Production of nitrogen containing chemicals from cyanophycin

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Thesis

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Chapter 1

General introduction

1.1 Transition towards renewable, carbon neutral and secure alternatives for fossil resources

Depletion, global warming and energy security issues are forcing us to make the transition from fossil resources towards renewable, carbon neutral and secure resources for energy and chemicals. In this respect plant biomass, defined as "a plant matter of recent (non-geologic) origin or material derived there from",[1] would be a suitable alternative.

1.1.1 Depletion of fossil resources

Currently, it is estimated that more than 85% of the world's total oil production is accounted for by crude oil, while the remainder is accounted for by natural gas liquids and non-conventional sources such as heavy oils and tar sands.[2] On average 35% of the crude oil in a field can be extracted as 'easy oil' by means of natural pressure (primary recovery).[3] From the initial extraction onwards oil production ramps up and finally arrives at a plateau as the result of decreasing oil pressure. Upon reaching this plateau the level of oil production is initially maintained by using pumps (secondary recovery) and eventually with the help of chemicals and other techniques (tertiary, enhanced recovery). Nonetheless, oil production will inevitably start to decline as the oil field is depleted.[2]

The concept that the extraction rate of fossil resources, like for most finite resources, grows, reaches a maximum, and then gradually declines to near zero was first expressed by Marion King Hubbert in 1956 and is referred to as the 'Hubbert's curve'.[4,5] The point at which the production of crude oil starts to decline is called 'peak oil' and upon reaching this point the demand for crude oil will start to outweigh its supply.

Following the most recent predictions by the International Energy Agency (IEA), peak oil has already occurred at presently producing fields.[6] Overall, peak oil might be delayed by development of recently discovered fields, fields that might be discovered in the near future, including unconventional oil (*e.g.* heavy oils and tar

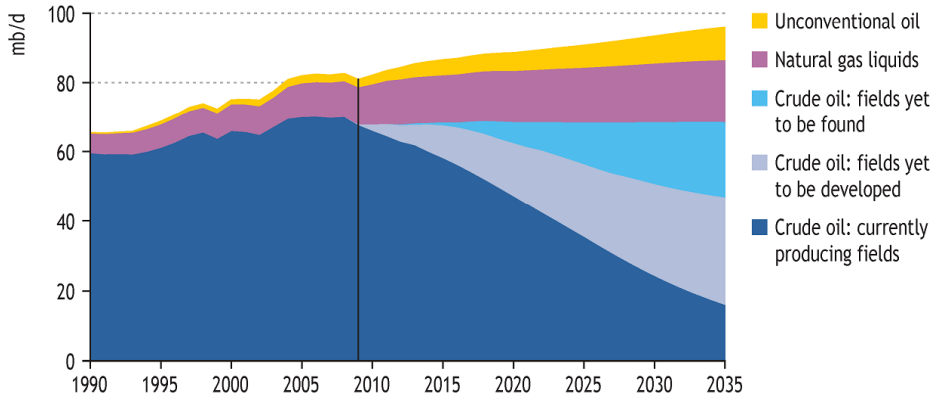


Figure 1.1. World oil production outlook as presented by the International Energy Agency in the World Energy Outlook 2010.[7]

sands) and increasing natural gas production (Figure 1.1).

However, depletion of fossil resources not only depends on oil production, but also on oil demand. The IEA estimated that worldwide oil demand will increase from 84 mb d^{-1} (million barrels per day) in 2009 to 99 mb d^{-1} in 2035.[6] The larger part of this increase is accounted for by emerging economies such as that of China (36%) and India (18%). Nonetheless, by 2035, the United States will still remain the world's second largest energy consumer behind China.

Taking the estimated global oil production (Figure 1.1) and global oil demand into account, it is clear that oil will become scarce in the near future. For this reason, a shift towards renewable resources for energy and chemicals will be necessary.

Plant biomass is considered a renewable feedstock. Over a time scale of weeks, plants can absorb radiation energy from the sun and store this energy by means of carbon dioxide fixation in the form of biomass. Both sunlight and carbon dioxide are widely available as will be so for billions of years.

