Inc, Burlington, Canada) according to the manufacturer's instructions. Codon-optimized versions of both genes were ordered from DNA 2.0 (Menlo Park, CA). Codon optimization was performed based on the codon table of Kazusa DNA Research Institute (Kisarazu, Japan). Electro transformation of *E. coli* was performed with the Gene pulser II using cuvettes with a 0.2-cm gap (Bio-Rad, Hercules, and CA). Isolation of plasmid DNA was performed using a GeneJET™ plasmid miniprep kit (Fermentas International Inc, Burlington, Canada) according to the manufacturer's instructions.

The plasmid pJet::*cphA*$_{7120}$ and pJet::*cphA*$_{6803}$ were digested using the restriction enzymes *Sph*I and *Pac*I (New England Biolabs, Ipswich, MA), subsequently ligated into the *Sph*I and *Pac*I sites of plasmid pPdcExPyrF. Plasmid pPdcExPyrF was a kind gift of Dr. C. D. Skory of the USDA, Peoria, IL. The nucleotide sequences of all fragments were verified by DNA sequence analysis (Baseclear BV, Leiden, The Netherlands).

3.3.3. Transformation of Rhizopus oryzae and stability assay of transformants

Transformation of *R. oryzae* M16 spores was achieved by particle bombardment. M5 tungsten particles (Bio-Rad, Hercules, CA) were coated with plasmid DNA according to the manufacturer's instructions. The particles were delivered by the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA), having a distance between the rupture disc and the particles of 1.6 cm. The distance between the particles and the spores was 6 cm. Spores (10^5) of *R. oryzae* M16 were plated on RZ medium and bombarded. The pressure was set with a rupture disc at 1,100 psi. To allow the formation of biomass and spores, the plates were incubated at 30°C for 5 to 7 days. After sporulation, the spores were harvested, and serial dilutions were made that were used to inoculate RZ plates with one spore. This process was repeated to ensure single progeny. In order to determine the stability of the transformants, serial dilutions of the spores grown from the glycerol stocks were made ranging from 8 to 8×10^9 spores per plate. The spores were plated on RZ medium containing 5-fluoroorotic acid (5-FOA) and 5-FOA complemented with 0.5 mg/ml uracil.

3.3.4. Transcript analysis

To determine the presence of *cphA* mRNA in the transformants, liquid cultures were grown as described in the previous section. Mycelium was harvested after 72 h, frozen in liquid nitrogen, and ground using a Braun micro-dismembrator (Braun, Melsungen, Germany). RNA was isolated and cDNA was generated as described by Oliveira and co-workers [41]. The cDNA served as the template for quantitative real-time PCR (qPCR). Primers were designed with the

Primer3 program to have a specific melting temperature of 60±1°C, GC content of 50±5%, and amplicon sizes between 139 and 150 bp. The primers used were 7120qF, 7120qR, 7120coqF, 7120coqR 6803qF, and 6803qR. Two reference genes were used; as an external reference gene kanamycin, the primers were kanqF and kanqR (Table 2) and as an internal reference gene pyruvate decarboxylase was used with the primers PDCqF and PDCqR (Table 2). The PCR mixes were pipetted with the CAS-1200 robot (Corbett Life Science, Sydney, Australia). Reaction mixtures for real-time PCR had a total volume of 16 µl and contained 4 µl cDNA (2.5 ng/µl), 1.2 µl of each primer (1.2 µM), and 10 µl ABsolute QPCR SYBR Green Mix (ABgene, Epsom, UK). The Rotor-Gene 3000 (Corbett Life Science) was used for thermal cycling and real-time detection of the DNA. The melting analysis feature was used to determine primer–dimer formation, and the comparative quantitation feature was used to determine take-off and amplification values. Relative expression was calculated by the Pfaffl method [42]. Normalization was performed on the basis of the added kanamycin external transcript proportional to the total RNA used for cDNA synthesis. Additionally, normalization was performed on the basis of pyruvate decarboxylase (PDC).

3.3.5. Cyanophycin synthetase activity assay

CphA activity was determined in cell-free extracts by a scintillation assay performed as described by Aboulmagd and co-workers [18] using L-[2,3,4,5-^{3}H] arginine monohydrochloride (GE Healthcare, Piscataway, NJ) with a specific activity of 1.59 TBq/mmol. The cell-free extract was obtained by centrifugation of a suspension of 100 mg grinded mycelium in 500 µl 20 mM Tris/HCl buffer pH 7.5 (4ºC, 16,100 g). To determine the effect of protease activity, 12.5 µl protease inhibitor cocktail (P8215) (Sigma-Aldrich, St. Louis, MO) was added to the cell-free extract. The activity was measured by adding the reaction mixture in a 1 to 10 ratio to Ultima gold scintillation liquid (Perkin-Elmer Life Sciences, Boston, MA) and scintillation counting in a model 1600 TR Tri-Carb liquid scintillation counter (Packard Instrument Company, Meriden, CT). To determine the specific activity, the protein concentrations in the cell-free extract were determined with a Bradford protein assay (Bio-Rad, Hercules, CA). The protein standards were prepared with bovine serum albumin.

All experiments were performed in triplicate. The specific enzyme activity was expressed in units per milligram, which represents the incorporation rate of L-arginine in nanomoles per minute per milligram of total protein in the cell-free extract.

3.3.6. Isolation and analysis of cyanophycin

Water-soluble and water-insoluble CGP was extracted using a modified method of Ziegler and co-workers [10]. Mycelium was harvested and disrupted as described in a previous section and 1 g (wet weight) was resuspended in 10 ml 20 mM Tris/HCl buffer pH 7.5. The suspension was centrifuged for 20 min at 4,600 g and 4°C. The cell-free extract was separated from the pellet, and water-soluble CGP was extracted from the cell-free extract. The soluble fraction was incubation at 65°C for 20 min and centrifuged for 20 min at 4,600 g at 4°C. The supernatant was incubated overnight with proteinase K after which the proteins were precipitated with ice-cold ethanol, washed with acetone, and air-dried.

Water-insoluble CGP was isolated from the biomass pellet obtained during the isolation of water-soluble CGP. This pellet was resuspended in 0.1 M HCl until the $CaCO_3$ was dissipated and the pH of the sample was 1. The sample was centrifuged for 20 min at 4,600 g at 4°C. The supernatant was neutralized with 0.1 M NaOH and re-centrifuged. The pellet was washed twice with demineralized water and dried. At each step, the dry weight of the removed material was gravimetrically determined to calculate the weight percentage of the accumulated CGP. The molecular mass of the CGP was determined by SDS-PAGE analysis using an 11.5% (*w*/*v*) polyacrylamide gel as described by Laemmli [43] with 50 µg of dried protein per slot. The protein marker used was a precision plus protein standard from Bio-Rad (Bio-Rad, Hercules, CA). The gels were stained with Coomassie Brilliant Blue R-250. To determine the amino acid composition, the isolated protein was hydrolysed in 6 M HCl with 1% *w*/*v* of phenol at 110°C for 24 h under nitrogen atmosphere. The individual amino acids were derivatised with OPA-reagent (*o*-phthaldialdehyde) and FMOC (9-fluorenylmethoxycarbonyl chloride) (Sigma-Aldrich, St. Louis, MO). The amino acid analysis was done using the Dionex rapid separation liquid chromatography system (Dionex Corporation, Sunnyvale, CA) with an Acquity UPLC® BEH C18 reversed phase

column (Waters, Milford, MA) using an Ultimate 3000 variable wavelength detector (Dionex Corporation, Sunnyvale, CA).

3.4. Results

3.4.1. Transformant stability

The stability of transformants generated in this study for the *pyrF* phenotype was determined with the aid of 5-fluoroorotic acid (5-FOA) and uracil selection. If the selection marker is lost, *pyrF* transformants are unable to metabolize 5-FOA, thereby preventing cell death, and uracil in the plates would facilitate cell growth. None of the transformants or the wild type were able to grow on the plates containing 5-FOA and uracil. This shows that all generated transformants were stable for the *pyrF* phenotype.

3.4.2. CphA expression in Rhizopus oryzae transformants

The *cphA*-encoding genes from *Anabaena* sp. strain PCC7120 ($cphA_{7120}$) and *Synechocystis* sp. strain PCC6803 ($cphA_{6803}$) were selected for expression in *R. oryzae* 99-880 on basis of their close codon usage. The GC difference in the first three bases for $cphA_{7120}$ was 3%, 3%, and 13% for $cphA_{6803}$, the difference was 2%, 2%, and 2%. To further increase the efficiency of gene translation, codon-optimized genes were designed and cloned by DNA 2.0 (Menlo Park, CA). All the genes were cloned into the *R. oryzae* expression vector pPdcExPyrF (Table 1). It was impossible to obtain a vector containing the codon-optimized gene of $cphA_{6803}$, despite the fact that several *E. coli* strains were used as a host for plasmid propagation. The transformation of *R. oryzae* spores with the expression vectors was accomplished by the biolistic transformation method. In total, 40, 14, and 38 transformants were isolated with the unmodified $cphA_{7120}$, the codon optimized $cphA_{7120}$ and the $cphA_{6803}$-encoding genes, respectively (Table 3). The total number of transformants isolated directly after the transformation with the codon-optimized gene was 60, yet many failed to grow or sporulate in the isolation process for single progeny.

All generated transformants were grown in liquid RZ medium for 72 h after which the mycelium was harvested for RNA extraction and protein analysis. The transformants were screened with quantitative real-time PCR (qPCR) for the presence and the amount of the specific *cphA* transcript. Not all isolated transformants expressed the *cphA*-encoding 8 out of 40 for cphA7120, 1 out of 14 for codon optimized $cphA_{7120}$, and 14 out of 38 for $cphA_{6803}$ (Table 3). The transcript levels for the $cphA_{7120}$-encoding genes represented 0.01% to 0.4% of the PDC transcript. For the transformants, expressing the $cphA_{6803}$-encoding gene was much higher, ranging from 0.8% to 39.5% of the PDC transcript. In the wild-type strain, the apparent transcript level of the *cphA*-encoding genes represented 1‰ of the PDC gene; this was considered to be an a-specific transcript.

Table 3. Number of transformants generated and tested positive at various stages in the experiment.

Cod opt; codon optimized.

cphA variant	Number of generated transformants	Transformants with transcript level	Transformants with CphA activity	Transformants with CGP accumulation
$cphA_{7120}$	40	8	0	0
$cphA_{7120}$ cod opt	14	1	0	0
$cphA_{6803}$	38	11	2	1

3.4.3. CphA activity in Rhizopus oryzae transformants

Transformants in which a *cphA* transcript was detected were further analyzed for CphA activity as determined with the scintillation assay. In two transformants, both expressing the $cphA_{6803}$ gene, specific enzyme activity was detected. These transformants were named transformant $cphA_{6803}$#1 and transformant $cphA_{6803}$#2. Both transformants had a specific enzyme activity of 1.5 mU/mg proteins, which was much higher than the wild-type in which an activity of 0.27 mU/mg protein was measured (Table 4). To determine if protease activity was the cause of the low specific enzyme activity, a broad-spectrum protease inhibitor cocktail was added to fresh cell-free extract. This did not lead to an increase in the enzyme activity (data not shown).

Table 4. Specific activities of cell free extract.

All assays were performed in triplicate. Transcript levels are relative to the PDC gene transcript. One unit is defined as the amount of L-arginine incorporated in nmol in one minute per mg protein [4]. SD; standard deviation.

Strain and transfor-mants	Relative cphA transcript	Mean DPM ± SD	Mean CphA sp. act. (mU/mg) ± SD	% dry weight water-soluble CGP/ insoluble CGP
wild-type	1.42E-05	448 ± 317	0.27 ± 0.066	0/0
cphA$_{6803}$#1	1.2E+01	1838 ± 436	1.5 ± 0.36	0.5/0
cphA$_{6803}$#2	2,5E+01	2220 ± 504	1.5 ± 0.35	0/0

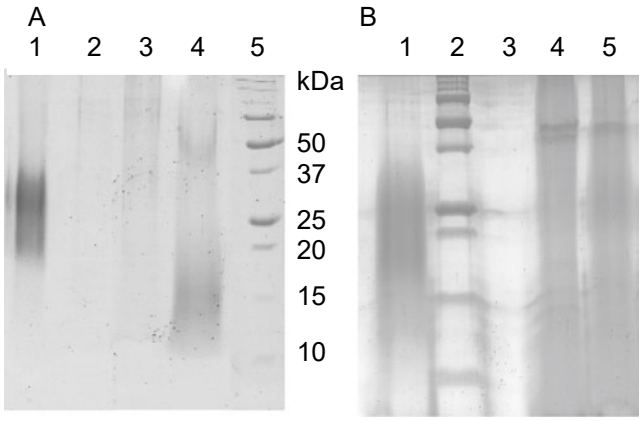

Figure 2. Water-soluble and insoluble CGP extracted from *R. oryzae* transformants.

SDS-PAGE analysis of the CGP accumulated in transformants of *R. oryzae* M 16 expressing *cphA*-encoding gene from *Synechocystis* sp. Strain PCC6803. Panel A shows the water-soluble CGP samples and panel B the water-insoluble samples. Per lane 50 µg sample of each of the different transformants was loaded.

Panel A lane 1; 20 µg CGP from *S. cerevisiae* G175 expressing the *cphA*-encoding gene from *Synechocystis* sp. strain PCC6308 [23], lane 2; *R. oryzae* 99-880, lane 3; *R. oryzae* M16 transformant $cphA_{6803}$#2, lane 4; *R. oryzae* transformant M16 $cphA_{6803}$#1; lane 5, protein marker

Panel B, lane 1; 20 µg CGP from *S. cerevisiae* G175 expressing the *cphA*-encoding gene from *Synechocystis* sp. strain PCC6308 [23]; lane 2; protein marker, lane 4; *R. oryzae* 99-880, lane 5; *R. oryzae* M16 expressing $cphA_{6803}$# 1.

3.4.4. Determination of the presence of CGP

CGP was extracted using the protocols for water-soluble and insoluble CGP. Only in transformant $cphA_{6803}$#1 water-soluble and insoluble CGP was detected by SDS-PAGE analysis (Figure 2). This transformant accumulated mainly water-soluble CGP to a maximal amount of 0.5% dry weight of the biomass (Table 4). The water-insoluble CGP was accumulated in such small quantities that it was impossible to accurately determine the dry weight percentage. The protein fractions were analyzed on SDS-PAGE gel. Both forms of CGP appeared to be poly-disperse in molecular mass. The molecular mass of the water-soluble CGP ranged between 10 and 20 kDa whereas the molecular mass of the water-insoluble CGP ranged from 25 to 37 kDa (Figure 2).