1.1.2 Global warming

For heat, the earth is largely dependent on solar radiation energy from the sun. The radiation energy emitted to earth is mainly absorbed by the earth's surface and for a smaller part by its atmosphere. If it was not for the earth's atmosphere,

the absorbed radiation energy would be relatively quickly emitted back to space, leaving the earth at an estimated temperature of -18°C.[8] Instead, the radiation energy emitted by land and ocean is absorbed by the atmosphere and emitted back to earth before it can reach space. This process by which energy is recycled in the atmosphere to warm the earth's surface is known as the greenhouse effect and keeps our planet at an average temperature of 14°C, making life possible.[9]

The greenhouse effect is mainly caused by the so-called greenhouse gasses that are present in the atmosphere and of which water vapor is the most important, followed by carbon dioxide.[10] Human activity since the Industrial Revolution, mainly through the use of fossil resources and deforestation, has led to a significant increase in levels of atmospheric greenhouse gasses such as carbon dioxide and methane.

The increase in atmospheric greenhouse gas levels has been linked to an observed overall increase of the earth's surface temperature by 0.74 ± 0.18 °C in the 20th century and it is expected to rise another 1.1 to 6.4 °C in the coming century.[11] This phenomenon is commonly known as 'global warming'. Although relatively small, this increase in temperature is probably enough to disturb the global climate resulting in rising sea levels, increasing occurrence of extreme weather events, extinction of species and decreasing agricultural yields.[12-15]

Although the cause and existence of global warming are debated by some authors,[16-18] most scientists agree that there is a scientific basis to assume that global warming is the result of man's emission of greenhouse gasses into the earth's atmosphere.[11] To address the problem of global warming on the long term it will be necessary to reduce its major cause: the emission of carbon dioxide into the atmosphere due to the application of fossil resources.[19] For this reason, a shift towards a carbon neutral resource for energy and chemicals will be necessary.

Theoretically, the application of plant biomass can be considered as carbon neutral. For example, trees absorb radiation energy from the sun and *via* photosynthesis this energy is stored by means of carbon dioxide fixation in the form of biomass. When wood is incinerated the stored 'solar' energy is released

in the form of heat while biomass decomposes to carbon dioxide. Subsequently, the carbon dioxide released in the atmosphere is taken up and fixated by newly growing trees. In turn these newly grown trees can be incinerated and so on.

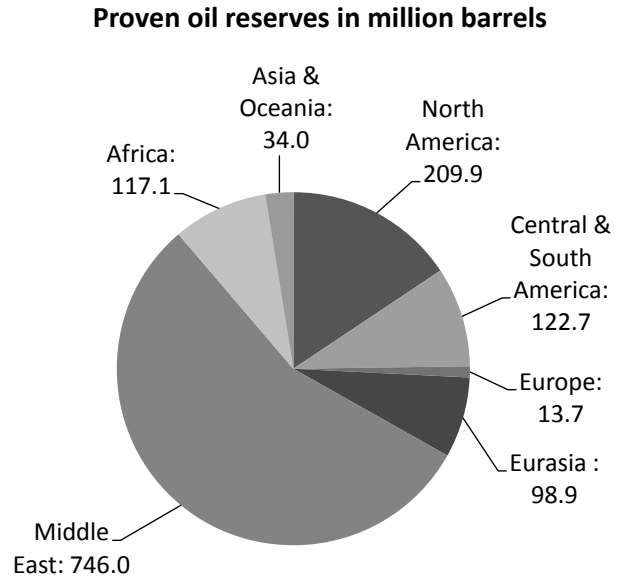
1.1.3 Energy security

Energy security is commonly defined as a reliable and consistent availability of various forms of energy at reasonable prices.[20,21] Since the energy crises of the 1970s, the lack of an adequate energy security policy has resulted in a sustained reliance on crude oil supplies from unstable regions.[22] This problem is captured by the graphs presented in Figure 1.2.

Most of the crude oil reserves are in the Middle East (Figure 1.2, top) and as a result within approximately 15 years from now the Middle East will be the only major reservoir of abundant crude oil.[20] At the same time, the Middle East is also the region with the highest frequency of crude oil supply interruptions since 1950 (Figure 1.2, bottom).

Not only instabilities in crude oil supply, but also the expected increase in crude oil demand from emerging economies such as China and India, as previously described in section 1.1.1, raises concerns about energy security due to increasing energy prices as the result of scarcity.[22]

To resolve the threats to energy security as a result of unreliable supply chains and an increasing demand, a renewable and more local alternative for crude oil should be applied. Such an alternative would decrease the dependency on the volatile global energy market and thus increases energy security. In this respect, plant biomass could be a suitable alternative for crude oil. Plant biomass can not only be sourced locally, it is also abundantly available as a residue from food, feed and forest industries.



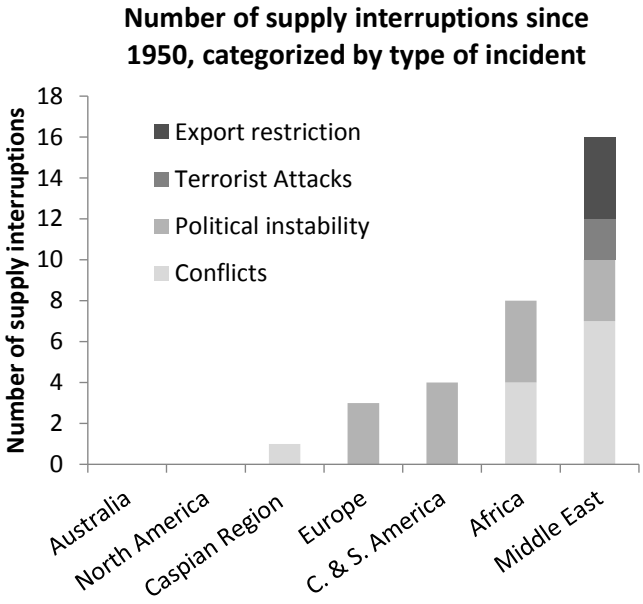


Figure 1.2. Top. Proven oil reserves in million barrels (2009) per region of the world, based on data from the U.S. Energy Information Administration.[23,24] **Bottom.** Number of supply interruptions since 1950, categorized by type of incident, based on data from Flouri *et al.*[25]

1.2 Plant biomass as an alternative to crude oil

1.2.1 Renewable and carbon neutral?

Although plant biomass is sometimes simply stated to be a renewable and carbon neutral feedstock, one should also take into account the use of fossil energy and subsequent carbon dioxide emissions involved in planting, harvesting, processing, transport and the production and application of fertilizers and pesticides.

In 2010, the largest part of the worldwide biofuel production, an estimated 90 billion liters bioethanol and 15 billion liters biodiesel, was accounted for by US corn and Brazilian sugar cane based ethanol, respectively 50 and 30 billion liters.[26]

The majority of the currently produced bioethanol is first generation bioethanol, which means that it is based on starch. In general, US corn ethanol is produced from the starch present in the corn kernels. To obtain the kernels the ears are harvested and the remainder of the corn plant, also known as corn stover, is left on the field.[27] In this way, it is estimated that 20.4 MJ fossil energy is required in the production of one liter bioethanol with an energy content of 21.2 MJ and 4.3 MJ worth of distillers' dry grain with solubles (DDGS), which can be applied as animal feed (Figure 1.3).[28] This means that corn based bioethanol, produced under current US farm and biofuel production standards, would yield a mere 5.1 MJ of renewable energy per liter bioethanol produced. Due to the required fossil energy input, the application of first generation US corn ethanol is neither a renewable, nor a carbon neutral alternative for fossil fuel, leading to only 12% reduction in greenhouse gas emissions.[28]

Brazilian sugar cane ethanol is currently also produced as a first generation biofuel, but nonetheless it is produced with a good net renewable energy yield of 22.6 MJ L^{-1} (Figure 1.3).[29] This is mainly due to the general practice to use the residue of sugar cane (bagasse) for generation of the required process energy. Similarly, the net renewable energy yield of US corn ethanol could be improved by combustion of corn stover.