3.4.5. Amino acid composition of CGP

The amino acid composition of the isolated CGP was determined by rapid separation liquid chromatography (RSLC). The samples were first hydrolysed by acid hydrolysis, and as a result, it was not possible to discriminate between aspartic acid and asparagine, nor between glutamic acid and glutamine. The amino acid composition of the water-soluble CGP comprised of equimolar amounts of arginine and aspartic acid/asparagine. These fractions represented 70.4 mol% of the total protein fraction. The remainder of the sample consisted primarily of leucine, glycine, serine, and lysine, with trace amounts of other amino acids. A sample from the wild-type strain extracted by the same method was also analyzed as a reference sample. In this sample, the combined fractions of aspartic acid/asparagine and arginine represented only 16.4 mol% of the total protein present in the sample. The most abundant amino acid fractions were glutamic acid/glutamine, aspartic acid/asparagine and alanine representing 11.4, 11.2, and 10.8 mol%, respectively. Lysine represented 7.2 mol% of the protein fraction in the wild-type sample and only 5.7 mol% in the CGP sample. The amino acid composition of the water-insoluble CGP was not accurately determined due to the low CGP content in the sample.

3.5. Discussion

3.5.1. CGP production in Rhizopus oryzae

The goal of this study was to produce the polypeptide CGP in the filamentous fungus *R. oryzae*. The transcript levels of the different *cphA*-encoding genes varied strongly in the transformants and in only two transformants expressing $cphA_{6803}$ was specific enzyme activity detected. The specific activity of CphA in the two transformants was 1.5 mU/mg protein. This is about 1,000 times lower than described for other eukaryotic microorganisms. In *S. cerevisiae* [23] and *P. pastoris* [24] both expressing $cphA_{6308}$, the CphA activity was 0.91 and 2.01 U/mg, respectively.

In addition, no correlation was detected between transcript levels and the measured enzyme activity for the *R. oryzae* transformants.

This in contrast to the results described in a study by Mertens and co-workers [44] with *R. oryzae* NRRL395 using the same promoter. Here, a clear correlation was observed between transcript levels and Green Fluorescence Protein (GFP) accumulation. Furthermore, CGP only accumulated in one of the two transformants which displayed enzyme activity.

There are several possible explanations for the low enzyme activity and CGP accumulation in the *cphA* transformants of *R. oryzae*. One option is a putative instability of the introduced construct. Kroll and co-workers [45] reported a much lower CGP production by engineered *E. coli* cells grown on mineral medium compared to those grown in complex media. It was hypothesized that this was due to the instability of the introduced plasmid. Apparently, the production of CGP in mineral medium, also applied in our study, is a strong metabolic burden resulting in an unstable genotype. A comparable phenomenon was observed in CGP producing transformants of *S. cerevisiae*. Here, a loss in enzyme activity and CGP accumulation occurred in transformants after several cultivation rounds, whereas the gene itself remained present in the cells (Dr. A. Bröker, personal communication). The isolation of single progeny transformants and biomass generation with *R. oryzae* in this study requires three consecutive sporulation events and are equivalent to many cultivation rounds. A loss comparable to that of *S. cerevisiae*'s enzyme activity after prolonged cultivation can explain the absence or low amounts of active enzyme and CGP. Another option can be a putative toxicity of the *cphA*-encoding gene products. The putative toxicity can also be a reason why there is a discrepancy between the mRNA levels and enzyme activity. Yet the discrepancy can also be a result of the direct inactivation of the CphA protein by, e.g., proteolytic activity.

Currently, a limited number of heterologous genes were successfully expressed in *R. oryzae*. These are genes coding for GFP [44] and lactate dehydrogenase A from *R. oryzae* NRRL395 in *R. oryzae* in 99-880 [39]. These genes are either small or originate from closely related organisms. The $cphA_{6803}$-encoding gene from cyanobacteria is phylogenetically more distant and with a size of approximately 3 kbp

significantly larger than the previously expressed genes. This demonstrated that larger heterologous genes can also be successfully expressed in *R. oryzae*.

3.6. Acknowledgements

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Chapter 4.
A fast and accurate UPLC method for analysis of proteinogenic amino acids

4.1. Abstract

A reliable and inexpensive method for the measurement of proteinogenic amino acids was developed. It allows the screening of large sample volumes that is important for current food and feed applications, but also for future biobased applications. The method is based on acid hydrolysis and automated pre-column derivatisation in the injection needle of the autosampler using *o*-phthalaldehyde/ethanethiol reagent in combination with 9-fluorenylmethyl chloroformate. The calculated mean limit of detection and limit of quantification of pure amino acids were 2.3 µM and 4.6 µM respectively. With this method it was possible to accurately analyse the amino acid composition of bovine serum albumin, soy meal, Jatropha press cake, and cyanophycin. Mean reproducibility of all amino acids from bovine serum albumin was 6.8%, with a mean recovery of 95%. The sample run time is 16 minutes with a total cycle time from injection to injection of an acid hydrolysed sample is 22 minutes, resulting in a greener method due to a reduction in solvent consumption.

4.2. Introduction

Historically, proteins from biomass are mainly used for feed and food applications and are obtained from dedicated production processes. With the advent of the biobased economy large amounts of proteins will become available in the waste material from, for instance, biofuel production. An example is the biodiesel production in Indonesia. Indonesia has the intention to supply up to 5% of the total energy requirement by alternative sources in 2025 [1]. The energy crop of choice for Indonesia is *Jatropha curcas L.* or Jatropha. A side product of this process is de-oiled Jatropha press cake which is rich in proteins. The production process is comparable to soy meal production from soy beans. Proteins from these processes will be available in large quantities and are heterogeneous in composition. Determination of amino acid composition in these samples is needed to estimate their value for e.g. feed.

In the biobased economy, increasing amounts of proteins, peptides or amino acids will also be produced using dedicated biotechnological processes. An example of such a process is the fermentative production of cyanophycin. Cyanophycin is a polypeptide produced in cyanobacteria and consists of an α-amino-α-carboxy linked aspartic acid backbone with arginine residues that are linked via the β-carboxyl group of each aspartic acid [2]. Currently, cyanophycin is formed in genetically modified ethanol producing microorganisms such as *Saccharomyces cerevisiae* [3] and *R. oryzae* [4] to develop the concept of duel production processes. In these specific processes, but also in e.g. the production of bulk chemicals from amino acids [5, 6] it is important to monitor reaction product formation. In these processes reliable, fast and cost-effective amino acid determinations are crucial.

Since most amines show neither natural UV absorption nor fluorescence, a derivatisation reaction is required prior to detection. Examples of derivatisation reagents are *ortho*-phthalaldehyde (OPA) in combination with a thiol compound, 9-fluorenylmethyl chloroformate (FMOC), phenylisothiocyanate (PITC), dansyl chloride, and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Of these derivatisation reagents OPA and FMOC are the most frequently used in papers describing derivatisation protocols from 2000 to 2011 [7].

This study focused on the derivatisation by OPA in combination with ethanethiol (ET) and FMOC. The main motivations for choosing OPA/ET-FMOC above other derivatisation reagents were; low costs, fast reactions at low temperatures, high selectivity, and high sensitivity [7, 8]. However, the OPA-derivatisation method has one drawback in the instability of the isoindoles formed after derivatisation [9]. Due to the instability it is not possible to derivatise the samples well in advance. To circumvent this problem an automated derivatisation reaction in the sample needle was performed. Principles of this derivatisation reaction are described in detail in various excellent review articles [7, 8].

In this study protein hydrolysis is performed according to the HCl hydrolysis described in the Eldex operator's manual [10] using the Pico Tag method. The derivatisation will occur in the needle of the autosampler and combined with the UPLC technique creates a fully automated derivatisation and separation method suitable for inexpensive analysis.

4.3. Experimental

4.3.1. Materials

Analytical grade OPA, FMOC, ET, potassium chloride, bovine serum albumin (BSA) standard (1 mg/mL), L-asparagine, L-glutamine, L-tryptophan, L-citrulline, γ-aminobutyric acid (GABA), L-ornithine, 1,4-diaminobutane (putrescine), 1,5-pentanediamine (cadaverine), and norleucine (internal standard) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard H contained exact amounts of each amino acid (2.5 µmol amino acid/mL and 1.25 µmol/mL cysteine), and was purchased from Pierce (Rockford, IL, USA). Milli-Q water (Millipore, Billerica, MA) was used for the preparation of all buffers and reagents.

Cyanophycin used in this study was produced in recombinant *Escherichia coli* cells grown on Protamylasse [11]. This sample was kindly provided by Prof. Dr. A. Steinbüchel from the Westfälische Wilhelms-Universität located in Münster, Germany.

Jatropha press cake was obtained from Energy Technology Center (B2TE) of the Agency for the Assessment and Application of Technology (BPPT), Serpong, West-Java, Indonesia. The press cake was de-oiled prior to use at the Pilot Pflanzenöltechnologie Magdeburg e.V. (PPM) in Magdeburg, Germany. Soy protein concentrate was obtained from Trades S.A. (Barcelona, Spain).

4.3.2. Standard solutions

The complete amino acid (AA) standard consisted of standard H supplemented with L-asparagine, L-glutamine, and L-tryptophan in the same concentrations as the other AAs (2.5 mM). The standard was diluted in Milli-Q water to 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mM and these calibrating solutions were used to determine the regression coefficients, limits of detection (LOD), and limits of quantification (LOQ) of the UPLC method. As an internal standard 1.5 mM norleucine was used.

4.3.3. OPA/ET and FMOC reagent solutions

The derivatisation procedure was partly based on reagent solutions described in the review of Hanczkó and co-workers [8]. Borate buffer was prepared by dissolving 1.24 g boric acid and 1.49 g potassium chloride in 50 ml Milli-Q water. The final pH was adjusted with sodium hydroxide (50% W/V, Merck, Darmstadt, Germany) to pH 10.5. ET solution was obtained by mixing 20 mL borate buffer solution with 80 mL methanol and 500 µL ET. Subsequently, 40 mg OPA was dissolved in 10 ml of the ET solution. FMOC reagent was obtained by dissolving 25 mg FMOC in 10 mL acetonitrile. All solutions should be freshly diluted every second day.

4.3.4. Sample hydrolysis

200 µL of a sample or BSA standard (1 mg/mL) was transferred to a 1.5 mL glass analysis vial and dried under vacuum using a Waters Pico Tag Workstation [10]. 500 µL of hydrochloric acid solution (6 M) containing 1% (w/v) phenol was added. The vial was sealed under vacuum after three alternating vacuum and flushing steps with nitrogen gas. Hydrolysis was performed at 110° C for 24 hours. After hydrolysis, the solution was dried under vacuum and the sample was suspended in 1 ml resuspension solution composed of 400 µL methanol, 100 µL

internal standard solution and 500 µL Milli-Q water. Undissolved particles were removed by filtration using a 0.2 µm regenerated cellulose syringe filter (Spartan 13/0.2 RC, product number 10 463 042, Whatman, Kent, UK).

4.3.5. Automation of OPA/ET-FMOC derivatisation

The derivatisation of the samples occurred in the needle of the Dionex UltiMate system at ambient temperatures by programming the robotic autosampler (Table 1). This method was derived from a Dionex protocol [12]. Injection diluent was added to stop the reaction and comprised of 48.5 mL eluent A (composition described in the next paragraph) and 1.5 mL 85% phosphoric acid. The entire derivatised sample was injected on the column with the sample needle in-line with the loop. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] were 23/9.9/3.2/1 using acid hydrolysed BSA with an average molar weight of 66.5 kDa as a standard.

Table 1. Sample derivatisation and injection scheme.

Autosampler programming instructions	
Position of vials	
Position 1:	Milli-Q water
Position 2:	OPA/ET reagent
Position 3:	FMOC solution
Position 4:	Injection diluents
Position X:	sample (e.g. amino acid standard)
	X is any other position of the autosampler tray.
Derivatisation/injection routine	
1	Draw 50 µL from vial 1 (water)
2	Draw 1 µL air
3	Draw 2 µL from vial 2 (OPA-ET reagent
4	Draw 2 µL from sample vial (e.g. position #12)
5	Draw 12 µL air
6	Mix five times by load/unload needle with 4 µL
7	Incubate for 60 s and unload 12 µL (air)
8	Wash needle externally 25 µL water/methanol (80/20% w/v)
9	Draw 2 µL from vial 3 (FMOC solution)
10	Draw 12 µL air
11	Mix five times by load/unload needle with 6 µL
12	Incubate for 15 s and unload 12 µL (air)
13	Wash needle externally 25 µL water/methanol (80/20% w/v)
14	Draw 4 µL from vial 4 (injection diluent)
15	Draw 12 µL air
16	Mix five times by load/unload needle with 10 µL
17	Unload 12 µL (air)
18	Inject the whole sample

Supplementary instructions: draw speed, 120 µL/min; eject speed, 600 µL/min.

4.3.6. Chromatography

Sample analysis was performed on a Dionex RSLC system with an UltiMate 3000 Rapid Separation pump and autosampler. Derivatised AAs were detected at 263 (FMOC derivative of proline) and 338 (OPA derivatives of AAs) nm with a Dionex Ultimate 3000 Variable Wavelength Detector. The AAs were separated using an Acquity UPLC BEH C18 reversed phase column (1.7 µm particle size, 2.1 x 150 mm) with a sample loop of 250 µL. The guard column was a VanGuard Acquity UPLC BEH C18 guard column (1.7 µm particle size, 2.1 x 5 mm). It is recommended replacing the guard column after 250 injections in order to obtain maximal results. The column temperature was maintained at 50º C. Eluent A consisted of 10 mM disodium phosphate, 10 mM sodium tetraborate, and 2 mM sodium azide pH 7.8. Eluent B consisted of methanol, acetonitrile, and Milli-Q water in a 20:60:20 ratios. Elution was performed at a flow rate of 0.4 ml/minute, using the following gradient (expressed as solvent B): initial composition: 20% B, 0.0-6.0 min: 55.3% B; 6.0-10.7 min: 55.3% B; 10.7-10.9 min: 100% B; 10.9-14.5 min: 100% B; 14.5-14.6 min: 20% B; 14.6-16.0 min: 20% B. When biological amines were present the run time was prolonged till 18 min: 20% B.

4.4. Results and discussion

4.4.1. Validation of the developed method using pure AAs

Blank samples and blank hydrolysis mixture were injected to determine if random peaks co-eluted with peaks of interest. The AAs and biological amines were successfully analysed as individual peaks which did not co-eluted with any of the random peaks (data not shown). Figure 1 shows the chromatogram of a standard solution after derivatisation and separation. The amino acids that contain a primary amino group were derivatised with OPA/ET; proline, however, contains a secondary amino group, and was derivatised with FMOC.

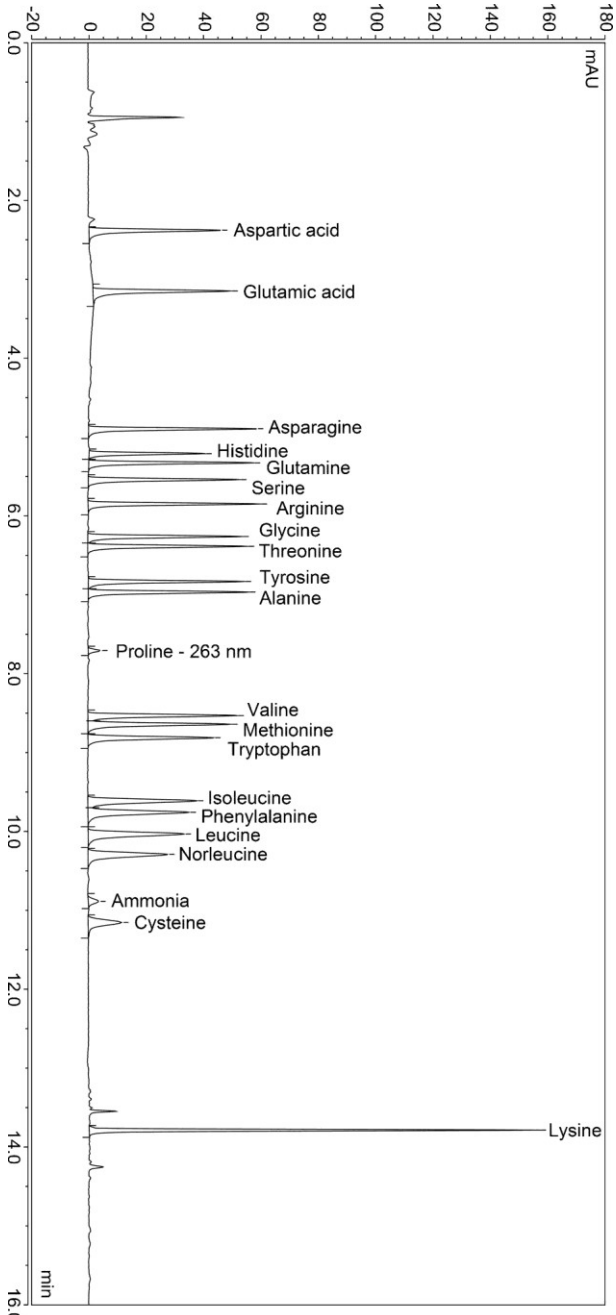

Figure 1. Elution profile of the pure amino acids (0.3 mM), derivatised with OPA/ET reagent in combination with FMOC measured at 338 and 263 nm, respectively. Amino acids were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured using a Dionex Ultimate 3000 Variable wavelength Detector.

The specificity of the method was determined by comparing the retention times obtained with the standard AA mixture (n=6) with those obtained from the reference BSA sample (200 mg/ml and n=12). The differences between the retention times were ≤ 0.1% (Table 2). The minimal retention time differences allow for a confident peak identification with a high specificity. Generally, a difference within 3% is considered acceptable between retention times of the same AAs present in the standard mixture and in the hydrolysed sample [13].

The linearity was determined using the correlation coefficients (r^2) for the measured AAs within the sample range of 0.01 to 0.50 mM. In general, the r^2 values were between 0.9991 and 1.0000. Cysteine formed an exception with an r^2 of 0.9973 and is partially destroyed by the acid hydrolysis that was used for the biological sample preparation. The mean r^2 of the measured AAs was 0.9997 (Table 2), demonstrating linearity of the detected response values within the measured concentration range. A major advantage of the use of ET in combination with OPA is the formation of one major derivatisation product per AA. In contrast, the response value after the derivatisation with OPA and 3-mercaptopropionic acid or N-acetylcysteine has to be calculated on the basis of 2 or 3 products [7]. Derivatisation with OPA and β-mercaptoethanol will also results in the formation of a single product. Yet these products are less stable than the ET derivatives and the fluorescent intensity is lower, particularly in a medium of high ethanol content [8]. The distinctive (unpleasant) odour of ET is a factor that should be accepted for this otherwise convenient thiol group donor.

The limit of detection (LOD) and limit of quantification (LOQ) for each of the AAs was determined using the calibration curves. The LOD was defined as a signal 3 times above the noise level and the LOQ was defined as the lowest concentration at which the signal could be analysed with an accuracy of 80-120%. The lowest LOD was measured for threonine and the highest for histidine with 1.1 and 6.2 µM, respectively. As expected, these two AAs also represented the lowest and highest LOQ with 2.1 and 12.4 µM, respectively. The mean LOD was determined to be 2.3 µM and the mean LOQ was slightly higher at 4.6 µM (Table 2).

Table 2. Retention times, calibration range, correlation coefficients, limits of detection (LOD), and quantification (LOQ).

AA	Retention time pure AAs from standard (min)	Retention time AAs originating from BSA (min)	Injected calibration range of sample (mM)	r^2	LOD (µM)	LOQ (µM)
Aspartic acid	2.32	2.32	0.01–0.50	0.9999	2.1	4.1
Glutamic acid	3.08	3.08	0.01–0.50	0.9998	2.7	5.3
Asparagine	4.87	N/A	0.01–0.50	1.0000	1.2	2.5
Histidine	5.21	5.20	0.01–0.50	0.9991	6.2	12.4
Glutamine	5.30	N/A	0.01–0.50	1.0000	1.4	2.9
Serine	5.51	5.51	0.01–0.50	1.0000	1.9	2.6
Arginine	5.95	5.95	0.01–0.50	0.9999	2.3	4.5
Glycine	6.23	6.23	0.01–0.50	0.9999	2.2	4.5
Threonine	6.35	6.36	0.01–0.50	1.0000	1.1	2.1
Tyrosine	6.82	6.82	0.01–0.50	0.9999	1.7	3.4
Alanine	6.94	6.94	0.01–0.50	1.0000	1.3	2.5
Proline[a]	7.72	7.72	0.01–0.50	0.9999	2.2	4.4
Valine	8.50	8.50	0.01–0.50	0.9999	2.1	4.2
Methionine	8.61	8.61	0.01–0.50	1.0000	1.5	2.9
Tryptophan	8.86	N/A	0.01–0.50	1.0000	1.4	2.8
Isoleucine	9.57	9.57	0.01–0.50	0.9999	2.2	4.3
Phenylalanine	9.72	9.72	0.01–0.50	0.9999	2.1	4.1
Leucine	9.98	9.98	0.01–0.50	0.9999	1.8	3.5
Cysteine	10.23	10.24	0.005–0.25	0.9973	4.5	9.0
Lysine	13.73	13.73	0.01–0.50	0.9994	5.0	10.0
Mean				0.9997	2.3	4.6

The high correlation coefficients, combined with the low LOD and LOQ values of the individual AAs indicate that the derivatisation in the injection needle and separation by the UPLC were both very precise and sensitive. With the developed method, it is also possible to qualitatively detect the biological amines GABA, putrescine, cadaverine, citruline, ornithine and norleucine as individual peaks (data not shown).

Amino acid (AA) samples originated from the standard solution using pure AAs and acid hydrolysed bovine serum albumin. AAs were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured at 338 nm using a Dionex Ultimate 3000 Variable wavelength Detector. Derivatised with FMOC and absorbance was measured at 263 nm

4.4.2. Amino acid analysis in bovine serum albumin

Acid hydrolysis is the most common protein hydrolysis method used for the determination of single AAs and employs 6 M of HCl at 110ºC *in vacuo* for 24 hours [14-16]. Not all AAs survive this hydrolysis process. Asparagine and glutamine are completely converted into aspartic and glutamic acid, respectively. Tryptophan is completely destroyed [17] and it is impossible to accurately determine cysteine levels in acid-hydrolysed samples. There are alternative hydrolysis methods that enable the determination of methionine and cysteine in samples, such as methanesulfonic acid hydrolysis [18] or performic acid oxidation [19]. Tryptophan levels can be determined after the hydrolysis of a sample with 4.2 M NaOH. In an extensive study the mean recovery of tryptophan was 85% with a variation of 59-102% [20]. A major drawback of this alkaline hydrolysis is the destruction of arginine, cysteine, serine, and threonine. In order to obtain a complete analysis of the AA content in the protein samples the alkaline hydrolysis was performed in our laboratory. The obtained results had a poor recovery and accuracy although it was possible to obtain indicative results in line with the available AA composition of BSA (unpublished data).

After the determination of the calibration range with single AAs, accuracy and precision of the method were determined by using acid hydrolysed BSA. BSA was chosen due to its known composition and purity of the commercial product. Total time between injections was 22 minutes with a runtime of 16 minutes. Sharp individual peaks were observed (Figure 2). Hydrolysis and analysis of the BSA samples was performed on 3 individual days (k=3) at 2 different concentrations (100 and 200 mg/ml BSA) instead of the desired 3 due to the limited sample positions in the hydrolysis station. The hydrolysis station can hold 4 reaction vial assemblies each capable of holding 3 reaction vials leading to a total of 12 spots, and since each concentration was measured with 6 samples per day (n=6), all 12 spots were then accounted for. The samples were measured in duplicates. The responses were expressed as ratios of the AA peak areas to the internal standard (norleucine) peak area.

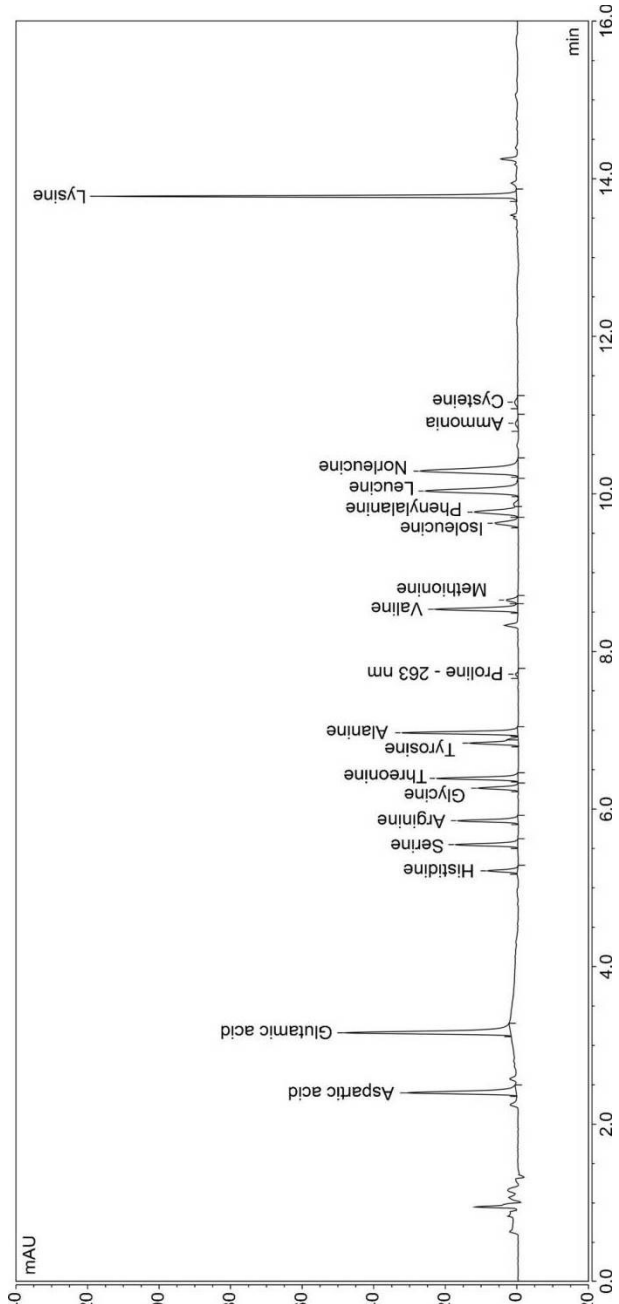

Figure 2. Elution profile of acid hydrolysed bovine serum albumin, derivatised with OPA/ET reagent in combination with FMOC measured at 338 and 263 nm, respectively. Amino acids were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured using a Dionex Ultimate 3000 Variable wavelength Detector.

Accuracy was defined as the agreement between the found value and the true, independently determined, concentration value. The accuracy of the method was evaluated using the recovery and the relative standard deviation

The absolute recovery (k=3) was lowest for serine and highest for lysine with 80.7 and 100.8% recovery, respectively. This was expected since the acid hydrolysis partially hydrolyses serine. The mean absolute recovery was 94.7% which is within the 90-110% range that is considered acceptable [13]. The relative standard deviation (RSD) for most samples was well below 2.0% with the exception of proline and methionine representing 3.7 and 3.2%, respectively (Table 3). Cysteine is included in the table but excluded from the calculations since it was detected after derivatisation. Yet it is destroyed to such an extent that no accurate data could be obtained. The accuracy (n=6, k=1) for most AAs was over 95% on average. The confidence interval was largest for proline with 0.9% and as little as 0.1% for the glutamic acid/glutamine fraction, serine, arginine, glycine, and methionine. This shows very high accuracy of our method.

The precision of the method was measured as the repeatability and the intermediate precision. Reproducibility, which refers to the use of the analytical procedure in different laboratories, was beyond the scope of the present study. The method has a repeatability (r) (n=6 and k=3) well below 4.0% for most AAs. The lowest percentage was measured for the glutamic acid/glutamine fraction (0.4%). The highest percentages were obtained for proline followed by methionine with 9.9 and 7.2%, respectively. The mean repeatability for the measured AAs was 3.0%. The intermediate precision (R) (n=18) was very diverse ranging from 0.45 to 14.1% for the glutamic acid/glutamine fraction and methionine, respectively. The mean reproducibility of all AAs was 6.8% (Table 3).

Table 3. Mean absolute recoveries (percentage), accuracy (percentage), and precision obtained for each amino acid (AA) after acid hydrolysis of bovine serum albumin.

AA	Absolute Recovery (k= 3)		Accuracy (n=6, k= 1)		Precision (k= 3)	
	Mean (%)	RSD (%)	Mean (%)	Confidence interval (%)	Mean r (n=6)	Mean R (n=18)
Asx[a]	99.3	1.0	100.5	0.3	1.4	7.0
Glx[b]	100.2	0.1	100.2	0.1	0.4	0.4
Histidine	97.9	1.7	97.6	0.2	2.8	9.7
Serine	80.7	0.6	81.0	0.1	1.7	2.6
Arginine	88.6	0.5	89.1	0.1	1.0	3.1
Glycine	94.6	1.0	95.3	0.1	2.3	4.0
Threonine	93.7	0.7	94.1	0.2	1.8	3.2
Tyrosine	98.8	1.8	98.0	0.3	4.2	9.4
Alanine	99.6	0.6	99.7	0.2	1.6	2.0
Proline[c]	96.8	3.7	98.1	0.9	9.9	13.9
Valine	92.4	1.7	93.7	0.4	3.1	8.5
Methionine	95.5	3.2	96.0	0.1	7.2	14.1
Isoleucine	89.7	1.9	91.5	0.2	3.4	11.3
Phenylalanine	90.5	1.0	90.8	0.2	2.4	4.7
Leucine	94.9	1.4	96.3	0.4	2.0	9.9
Cysteine	25.1	22.4	20.2	1.6	53.5	95.5
Lysine	100.8	1.1	101.1	0.5	2.7	4.4
Mean	94.7	1.4	95.2	0.3	3.0	6.8

AAs were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/ 9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured at 338 nm using a Dionex Ultimate 3000 Variable wavelength Detector. Cysteine was not included in the mean values due to the fact that this AA is largely destroyed during the acid hydrolysis k number of days of analysis, n number of repetitions, r repeatability coefficient, and R reproducibility coefficient

a After acid hydrolysis, asparagine is converted into aspartic acid resulting in a combined peak

b After acid hydrolysis, glutamine is converted into glutamic acid resulting in a combined peak

c Derivatised with FMOC and absorbance measured at 263 nm

In our study, an UPLC system was used for the separation of the derivatives, resulting in a run time of only 16 minutes for AAs or 18 minutes including biological amines. This greatly reduced the time for analysis compared to a study by Körös and co-workers [21] where the same derivatisation agents were used but separation took 45 minutes because an HPLC system was used. This reduction was possible due to the use of smaller sized particles as filling material of the HPLC columns. With the UPLC system a great reduction in solvent use was achieved; from 17.5 ml acetonitrile and 23.0 ml methanol to 0.8 and 2.4 ml, respectively. Another HPLC method used in the separation of proteinogenic AAs required 18.3 ml for both acetonitrile and methanol [12]. Compared to other UPLC methods our method does not vary greatly in the consumption of solvents [12].

4.4.3. Analysis of biological samples

Data from the analyses of pure AAs in combination with the BSA sample proved the robustness of the employed method. However, these samples did not contain any of the impurities such as iron, copper or carbohydrates that can be present in biological samples. These contaminations can result in unusual artefacts and/or the loss of AAs. Soy meal, de-oiled Jatropha press cake, and cyanophycin were selected as biological protein samples. Three individual samples per test substance were acid hydrolysed and injected one time per sample. The samples and AA standards were eluted from the column using the biological amine elution program.

According to our analysis of the single AAs the soy meal sample contained 49% protein. This is comparable to the data (45%) provided by the Nutrient Data Laboratory from the United States Department of Agriculture [22]. When the individual AA concentrations were compared between the samples, only minor differences were observed. These differences can be contributed to the fact that biological samples are compared and the displayed values are rounded off (Table 4, Figure 3).

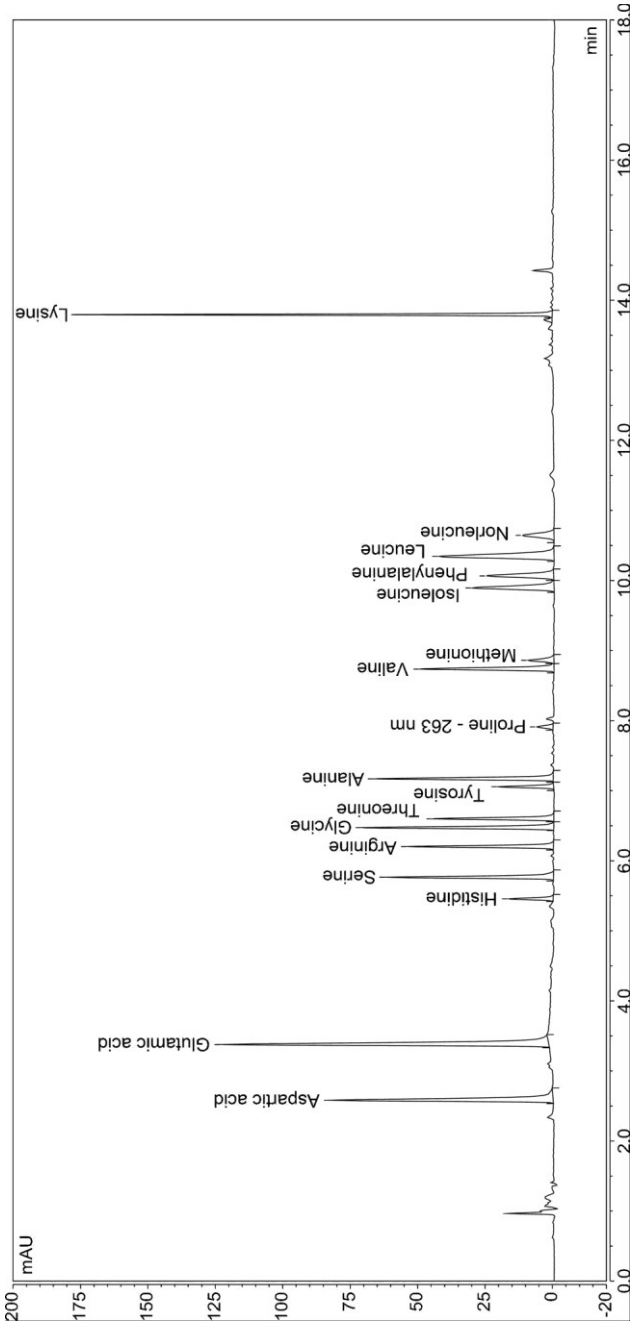

Figure 3. Elution profile of acid hydrolysed soy meal, derivatised with OPA/ET reagent in combination with FMOC measured at 338 and 263 nm, respectively. Amino acids were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured using a Dionex Ultimate 3000 Variable wavelength Detector.

The Jatropha press cake contained 15% protein according to our analysis of the single AAs (Table 4, Figure 4). In a different study the protein content (determined with the Kjeldahl method) of a sample obtained from the same supplier was estimated at 27% protein [23]. However, Lestari and co-workers employed nitrogen to protein conversion factor of 6.25, which is the conversion factor for pure protein. Calculating the protein content on the basis of total nitrogen in samples that also have other nitrogen-containing components leads to an overestimation of protein content. While calculating the protein content on the basis of acid hydrolysed sample leads to an underestimation due to the loss of AAs.

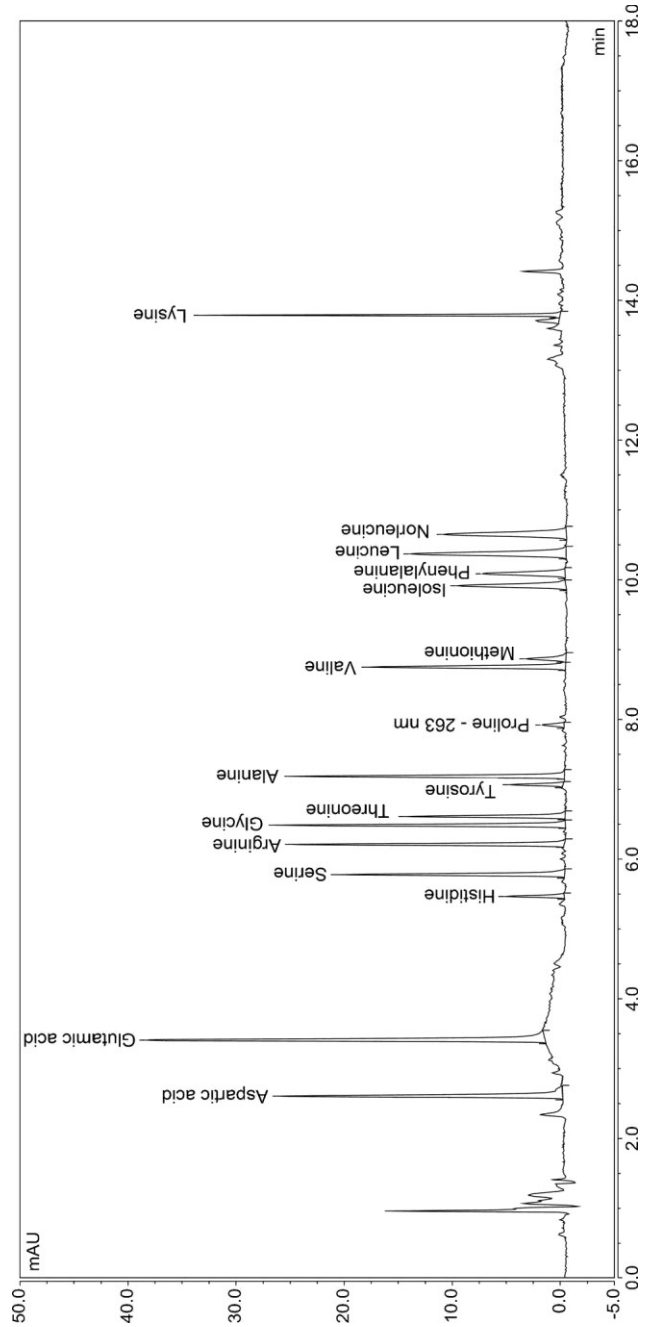

Figure 4. Elution profile of acid hydrolysed Jatropha press cake, derivatised with OPA/ET reagent in combination with FMOC measured at 338 and 263 nm, respectively. Amino acids were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/ [OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured using a Dionex Ultimate 3000 Variable wavelength Detector.

The cyanophycin sample comprised primarily of the aspartic acid/asparagine fraction and arginine representing 392, and 461 mg/g respectively (Table 4, Figure 5). A smaller fraction was formed by lysine with 6 mg/g. This is in accordance with the composition of this particular batch described by Elbahloul and co-workers [11]. The comparability of previous results to the results of the new method indicates that the AA composition of proteogenic samples can reliably be determined.

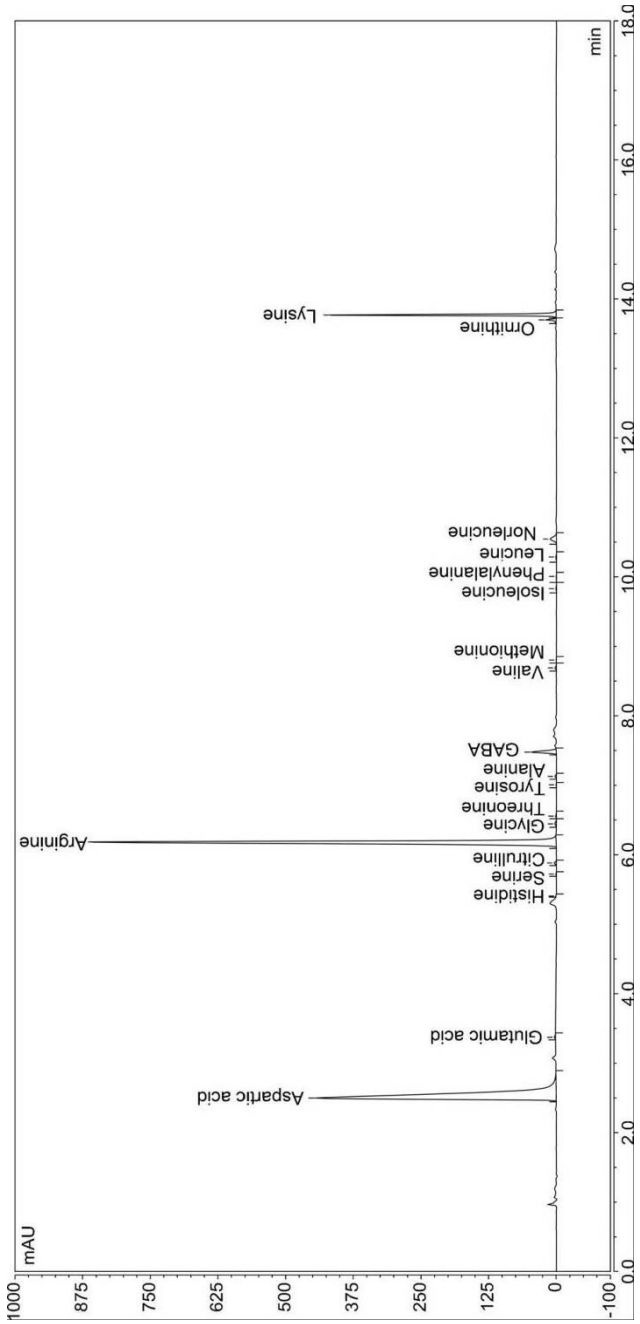

Figure 5. Elution profile of acid hydrolysed cyanophycin, derivatised with OPA/ET reagent in combination with FMOC measured at 338 and 263 nm, respectively. Amino acids were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured using a Dionex Ultimate 3000 Variable wavelength Detector.

Table 4. AA analysis of various biological samples.

AA	Soy meal mg/g	Soy meal mg/g reference values[a]	Jatropha Press Cake mg/g	Cyanophycin mg/g	Retention time Soy/ Jatropha/ Cyanophycin
Asx[b]	58	57	17	392	2.59/2.63/2.62
Glx[c]	91	87	26	0	3.37/3.41/3.37
Histidine	13	12	4	0	5.45/5.46/5.41
Serine	26	26	9	0	5.76/5.77/5.72
Arginine	34	35	15	461	6.20/6.21/6.18
Glycine	22	21	8	0	6.47/6.48/6.44
Threonine	21	20	7	0	6.59/6.61/6.56
Tyrosine	14	17	3	0	7.05/7.05/7.01
Alanine	23	21	9	0	7.16/7.18/7.13
Proline[d]	29	26	8	0	7.94/7.95/7.89
Valine	25	22	9	0	8.73/8.74/8.69
Methionine	7	6	3	0	8.85/8.86/8.80
Isoleucine	24	22	8	0	9.88/9.90/9.83
Phenylalanine	26	23	8	0	10.05/10.07/10.01
Leucine	40	37	12	0	10.33/10.35/10.28
Lysine	32	30	6	6	13.79/13.79/13.77
Total	485	450	149	859	

The samples were acid hydrolysed in triplicates and injected once. AAs were derivatised with OPA/ET with the order based on ascending elution time. The final molar ratios of [ET]/[OPA]/[FMOC]/[AAs] are 23/9.9/3.2/1. Separation was achieved with a Dionex RSLC system using an Acquity UPLC BEH C18 reversed-phase column. Absorbance was measured at 338 nm using a Dionex Ultimate 3000 Variable wavelength Detector.

a. Reference values obtained from the United States Department of Agriculture [22]
b. After acid hydrolysis, asparagine is converted into aspartic acid resulting in a combined peak
c. After acid hydrolysis, glutamine is converted into glutamic acid resulting in a combined peak
d. Derivatised with FMOC and absorbance measured at 263 nm

4.5. Conclusions and outlook

The developed method is comprised of two parts. The first part consists of the acid hydrolysis and derivatisation of the individual AAs and biological amines with OPA/ET-FMOC in the injection needle, followed by separation and subsequent detection of the derivatives using UPLC. The specificity of the method was demonstrated by comparing the retention times of the AAs from the hydrolysed BSA to standard solutions. The linearity was demonstrated for the single AAs by high correlation coefficients above 0.999. The mean LOQ and LOD were 2,5 and 5,0 µM, respectively. The accuracy was determined by comparison of the found AA amounts to the known sample composition of BSA. The mean accuracy was calculated using 6 measurements in one day and was 95% with a mean confidence interval of 0,3%. The precision was calculated based on 18 measurements from 3 days, with a mean of 6.8%. The determined validation parameters are in the commonly acceptable ranges for this kind of analysis.

Additionally, the AA composition of protein samples containing naturally occurring contaminants could be determined with good recoveries. Furthermore, proline levels could be determined using the FMOC derivatisation.

Full separation occurred within 16 minutes runtime. Combined with the automated derivatisation method, this led to a total time between injections of only 22 minutes for single AAs. The method can be extended to 24 minutes to include biological amines. With the current techniques, it is not possible to significantly reduce the separation time any further. The use of an UPLC over HPLC analysis resulted in a significant reduction in the organic solvents required, thereby resulting in much greener method.

The optimized method for AA analysis in complex mixtures presented here is reliable, fast, inexpensive and greener due to a reduction in solvent consumption. It was shown to be applicable to a wide range of biological samples and is very suitable for the screening of large amounts of samples. Furthermore, an extended method can be employed for the detection of biological amines GABA, putrescine, cadaverine, norleucine.

These compounds form intermediates in reactions used to form compounds of interest in the biobased economy [5, 6].

The remaining disadvantage of the method is formed by the time required for full acid hydrolysis. In theory it should be possible to reduce the hydrolysis time for this method to less than one hour if micro-wave radiation is employed [14]. However, this still requires manual handling of the hydrolysed samples prior to derivatisation.

4.6. Acknowledgment

This project was supported by a grant (EOSLT02034) provided by SenterNovem (Utrecht, the Netherlands).

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Chapter 5.
Introduction of β-1,4-endoxylanase activity in *Rhizopus oryzae*

5.1. Abstract

Rhizopus oryzae 99-880 is not able to hydrolyse the plant polysaccharide xylan, which forms a large fraction of hemicellulose. In order to enable xylan hydrolysis a β-1,4-endoxylanase encoding gene from *Aspergillus niger* CBS 513.88 was introduced. A stable transformant was obtained with a xylanase activity of 3.5 IU/ml culture broth. The correlated specific activity was 1.5 U/µg protein. The transformed strain was able to grow – albeit slowly - on xylan.

5.2. Introduction

It is estimated that in the coming decades fossil fuels such as coal, gas and oil will become scarce. As a result it will be economically unfeasible mining these resources for bulk use. Utilization of alternative energy sources in the form of geothermal, water, wind and solar energy can provide a large part of the energy requirement. These resources do not form an alternative for production of platform chemicals or liquid transportation fuels. A feasible alternative feedstock for these chemicals is biomass [1]. In first generation biofuel production processes, edible sugars, vegetable oils or starch are directly converted to the end product by biological or chemical conversion processes. A good example is the production of bioethanol form corn starch in the United States. In the past the starch-producing plants were mainly grown for feed or food production. With the rise of first generation biofuels the “food versus fuel” debate started. To reduce competition for edible resources between food and feed on one side and fuel and chemicals on the other side, second generation production processes are being developed. Using the second generation process the production potential of a land area is increased by using inedible lignocellulosic biomass as well [2]. As a result additional resources for production of fuels and chemicals become available without compromising food production. Lignocellulosic biomass is rich in polysaccharides such as cellulose (35-50%) and hemicellulose (5-20%) and contains a significant lignin fraction [3].

Hemicellulose is heterogeneous and the precise composition depends on plant species, tissue and growth conditions [4]. It is classified according to the main sugar in the backbone: mannan (β-1,4-linked D-mannose), xyloglucan (β-1,4-linked D-glucose) and xylan (β-1,4-linked D-xylose). The backbone can be decorated with side groups such as D-galactose, D-xylose, L-arabinose, and D-glucuronic acid. The complete degradation of hemicelluloses requires a large repertoire of enzymes.

Microorganisms containing this repertoire and utilizing the constituent monosaccharides for the production of platform chemicals are of commercial interest. The filamentous fungus *R. oryzae* is such a microorganism. This organism is able to grow on a wide range of biomass-derived feedstocks *e.g.* wheat bran, potato powder, dried cereals, cellulose and hydrolysates of rice straw, corn straw,

wheat bran and corn cob [5-12]. When grown on D-glucose L-(+)-lactic acid or fumaric acid in combination with ethanol are produced in excess of 85% of the theoretical yield. Also D-xylose can be used as substrate [13, 14]. The specific production routes for these platform chemicals and corresponding yields were reviewed by Meussen and co-workers [15].

The genome of *R. oryzae* 99-880 was sequenced [16] and an extensive analysis of the genome shows a different set of hydrolysing enzymes in comparison to Ascomycetes and Basidiomycetes [17] . Less putative enzymes were detected involved in the degradation of complex carbohydrates like hemicellulose but more towards utilization of simple carbohydrates.

Although *R. oryzae* 99-880 is able to utilize xylose as carbon source, it is not able to hydrolyse xylan. Introducing this ability would enhance the applicability of the fungus. *R. oryzae* is however difficult to engineer and only two genes have been successful functional heterologous expressed far [15, 18]. In this study a β-1,4-endoxylanase e*ncoding gene* from *A. niger* CBS 513.88 was introduced in *R. oryzae* 99-880 with the aim to utilize xylan as carbon source.

5.3. Materials and Methods

5.3.1. Strains, media, growth conditions and methods

The One Shot® Mach1™ T1 Phage-Resistant *Escherichia coli* strain obtained from Invitrogen (Carlsbad, CA) was used for plasmid maintenance and propagation. The cells were grown in Luria-Bertani (LB) medium containing 50 µg/ml ampicillin at 37°C while agitated at 250 rpm in an orbital shaker. *R. oryzae* M16 [19] was used as a host organism in transformation. *R. oryzae* M16 was a kind gift of Dr. C. D. Skory of the United States Department of Agriculture, Peoria, IL. This strain is derived from the sequenced strain *R. oryzae* 99-880 (Fungal Genetics Stock Center FGSC 9543) and contains a point mutation in the orotate phosphoribosyltransferase (*pyrF*) encoding gene of *R. oryzae* 99-880 [16]. The transformants were maintained on synthetic *Rhizopus* (RZ) medium [20] containing 1.5% (wt/v) Oxoid agar number 1 and 2.0% D-glucose.

For growth of *R. oryzae* M16 the medium was supplemented with 0.5 mg/ml uracil. The plates were incubated for 4 days at 30 °C. Spores were harvested using a saline Tween-80 solution [0.9% (w/v) NaCl, 0.005% (v/v) Tween-80] and a glass spatula under mild agitation. Biomass of *R. oryzae* transformants and wild-type was generated by cultivation in shake flasks containing 100 ml RZ medium using 100 g/l of D-glucose, D-xylose, 2% beech xylan or birch xylan as carbon source. The initial spore concentration in the shake flasks was 10^6 spores per ml. The cultures were incubated for 96 hours at 30°C under constant agitation in an orbital shaker at 200 rpm. To maintain a stable pH, 10 g/l $CaCO_3$ was added after 18 hours. When the $CaCO_3$ was almost dissipated fresh $CaCO_3$ was added. All experiments were performed with biomass obtained from liquid cultures unless stated otherwise. *A. niger* CBS 513.88 [20] was grown on minimal medium [21]. This strain can be obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. All experiments were performed with biomass or culture filtrate obtained from liquid cultures unless stated otherwise.

5.3.2. DNA techniques

A β-1,4-endoxylanase e*ncoding gene (*xynB GenBank: CAK43456.1) from *A. niger* CBS 513.88 was cloned from cDNA by PCR amplification using Phusion DNA polymerase according to the manufacturer's instructions (Finnzymes, Espoo, Finland). The cDNA was a kind gift of Dr. J.M. Oliveira from the Wageningen University, the Netherlands. The oligonucleotide primers used to clone the gene were xynBF and xynBR (Table 1). These primers contained the restriction sites for *Sph*I and *Pac*I in the start and stop codon, respectively. The correct size of the DNA fragments was verified by agarose gel electrophoresis using 0.8% agarose with 0.5 times Tris-Acetate-EDTA buffer. The amplicons were ligated into the plasmid pJET1.2/blunt using the CloneJET™ PCR Cloning Kit (Fermentas international Inc, Burlington, Canada) according to the manufacturer's instructions. Electro-transformation of *E. coli* was performed with the Gene Pulser II using cuvettes with a 0.2 cm gap (Bio-Rad, Hercules, CA). Isolation of plasmid DNA was performed using a GeneJET™ plasmid miniprep kit (Fermentas international Inc) according to the manufacturer's instructions. Plasmids pJET::*xynB* and pPdcExPyrF were digested with restriction enzymes *Sph*I and *Pac*I (New England Biolabs, Ipswich, MA) and the

isolated *xynB* gene was subsequently ligated into the pPdcExPyrF vector to yield plasmid pPdcExPyrF::*xynB*. The nucleotide sequence of the *xynB* gene was verified by DNA sequence analysis (Baseclear BV, Leiden, The Netherlands).

Table 1. Oligonucleotide primers used in this study.
Characters in bold indicate the restriction sites that were introduced.

Oligonucleotide primer	Oligonucleotide primer sequence 5' to 3' orientation
xynBF	**GCATGC**TCACCAAGAACCTTCTCCTC
xynBR	**TTAATTAA**TGAACAGTGATGGACGAAGATC
kanqF	AGCATTACGCTGACTTGACG
kanqR	AGGTGGACCAGTTGGTGATT
pdcqF	ACAGCCGAATTTGCTTCACT
pdcqR	GATAGCGGCCCTACAGAGG
xynBqF	CTACCCGTACCAATGCTGCT
xynBqR	ATGGACGAAGATCCACTGCT

5.4. Transformation of *Rhizopus oryzae*

Transformation of *R. oryzae* M16 spores was achieved by particle bombardment. M5 tungsten particles (Bio-Rad, Hercules, CA) were coated with plasmid DNA according to the manufacturer's instructions. The particles were delivered to 10^5 spores dispersed on the surface of an RZ plate using the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA). The distance between the rupture disc and the particles and the particles and the spores was 1,6 and 6 cm, respectively. The pressure was set with a rupture disc with a failure pressure of 1,100 psi. The plates were incubated at 30°C for 5 to 7 days to allow the formation of mycelium and spores. After sporulation the spores were harvested and serial dilutions were made to ensure the inoculation of a single spore on each plate. This process was repeated to ensure single progeny since *R. oryzae* cells are multinucleated.

5.5. Transcript analysis

To determine the presence of transcript of the gene of interest (*xynB*) liquid cultures were grown as described before using RZ medium and 10% D-glucose and D-xylose. Mycelium was harvested over a period of 4 days, snap frozen in liquid nitrogen and ground using a Braun micro-dismembrator (Braun, Melsungen, Germany). Total RNA was isolated and cDNA synthesized as described by Oliveira and co-workers [23]. The cDNA served as the template for quantitative real-time PCR (qPCR). Primers were designed for *xynB*, *pdcA* genes with the Primer3 program. The amplicons had a specific melting temperature of 60 °C±1°C, GC content 50% ± 5% and size was between 139 and 150 base pairs. Primers used for *xynB* encoding gene were; xynBqF and xynBqR, for *pdcA*; pdcqF and pdcqR and for the kanamycin transcript; kanqF and kanqR (Table 1). The PCR mixtures were pipetted with the CAS-1200 robot (Corbett Life Science, Sydney, Australia). Reaction mixtures for real-time PCR had a total volume of 16 µl and contained 4 µl cDNA (2.5 ng/µl), 1 µl of each primer (1.5 µM) and 10 µl ABsolute QPCR SYBR Green Mix (ABgene, Epsom, UK). The Rotor-Gene 3000 (Corbett Life Science) was used for thermal cycling and real-time detection of the DNA. The melting analysis feature was used to determine primer–dimer formation. The comparative quantitation feature was used to determine take-off and amplification values. Relative expression was calculated by the Pfaffl method [24]. Normalization was performed on basis of the added kanamycin external transcript proportional to the total RNA used for cDNA synthesis. Additionally normalisation was performed on basis of the internal *pdcA* transcript.

5.5.1. Semi-quantitative analysis of β-1,4-endoxylanase activity

A semi-quantitative plate assay was used to screen for β-1,4-endoxylanase activity in the transformants and the fungal host strains [24]. Agar-plates contained of a bottom layer of synthetic medium (RZ or minimal medium) with 25 mM pure carbohydrate or 0.2% xylan and a top layer consisting of synthetic medium containing 0.1% azurine-cross-linked (AZCL) xylan from oat (Megazyme, Wicklow, Ireland). The plates were inoculated with 100 spores per plate and incubated at 30 ºC for a time period up to 4 days. Upon hydrolysis of the xylan the azurine is

released resulting in the formation of a blue halo. The growth of the mycelium and halo formation in the plates was monitored in order to semi-quantitatively determine the production of the β-1,4-endoxylanase in time.

5.6. β-1,4-endoxylanase activity assay

A modified version of the Megazyme AZO-WAX enzyme assay was used to determine enzyme activity. The original protocol was based on solid samples and made use of 5.0 ml sodium acetate buffer (100 mM, pH 4.7) and 0.2 ml enzyme solution. In the modified assay *44 µl* culture broth *was mixed with 438.6 µl s*odium acetate buffer. As a control either 17.5 µl enzyme solution from the kit or water was added to the assay. The assay was further performed as described by the manufacturer and the results are given in U/ml. The reducing sugar procedure is based on the Somogyi-Nelson procedure. The specific enzyme activity was determined as units of activity per µg extracellular protein (U/µg). The protein concentration in the medium was determined with the Bradford microassay procedure according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Bovine serum albumin (BSA) was used as standard to calculate protein concentrations.

5.7. Analysis of the xylan degradation pattern

Mycelium was grown in liquid medium containing D-glucose as carbon source. 48 Hours after inoculation a sample of the medium was concentrated 16 times using a Vivaspin column (cut-off value 10 kDa) (Sartorius AG, Goettingen, Germany). A suspension of 0.1% beech xylan in Megazyme reaction buffer was prepared containing ~8 U/ml of culture concentrate. This suspension was incubated for 8 hours at 50 °C after which the samples were heat inactivated for 5 minutes at 100 °C. The samples were spun down in an Eppendorf centrifuge at 12,100 g for 10 minutes. The supernatant was diluted 10 times in demineralized water and analyzed using a Dionex system (Sunnyvale, CA, USA) equipped with ED50 electrochemical detector. Separation of the mono and oligosaccharides was performed on an anionic exchange column (Carbopac PA 100, 25*4 mm).

Elution of the samples was achieved in a NaOH gradient starting for the first 10 minutes at 0.05 M, which increased to 0.90 M in the following 20 minutes. Reference samples were xylobiose, xylotriose, xylotetraose and xylopentaose (Megazyme) supplemented with D-glucose, D-xylose and D-arabinose.

5.8. Transfer experiment with different carbon sources

Mycelium was grown in liquid medium containing D-glucose as carbon source. Mycelium was harvested after 48 hours on a nylon filter under mild suction and washed twice with saline. RZ medium containing 2% beech or oat xylan was inoculated with this mycelium and incubated for 14 days at 30 ºC while agitated at 200 rpm.

5.9. Results

5.9.1. Introduction of β-1,4-endoxylanase in Rhizopus oryzae

In order to increase the hydrolytic capacity of *R. oryzae* 99-880 towards plant biomass a β-1,4-endoxylanase from *A. niger* CBS 513.88 was introduced. Spores of the *pyrF* auxotrophic mutant of *R. oryzae* 99-880 were transformed by the biolistic transformation system using plasmid pPdcExPyrF::*xynB*. In total 21 transformants were isolated which displayed auxotrophic growth on synthetic medium containing D-glucose. These transformants were screened for β-1,4-endoxylanase activity using an AZCL-xylan plate assay. Three transformants displayed β-1,4-endoxylanase activity as observed by the formation of a blue halo (data not shown), indicating that the *xynB* gene was expressed and the xylanase protein was active. The phenotype of two of these transformants was unstable during 2 consecutive germination and sporulation cycles indicated by the partial loss of the ability to hydrolyse the AZCL-xylan (data not shown) and were discarded from the experiment. Transformant number 8 (*R. oryzae* T8) showed a stable phenotype, indicating that the plasmid integrated into the genome.

5.9.2. Determination of the expression levels of xynB and xylanase activity

With the aid of quantitative real-time PCR (qPCR) the expression of *xynB* in T8 and a control was followed over a time period of 4 days. Gene expression rose strongly between 24 and 48 hours after which the expression levelled off (Figure 1). In the wild-type strain the transcript level of the *xynB* encoding gene represented less than 1‰ of the PDC encoding gene and this was considered a non-specific product.

The β-1,4-endoxylanase activity in liquid medium containing D-glucose as carbon source was determined in triplicate. The highest enzyme activity was measured in the sample taken after 48 hours and represented 3.5±0.2 IU/ml (Figure 1), with 1 unit defined as the amount of enzyme required to release 1 µM of xylose reducing equivalent per minute. The highest specific activity (1.5±0.1 IU/µg total protein) was detected at 24 hours and decreased in time; this as a result of an increase in protein concentration and an eventual decrease in enzyme activity. It should be noted that the total extracellular protein concentration is very low and in the order of µg/mL. In broths from the wild-type strain no β-1,4-endoxylanase activity was detected at any time point.

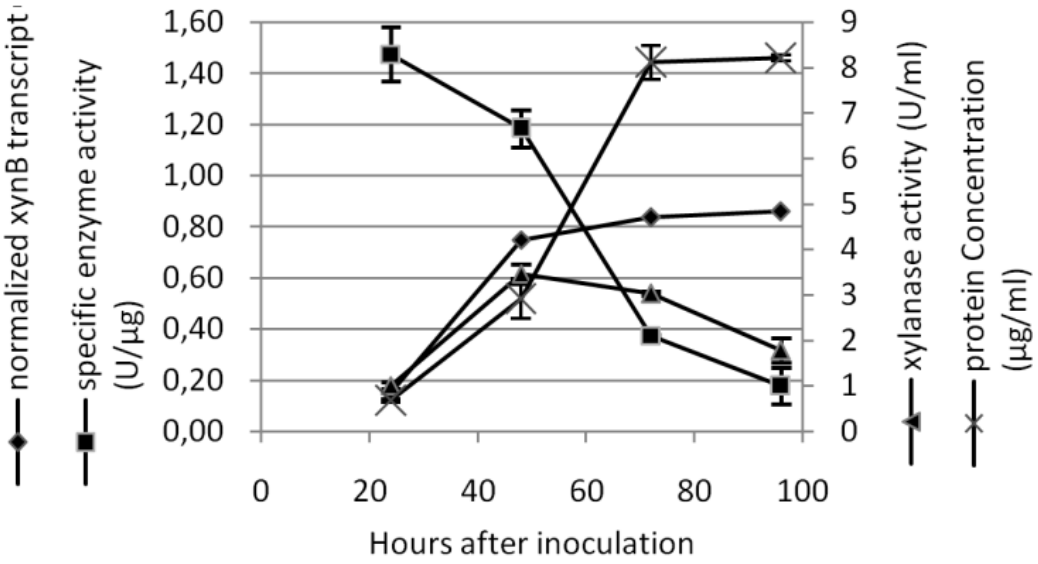

Figure 1. Evaluation of *R. oryzae* transformant T8 expressing *xynB* in respect to gene expression, enzyme activity and extracellular protein production.

5.9.3. Growth experiments on various carbon sources

To determine if the introduction of *xynB* improved growth on xylan, a semi-quantitative plate assay was performed. D-glucose, D-xylose, beech, birch and oat xylan were used as carbon sources (Figure 2). The mycelium grew well on D-glucose and D-xylose and slow growth was observed on the various xylan sources. Liquid medium was inoculated with spores using xylan as sole carbon source. Biomass was not formed and microscopical analysis of the spores revealed that the spores would not fully germinate (results not shown). To circumvent poor germination on xylan cultures were inoculated with mycelium. Biomass starter cultures were pregrown using D-glucose, the mycelium was carefully washed and transferred to fresh medium containing xylan as sole carbon source. Growth was observed only for T8 although it was very slow. Accurate quantification of growth was impossible due to the low growth rate and the inclusion of the undissolved xylan particles into the biomass. The inclusion of undissolved particles into the mycelium is a common problem when the biomass forms pellets or clumps.

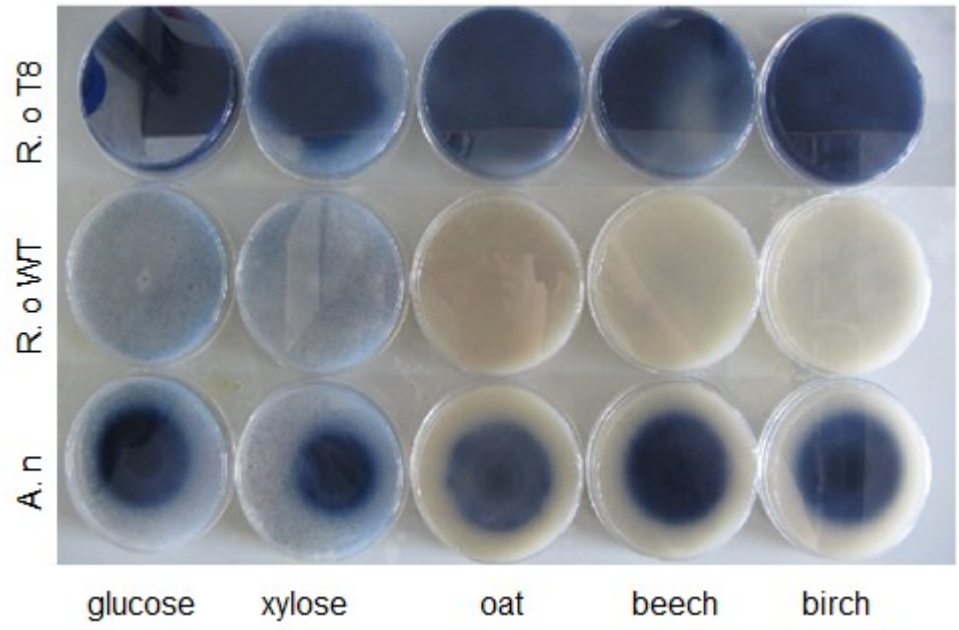

Figure 2. Evaluation of xylan degradation capacities of various strains. A. n; *Aspergillus niger* CBS 513.88, R. o WT; *R. oryzae* 99-880, R.o T8; *R. oryzae* T8. Grown on 25 mM glucose, 25 mM xylose and 0.2% oat, beech and birch xylan containing an AZCL-xylan top layer for 4 days.

5.9.4. Degradation patterns of xylan after hydrolysis

Beech xylan was digested by concentrated supernatant from strain T8 and analysed for the liberation of mono- and oligomeric-saccharides. No detectable amount of arabinose and low amounts of xylose were found. Oligo xylose sugars were detected with a predominance of xylobiose and xylotriose (Figure 3). Initially, the main hydrolysis products of xylan are D-xylopyranosyl oligomers and at later stage smaller molecules such as mono-, di- and tri-saccharides of D-xylopyranosyl may be produced [25].

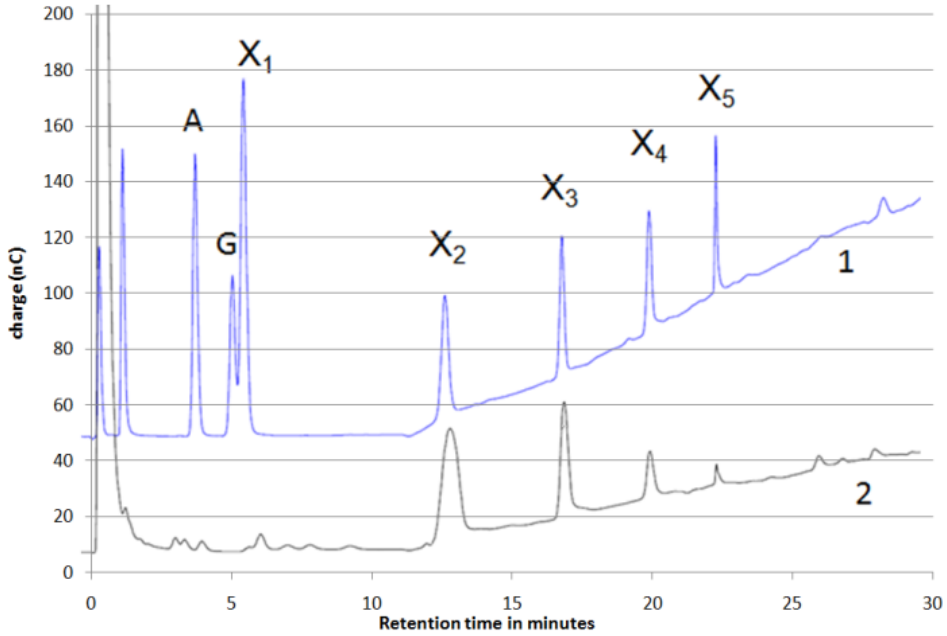

Figure 3. Hydrolysis profile of beech xylan digested by β-1,4-endoxylanase. Line 1 is a reference sample containing pure sugars (0.6 mM) and line 2 is a xylan sample hydrolysed by concentrated culture filtrate from *R. oryzae* T8. A; L-arabinose, G; D-glucose, X_1;D-xylose, X_2; xylobiose, X_3; xylotriose, X_4; xylotetraose, X_5; xylopentose.

5.10. Discussion

The auxotrophic mutant used in our study is derived from *R. oryzae* 99-880 and unable to degrade xylan and possesses few putative enzymes involved in the degradation of complex carbohydrates [17]. Many *R. oryzae* strains are known to grow on complex substrates but few studies were performed regarding detection of the specific enzyme β-1,4-endoxylanase. In one study 67 *Rhizopus* spp. were screened and three *R. oryzae* strains displayed β-1,4-endoxylanase activities of 306, 372 and 508 IU/ml [26]. In another study a β-1,4-endoxylanase from *R. oryzae* ATCC 9363 was partial purified and characterized [28]. The maximal enzyme activity for this strain was 260 IU/ml and the DNS method was employed in both studies. Subsequent measurements by Bakir and co-workers used the Somogyi-Nelson method and the initial enzyme activity in rich medium supplemented with xylan was 0.39 IU/ml. Extensive optimization of the medium, pH, and temperature resulted in a maximum activity of 20.5 IU/ml was obtained [27]. Transformant T8 generated in our study produced a maximal enzyme activity of 3.5±0.2 IU/ml in synthetic medium without optimization of the parameters. The maximal specific activity of 1.5±0.1 IU/µg protein was much higher compared to 0.93 IU/mg protein [28]. This higher specific activity is a result of the very low protein concentration in the medium. In conclusion the maximal β-1,4-endoxylanase activity by *R. oryzae* T8 is comparable, albeit in the lower levels, to wild type strains.

R. oryzae T8 has xylan hydrolysing capacities measured with AZCL-xylan plate assay and degradation products of xylan were detected using HPLC analysis. In the plate a small amount of biomass was also formed on the control plates lacking any carbon source. No biomass was formed when spores of T8 were placed in liquid medium containing xylan as sole carbon source. Microscopic analysis revealed that growth arrested after the formation of germ-tubes. When biomass was pregrown on D-glucose and transferred to medium containing xylan as sole carbon source slow growth was observed. When xylanases degrade xylan a small amount of D-xylose is liberated [25]. The D-xylose formed by the β-1,4-endoxylanase activity facilitates growth of the biomass.

It is therefore expected that the introduction of a β-xylosidase encoding gene into transformant T8 would result in full degradation of the formed oligo xylose residues, thereby facilitating prolific growth and opening up a large pool of inexpensive substrate.

Xylanases by nature are very stable enzymes able to survive a wide range of conditions for prolonged periods of time [28, 29]. The drop in the measured β-1,4-endoxylanase activity after 48 hours can be caused by enzyme inactivation or by degradation *e.g.* due to protease activity. In addition, the amount of extracellular protein is very low. It is unlikely that gene transcription forms a problem since the normalized transcript levels are stable after 48 hours. The amount of *xynB* transcript (starting at 15% and ending at 90% of *pdc* transcript) is comparable to another study using the same expression system [15].

In conclusion, a β-1,4-endoxylanase from *A. niger* CBS 513.88 was successfully expressed in *R. oryzae* increasing the number of functional heterologous proteins expressed in *R. oryzae* [15, 30, 31].

5.11. Acknowledgements

We would like to thank Dr. C. D. Skory for providing plasmid pPdcExPyrF and *R. oryzae* M16. Dr. J.M. Oliveira for providing a cDNA sample of *A. niger* CBS513.88. This project was supported by a grant (EOSLT02034) provided by SenterNovem (Utrecht, the Netherlands).

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Chapter 6.
General Discussion

6.1. Introduction

Traditionally, commercially available amino acids are obtained through microbial fermentations. The production volumes are generally low and the price is too high for bulk use, e.g. in the chemical industry. Therefore, alternative sources need to be investigated which can supply large volumes of amino acids at low cost. Residual protein streams from food, agro and biofuel industry can form a suitable source of amino acids. Additionally, these processes can become economically interesting since 40% of the integral cost price of nitrogen containing chemicals from fossil resources is formed by the energy and process costs [1]. These residual protein streams have a low economic value, are heterogenic in composition and are diluted. Finding a way to concentrate and reduce the heterogeneity of these amino acids would be valuable.

Cyanophycin (CGP) consists of an α-amino-α-carboxy linked aspartic acid backbone with arginine residues linked to the β-carboxyl group of each aspartic acid [2]. It is accumulated by cyanobacteria as an insoluble energy and nitrogen containing reserve material [3].

Because cyanophycin is a solid, crystalline material in cyanobacteria it can be easily recovered from the microbial biomass [4]. We postulated that converting dilute amino acids present in lignocellulosic hydrolysates into cyanophycin by microorganisms that simultaneously convert the sugars into ethanol, as a strategy to valorize both carbohydrates and amino acids at the lowest capital cost.

The amount of CGP produced (% of cell dry weight) varies strongly between different wild type strains. *Synechococcus* MA19 produced 3.5% [5], *Synechocystis* PCC 6803 produced 5% [6], *A. cylindrica* produced 7.8% [7] and *Synechocystis* PCC 6308, which possesses one of the most used CphAs produced up to 16.0% CGP [8]. Cyanobacteria require photo-bioreactors to grow and achieve a very low cell density after a very long cultivation time of 14 days or more. As a result it is not possible to supply the potential market demand using cyanobacteria [9].

In order to increase the production of CGP various *CphAs* have been expressed in prokaryotic and eukaryotic (micro)organisms. Commercially interesting organisms used to express *CphAs* are tobacco [10], potato [11], *Escherichia coli* [12], *Pseudomonas putida* [13], *Ralstonia eutropha* [13], *Saccharomyces cerevisiae* [14] and *Pichia pastoris* [15]. These microorganisms are readily genetically accessible, show high productivity and have favourable growth conditions. An overview of CGP production in various wild type strains and hosts is described in a review by Frommeyer and co-workers [9].

The suitability of cyanophycin production to concentrate and reduce the heterogeneity of dilute amino acid streams was investigated in the N-ergy project, funded by SenterNovem, on behalf of the Dutch Ministry of Economic Affairs as part of the Program “EnergieOnderzoeksstrategie Lange Termijn”. Three PhD projects were dedicated to this research. At the Westphalian Wilhelms University in Münster a PhD project was executed by Anna Bröker aiming at the production of CGP in *Saccharomyces cerevisiae* [16]. At the Wageningen University two PhD projects were performed. Paul Könst studied the conversion of cyanophycin into nitrogen-containing chemicals [17].

Most of the organisms currently studied for the heterologous produce of CGP use glucose as a carbon source which can be supplied in crude extracts or produced by the hydrolysis of starchy materials. These carbon sources are relatively expensive and using glucose for non-food applications can have a detrimental effect on the food supply. In the so called second generation processes the inedible parts of the food crops can be used as feedstock. First the biomass needs to be pre-treated by one of the pretreatment methods; addition of concentrated acid, sulfur dioxide, hydrogen peroxide or lime, steam explosion, ammonia fiber expansion, wet oxidation, liquid hot water extraction, carbon dioxide explosion and organic solvent treatments reviewed by [18]. In general, these pretreatment methods destroy some sugars and generate toxic compounds which reduce the efficiency of the fermentation process. When enzymatic hydrolysis is employed there is generally no production of toxic compounds although sugars are needed for the growth of the enzyme producing organism.

To overcome the need for glucose as a carbon source, genes are introduced in the production organisms to broaden the substrate specificity and hydrolytic capacity [19].

Alternatively, one could select an organism that is already capable of producing a wide range of hydrolytic enzymes and is capable of producing various platform chemicals. In our study the filamentous fungus *R. oryzae* was selected as the production organism. *R. oryzae* is a ubiquitous filamentous fungus found on decaying organic material. *R. oryzae* strains are capable of a wide range of extracellular enzymatic activities such as amylolytic [20], xylanolytic [21, 22], pectinolytic [23] and cellulolytic [24-26] capabilities. An overview of the hydrolytic capacity toward biomass combined with the specific activities is presented in various review articles and experimental comparisons [27-29]. It should be noted that there are large varieties in the hydrolytic capacities and product yields between strains.

Because the hydrolytic capabilities of *R. oryzae* may be advantageous for concentrating dilute amino acid streams originating from lignocellulose residue streams, it was decided that the third PhD student should study the heterologous production of cyanophycin in this microorganism.

6.1.1. Goal of thesis

The aim of the research described in this thesis was to confirm the suitability of *R. oryzae* as a cell factory for the production of platform chemicals from lignocellulosic side streams, and to develop genetic tools to enable cyanophycin production by this microorganism and enhance its saccharolytic potential.

6.2. Discussion of results

The results of this research are described in chapter Chapters 2 to 5. Below, these results are discussed and interpreted in the context of available scientific literature.

6.2.1. *Rhizopus oryzae as a cell factory for production of platform chemicals*

In **chapter 2** we reviewed the opportunities and challenges of using *R. oryzae* as a production organism for platform chemicals. The focus is on the molecular background and metabolic routes to produce ethanol, L-(+)-lactic acid and fumaric acid. Under aerobic conditions the carbon flow is channeled towards the production of the organic acids and under anaerobic conditions ethanol is formed. These fermentation products are produced in high yields in excess of 85 % of the theoretical yield for L-(+)-lactic acid and ethanol and over 65 % for fumaric acid. The organism is capable of using a wide range of sugars such as glucose and xylose [29]. *R. oryzae* strains are capable of producing a broad spectrum of hydrolysing enzymes which make it interesting to utilize lignocellulosic biomass side streams, such as cellulases and xylanases. In addition strains are able to produce pectinases, amylases, lipases, and proteases [27]. *R. oryzae* has been applied to convert hydrolysates of corncobs [30], wheat bran [31], distillers grain [31] and paper solid waste [32]. The interesting hydrolytic capacity and the fermentation end products make this organism an interesting candidate for the biobased economy.

R. oryzae has the GRAS status and is available as a biosafety classification 1 organism. It is however also recognized as an opportunistic human pathogen and has a high prevalence under mucormycosis infections [33]. Most mucormycosis cases have an underlying illness such as an elevated serum iron level, trauma, or a weakened immune system [34].

6.2.2. *Genetic engineering of Rhizopus oryzae*

The state-of-the-art of genetic engineering of *R. oryzae* at the start of the project is also described in **chapter 2**. Several transformation systems have been developed for organisms in the order of Mucorales. These include *Absidia glauca* [35], *Mucor circinelloides* [36], *Parasitella simplex* [37], *Phycomyces blakesleeanus* [38], *R. niveus* [39] and *Rhizomucor pusillus* [40]. In general, the main bottleneck in heterologous gene expression for Mucorales is formed by the recombination and replication mechanisms affecting the introduced DNA.

The DNA introduced by transformation will remain extra chromosomal and replicate autonomously since it does not require a defined origin of replication [38, 41]. As a result, transformants usually display a mitotically unstable phenotype which requires the screening of large amounts of transformants. In addition to autonomous replication, these plasmids will form high molecular weight concatenated structures. These structures will co-migrate with genomic DNA, resulting in incorrect conclusions regarding integration [42, 43].

The conclusion of this chapter was that *R. oryzae* is an interesting candidate for the conversion of lignocellulose into ethanol and CGP, and that the main challenge was the introduction of the capability to produce CGP by genetic engineering.

6.2.3. CGP production by Rhizopus oryzae

In **chapter 3** we described how CGP production was realized in *R. oryzae*. We selected strain 99-880 because of the available genome sequence and transformation system [44]. A protocol was developed based on protoplast formation using an enzyme cocktail from Dyadic, PEG based transformation and hygromycin selection. Before this method was completely developed a method based on particle bombardment was published (Skory and Ibrahim 2007) and adopted to realize CGP production. We expressed various *cphA* genes from *Synechocystis sp.* strain PCC6803 and *Anabaena* sp. strain PCC7120. It proved impossible to obtain a codon-optimized version of the $CphA_{6803}$ indicating the toxicity of the gene product for the numerous bacterial strains used for cloning. Out of the 92 isolated transformants 20 tested positive for the expression of *cphA* and only two displayed a specific enzyme activity of 1.5 mU/mg protein. This is about 1,000 times lower than described for other eukaryotic microorganisms. In *S. cerevisiae* [45] and *P. pastoris* [46], both expressing $cphA_{6308}$, the CphA activity was 0.91 and 2.01 U/mg, respectively. In addition, no correlation was detected between transcript levels and the measured enzyme activity of the *R. oryzae* transformants.

Expression of *cphA* in *R. oryzae* resulted in accumulation of CGP, but to low levels of 0.5% of the CDW. In other microorganisms much higher contents were obtained, e.g. up to 43.4% in *Pseudomonas putida* ATCC 4359, 48% in *Ralstonia*

eutropha H16, 36.2 % in *E. coli* and 15.3% in *S. cerevisiae* [9]. The low CGP production in *R. oryzae* is likely to be a consequence of the low activity of CphA. There are no clear indications why this activity is so low and how it can be enhanced.

CGP in cyanobacteria is insoluble in water and is comprised of equimolar amounts of arginine and aspartic acid. Water soluble CGP is observed in a wide range of heterologous transformants expressing various *cphA*s, summarised in a review by Frommeyer and co-workers [9]. In most cases this is a result of the incorporation of L-lysine in the CGP polymer [47]. The rapid growth of the cells and the accumulation of CGP might deprive the cells of arginine which results in the incorporation of lysine which is structurally similar. This assumption is further demonstrated in a mutant of *Synochocystis* 6803 containing a single point mutation in the PII signalling pathway which unlocks the arginine pathway. With an overflow of arginine more CGP is accumulated into the cells [48]. The L-lysine content in CGP depends on culture temperature [49] and is strongly depending on the host. For instance, *E. coli* expressing $CphA_{6803}$ accumulated CGP with a maximal L-lysine content of 25 mol% [50]. While using the same *CphA* no soluble CGP accumulated in *R. eutropha* and *P. putida* [51].

In *R. oryzae* the soluble form was the major fraction, and the insoluble form occurred only in traces. The soluble CGP_{6803} consisted of equimolar amounts of aspartate and arginine, the presence of L-lysine was not observed. The reason why this CGP is soluble is therefore unknown. It cannot be excluded that the very low CGP content in itself is unfavourable for the formation of insoluble particles. Poly cations are known to exhibit toxicity by interfering with cell membranes [52]. It cannot be excluded that CGP has a similar effect on the membranes of *R. oryzae*, especially in its soluble form.

6.2.4. Analysis of proteinogenic N-containing chemicals

During the project the cyanophycin content of samples had to be determined. Existing methods involve the acid hydrolysis of the protein, followed by HPLC analysis of the amino acids after derivatisation of the amine groups, requiring many pipetting actions and expensive derivatisation reagents and the analysis is time consuming. To speed up the detection process and decrease operating costs a reliable and inexpensive method for the measurement of proteinogenic amino acids and other N-containing chemicals was developed in **chapter 4**. The method is based on acid hydrolysis and automated pre-column derivatisation of the amine group in the injection needle of the autosampler using o-phthalaldehyde/ethanethiol reagent in combination with 9-fluorenylmethyl chloroformate. The calculated mean limit of detection and limit of quantification of pure amino acids were 2.3 and 4.6 µM, respectively. With this method, it was possible to accurately analyse the amino acid composition of bovine serum albumin, soy meal, Jatropha press cake, and cyanophycin. Mean reproducibility of all amino acids from bovine serum albumin was 6.8%, with a mean recovery of 95%. The sample run time is 16 min with a total cycle time from injection to injection of an acid hydrolysed sample of 22 min. This is roughly half the run time of a conventional HPLC method using the same derivatisation principle [53].

6.2.5. Engineering the saccharolytic capacities of *Rhizopus oryzae*

The functional expression of CphA is a disadvantage for the cells: amino acids that are otherwise used for growth, are now used for the synthesis of cyanophycin. Moreover, cyanophycin may be toxic, especially the soluble form, due to its polycationic nature. To assess the potential of genetic engineering in *R. oryzae* further, we opted for the introduction of a trait that clearly has advantages for growth.

Various *R. oryzae* strains are capable of hydrolysing hemicellulosic biomass and utilizing the sugars for its metabolism. *R. oryzae* 99-880 however, is not able to utilize xylans as carbon source. Genome sequence analysis indicated that it misses several genes necessary for the complete hydrolysis of xylans. In order to increase

its hydrolytic capacity a β-1,4-endoxylanase encoding gene (XynB) of *Aspergillus niger* CBS 513.88 was introduced (**chapter 5**). This enzyme is able to release a small amount of xylose from xylan, which can used be by *R. oryzae* for growth. Expression of xynB, is therefore beneficial when the cells are grown in the presence of xylan as single carbon source. In total 13 transformants were isolated which displayed growth and screened for β-1,4-endoxylanase activity using a plate assay containing AZCL-xylan. Three transformants were isolated which displayed enzyme activity and one was mitotically stable. When this transformant was grown in liquid medium using xylan as sole carbon source growth was observed – albeit slowly. The maximum enzyme activity in the culture broth was 3.5 U/ml, with a specific activity of 1.5 U/µg protein. Compared to literature these activities are low.

This study shows that efficient expression of heterologous genes in *R. oryzae* is possible, and that the low efficiency for the transformation with CphA using the identical expression system is most likely caused by the gene itself, enzyme function or the presence of its product CGP.

6.3. Recent developments and outlook

After the practical work described in this thesis was ended in 2012, other groups have continued working on the application and engineering of *R. oryzae* as a cell factory. This paragraph describes their relevance with respect to this thesis.

6.3.1. Application of Rhizopus oryzae as cell factory

A number of reviews have been published describing the progress made with *R. oryzae* as a cell factory, aiming at the production of chemicals and enzymes [27, 28]. The research describing the production of chemicals only covers the products naturally made by *R. oryzae* strains: L-(+)-lactic acid, fumaric acid and ethanol. No publications were found on the synthesis of products that require genetic engineering of *R. oryzae* strains.

6.3.2. Genetic engineering of Rhizopus oryzae

Since our studies on genetic engineering of *R. oryzae* ended in 2012, a few developments in this field have been published. The system by Yuzbashev and co-workers is based on a similar plasmid and transformation method as described by Skory [54] but additionally containing a genomic repeating sequence named H (rptH). This resulted in the integration of 25 copies arranged in tandem that can be used for multicopy gene insertion and expression.

Xu et al. performed electroporation of germinated spores [55]. With this system enhanced-GFP was expressed. According to the authors this method had high efficiency, short handling time, and reliable reproducibility. Another transformation system is based on the formation of protoplasts and PEG medium transformation and was successful in expressing GFP tagged proteins in spores of *R. oryzae* [56].

An RNAi method was developed and used to silence the high affinity iron permease ftr1 gene and study its effect. A construct was generated with a sense and antisense (both 450 bp) of the ftr1 gene held apart by a spacer element [34]. After transcription of the construct, a hairpin structure was formed (hpRNA) which initiated the RNAi process. This method was successful in silencing the gene. The iron uptake of the transformants generated with this construct was reduced by roughly 50 %.

Finally, a CRISPR/Cas9 method was developed and used to successfully disrupt a toxin-encoding gene resembling ricin [57].

These studies are mostly aimed at tackling the pathogenic character of *Rhizopus* species, and not on increasing the production of chemical compounds.

6.3.3. Lignocellulosic hydrolysates as substrate for chemical production

The biorefinery concept is intensively studied and the second generation processes can supply the raw material without competing with the food supply [58]. In these processes lignocellulosic materials are converted. There is a great variation

in the feedstock, conversion routes (microbial, flash pyrolysis and thermochemical) and end products. The microbial conversion route has the advantage that it can use the natural flexibility of microorganism. This is ideal since the exact composition of the feedstock may change over the course of the year and geographical locations. A good example is the production of second generation bioethanol. One factory (POET/DSM) is operational but several other initiatives were stopped. Major reason is the high capital intensiveness urging for large units in order to benefit from economy of scale. Large scale however has the disadvantage of high logistic and higher raw material costs. But further investments and research are on hold until second generation bioethanol production becomes a commercial success.

Higher value chemicals can improve the economics starting from lignocellulosic raw materials, but only if the substrates required can compete economically with first generation substrates, since little bonus value for second generation products is expected. While lignocellulose derived substrates can contribute lower costs if the microorganism will secrete (hemi)cellulases and C5 sugars can be used by the microorganism also some drawbacks versus first generation being less pure and less concentrated have to be compensate by other advantages from lignocellulose raw materials, in order to become attractive.

Nitrogen containing substrates from second generation raw materials might offer a competitive position provided that the molecular structure of e.g. amino acids can be converted to known (specialty or bulk) chemicals by enzymatic and-or chemical conversion. Since 2012 a limited number of research projects were dedicated on valorising protein in lignocellulosic streams. The MIMOSA project was focussed on utilising small volume heterogeneous substrate input which was converted to multiple end products and included the extraction and valorisation of the proteins [59]. Another project was focussed on the isolation of proteins from the rubber seed presscake [60].

The technical solutions for the production of chemicals from lignocellulosic hydrolysates and proteins in these streams are available. Unfortunately, the costs involved in producing these compounds are too high to compete with the classical petrochemical processes.

6.4. Conclusions

The goal of this thesis was to confirm the suitability of *R. oryzae* as a cell factory for the production of platform chemicals from lignocellulosic side streams, and to develop genetic tools to enable cyanophycin production by this microorganism.

The expression experiments performed in this work demonstrate that it is possible to functionally express heterologous genes in *R. oryzae.* Although more work should be performed to understand the bottlenecks in expression since the accumulation of CGP and the xylanase activity is lower than expected.

Cyanophycin production was successful. The minimal activity of the CphA enzyme however resulted in a low CGP content of the cells. At the beginning of the project we anticipated that we could use the production of insoluble CGP by *R. oryzae* as a way to concentrate dilute amino acid solutions. The absence of insoluble CGP could be a significant drawback for that concept.

In the N-ergy project, also the combined production of ethanol and cyanophycin by S. cerevisiae was studied. Significantly higher cyanophycin production was realized in this yeast. Although wild-type *S. cerevisiae* is limited in the utilisation of C5-sugars and the hydrolysis of polymeric sugars, recent developments [61] show that these challenges can be successfully tackled by means of genetic engineering. *S. cerevisiae* may be the better cell factory for the combined production of cyanophycin and ethanol from lignocellulose

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Summary

Fossil fuels, including oil, natural gas, and coal are primary energy sources and account for 86% of the world's energy consumption. These fossil fuels are also used as a feedstock for the production of chemicals and materials. It took millions of years to form these fuels from biomass and their consumption is therefore considered non-renewable. At a certain moment the reserves of these fossil resources will be depleted and prior to that moment mining for bulk use will be economically unfeasible. Beside the finite reserves there are also many negative effects on the environment. These include the pollution of the environment and global warming. It is generally considered that global warming is caused by the release of greenhouse gasses, such as CH_4, NO_x, water vapour and CO_2 into the atmosphere. Alternative resources for the generation of heat and power can be formed by geothermal, water, wind, and solar energy. However, these resources do not form an alternative for the production of chemicals and materials since this requires (chemical) building blocks.

Biomass can form a suitable alternative resource for the formation of these building blocks. Biomass in its pure form is complex and can in most instances not directly be used for the production of renewable compounds. Just like fossil fuels it should be processed or biorefined to obtain building blocks for chemicals and materials. In the biorefining process all the non-food fractions such as the oils, gums, carbohydrates, proteins, and lignin should be used for their most energetically favourable applications.

Nitrogen containing molecules are used in many chemical applications like the production of nylon, polyurethane and acrylonitrile. Naphtha does not comprise nitrogen-containing molecules and they have to be synthesised by binding nitrogen derived from air, a very energy intensive process. Forty percent of the integral cost price of nitrogen containing chemicals from fossil resources is formed by the energy and process costs.

Biomass, on the other hand, contains nitrogen containing chemicals in the form amino acids. Traditionally, commercially available amino acids are obtained through microbial fermentations. These production volumes are generally low and the price

is too high for bulk use. Therefore, alternative sources need to be investigated which can supply large volumes of amino acids at low cost. Interesting sources are the residual protein streams from the food industry or the production of biofuels. These streams are however heterogeneous in composition and result in low amino acid concentrations. Cyanophycin (CPG) is naturally accumulated by cyanobacteria as insoluble granules which form an energy and nitrogen reserve material and consists of equimolar amounts of arginine and aspartic acid. The insoluble nature can be used to isolate and concentrate the granules. The wish to produce bioethanol from (lignocellulosic) waste streams coupled to the concentration and subsequent conversion of amino acids into CGP culminated into the N-ergy project. The research described in this thesis was focused on the production of cyanophycin (CGP) in the filamentous fungus *Rhizopus oryzae*.

Chapter 2 is an in-depth analysis of the potential of *R. oryzae* for the production of platform chemicals and the genetic accessibility. Strains of *R. oryzae* are capable of producing a wide range of lignocellulosic hydrolysing enzymes and grow on a wide range of carbon sources. Using these carbon sources it is capable of producing L-(+)-lactic acid, ethanol with a fumaric acid. The yields using D-glucose are in excess of 85% of the theoretical yield for L-(+)-lactic acid and ethanol and over 65% for fumaric acid. The hydrolytic capacity and high yields of fermentation end products make this an interesting organism for the biobased economy. Though, genetic modification is challenging since the introduced DNA rarely integrates in the genome leading to mitotically unstable phenotype.

In order to produce cyanophycin several cyanophycin synthetase encoding genes were introduced in the sequenced strain *R. oryzae* 99-880. The genes originated from *Synechocystis* sp. strain PCC6803 and *Anabaena* sp. strain PCC7120. Also, a codon optimized version of the latter gene was introduced (**chapter 3**). Only one transformant (expressing $chpA_{6803}$) out of a total of 92 isolates was able to produce water-soluble CGP (0.5%) with traces of water-insoluble CGP. There was no correlation between transcript levels of *cphA*, enzyme activity, and CGP accumulation. In addition, the water soluble CGP did not contain L-lysine which is generally assumed to be the cause for the soluble behaviour. The total amount of

CGP and enzyme activity is low in comparison the other genetically modified microorganisms expressing *cphA*'s.

The common methods to determine the amino acid composition of protein samples require an acid hydrolysis followed by a derivatisation step with costly reactants. Cost effective derivatisation methods are available yet these require manual handling prior to the analysis of each individual sample. In order to analyse large volume of samples, a cost effective and fast new method was required. A new method was developed using acid hydrolysis and automated pro-column derivatisation using o-phthalaldehyde/ethanethiol reagent in combination with 9-fluorenylmethyl chloroformate (**chapter 4**). Due to the automated derivatisation in the injection needle the handling time is greatly reduced. Additionally, the run time for is sample using the UPLC method was 16 minutes which is a reduction of roughly 50% in comparison to a HPLC separation. The method was used for the analysis of proteins like CGP and has a mean recovery of the amino acids of 95%. With the current available techniques, it is not possible to further reduce the time required for the derivatisation and analysis.

Introduction of CphA is *R. oryzae* was successful but resulted only in the accumulation of trace amounts of soluble cyanophycin. A possible explanation for the low accumulation is the fact that the production of soluble cyanophycin has a detrimental effect on growth of *R. oryzae*. We therefore tested the introduction of heterologous genes that should have a beneficial effect on growth. *R. oryzae* 99-880 has no xylanolytic capacity although it can grow on xylose. In order to increase the xylanolytic activity a β-1,4-endoxylanase encoding gene (xynB) of *Aspergillus niger* CBS 513.88 was introduced (**chapter 5**) using the same method as employed for the introduction of *cphA*'s. In total 13 transformants were isolated of which three displayed enzyme activity and one was mitotically stable. In this experiment there is a clear correlation between transcript levels of *xynB* and enzyme activity. The stable transformant was able to grow on xylan as carbon source. This demonstrated that metabolic engineering using the selected transformation system can be performed successfully. The large difference in numbers between transformants that successful

produce β-1,4-endoxylanase in comparison to cyanophycin is indicative that the bottleneck in CGP production is product related.

In recent years little development is published in respect to the genetic engineering of *R. oryzae* **(chapter 6)**. The systems that are published are not always used to express genes and some are developments from the system that was also used in this study. Still *R. oryzae* is widely studied as an organism of interest for the biobased economy due to the hydrolytic capacity and range of product that can be formed by the various strains.

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Curriculum Vitea

Bas Johannes Meussen was born in Zwolle, the Netherlands, on the 13th of September 1981. He obtained his H.A.V.O diploma in 2000 at Thorbecke Scholengemeenschap in Zwolle. Following this, he completed a BSc course in Biology and Medical Laboratory research at Saxion University of Applied Sciences in Deventer, the Netherlands. For his BSc thesis he went to TNO-Environment Energy and Process innovation where he worked under the guidance of Dr. Jan Gerritse on the bacterial ecology in microbial fuel cells and the proof of principle of novel models of microbial fuel cells. In 2004 he continued with an MSc course in Biotechnology at Wageningen University, the Netherlands. The MSc thesis was performed at Wageningen University at the Fungal Genomics section of the Laboratory of Microbiology under the supervision of late Dr. Leo de Graaff. The research was focused on the conversion of a waste product from the wheat industry into ethanol or L-(+)-lactic acid using *Rhizopus oryzae*.

He started the work for this PhD-thesis in 2006 at Valorisation of Plant Production chains in collaboration with the Fungal Genomics section of the Laboratory of Microbiology. It focused on the metabolic engineering of *R. oryzae* for the production of cyanophycin and the co-production of ethanol. The practical work on the PhD-thesis was stopped in 2010. In 2011 he started working at AKZO-Nobel at the Ecotoxicology and Environmental testing group in Arnhem, the Netherlands. Here he worked on assessing the biodegradability of (novel) chemical compounds. In 2014 he started a new career as a lecturer in the field of molecular biology at Saxion University of Applied Sciences, the Netherlands.

List of Publications

Meussen, B.J., L.H. De Graaff, J.P.M. Sanders, and R.A. Weusthuis, Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals. Applied Microbiology and Biotechnology, 2012. 94(4), p. 875-886.

Meussen, B.J., R.A. Weusthuis, J.P.M. Sanders, and L.H. de Graaff, Production of cyanophycin in *Rhizopus oryzae* through the expression of a cyanophycin synthetase encoding gene. Applied Microbiology and Biotechnology, 2012. 93(3): p. 1167-1174

Meussen B.J.[*], A.N.T. van Zeeland [*], M.E. Bruins, and J.P.M. Sanders, A fast and accurate UPLC method for analysis of proteinogenic amino acids. Food Analytical Methods, 2014. 7(5): p.1047–1055

**both authors contributed equally*

Overview of Completed Training Activities

Discipline specific activities

Renewable Resources for the Bulkchemical Industry, Wageningen University (Wageningen, NL)	2006
RRB3 conference (Ghent, BE)	2007
RRB4 conference (Rotterdam, NL)	2008
IP Biorenewable Resources (Graz, AT)	2008
Domein Applied Sciences congress (Utrecht, the Netherlands)	2018

General courses

VLAG PhD week, VLAG (Bilthoven, NL)	2007
Writing and presenting scientific papers, Wageningen University (Wageningen, NL)	2007
Training networking skills, Wageningen University (Wageningen, NL)	2010
Coaching in SLB-groepen, Saxion (Enschede, NL)	2016
Didactiek in een Digitale Leeromgeving, Saxion (Enschede, NL)	2016
Basis Kwalificatie Examinering, Saxion (Enschede, NL)	2018

Optionals

Preparation of research proposal (Wageningen, NL)	2006
PhD Study trip to California (California, USA)	2006
Group meetings, VPP (Wageningen, NL)	2006-2010
Theme meetings, VPP (Wageningen, NL)	2006-2010
Weekly/biweekly Fungal genomics meeting, Laboratory of Microbiology (Wageningen, NL)	2006-2010
Biweekly PhD/Postdoc meetings (Laboratory of Microbiology) (Wageningen, NL)	2006-2010
Scintillation assays at WWU Münster (Münster, DE)	2008-2010

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The cover design is a production of G.M. Meussen-Selhorst and depicts mycelium of *R. oryzae* 99-880 growing on potato glucose agar photographed using a sample preparation microscope with 300 times magnification.