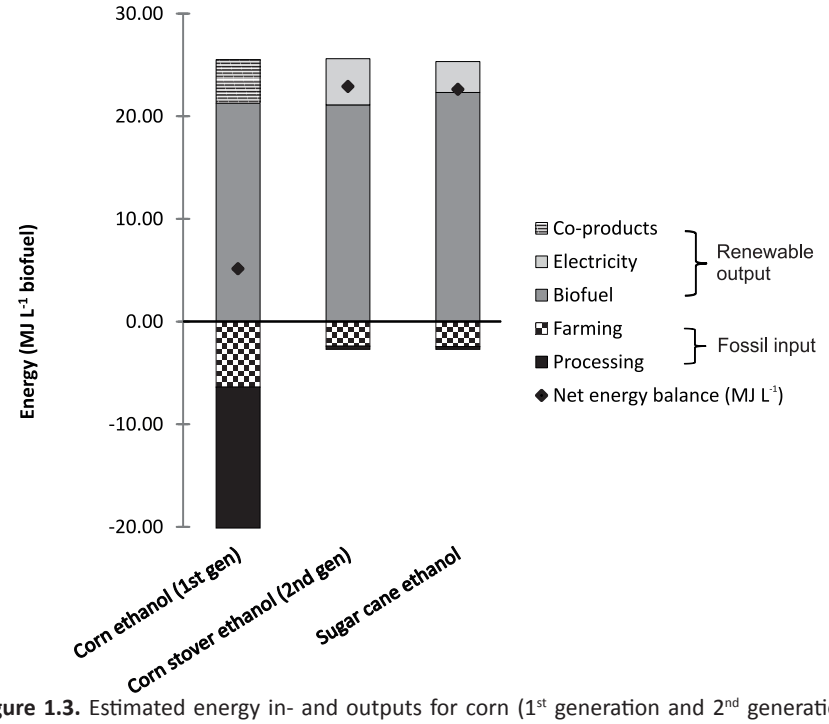


Figure 1.3. Estimated energy in- and outputs for corn (1st generation and 2nd generation in the US) and sugar cane (Brazil) based ethanol production, based on work of Hill *et al.*[28], Macedo *et al.*[29] and Lavigne and Powers[30]. The estimated net energy balance (the sum of the outputs minus the sum of the inputs) for each biofuel is represented by black dots.

Alternatively, the net renewable energy yield of US corn ethanol could be improved upon using second generation production processes. These processes are based on the application of cellulosic residues of agricultural crops. In case of corn that would be corn stover. Corn stover would then be harvested and subsequently pretreated and hydrolyzed to yield fermentable sugars. While the sugar content of corn stover is used for fermentation, the remainder is used to generate process energy. Using second generation production techniques, a net energy yield of 22.9 MJ L^{-1} renewable energy could be achieved, while the ears could be applied for food or feed.[30]

These examples show that replacement of fossil fuels by corn or sugar cane based ethanol involves the input of fossil energy and is therefore neither completely renewable nor carbon neutral. Nonetheless, the examples also show that

improvements in the production process can increase the net renewable energy yield. Another effective way to increase the renewable and carbon neutral character of plant biomass would be to apply it following the biorefinery approach.

1.2.2 The biorefinery approach

Using nature's functionalities to save fossil energy

Similar to oil based refineries, where many petrochemicals are produced from crude oil, biorefineries will produce many different products from biomass.[31] More specifically, a biorefinery is a facility that integrates separation and conversion processes to produce an array of products such as food, feed, fuel, heat, power and chemicals from biomass.[1]

Via a biorefinery all components present in non-food biomass residues, such as carbohydrates, lignin, proteins and oils, could be used for their energetically most favorable application, such as fuel, heat, power and chemicals. In this way, energy stored in plant biomass is most efficiently used and as a subsequence the fossil energy input can be significantly reduced.[32]

For example, many nitrogen containing bulk chemicals are produced from naphtha. To achieve this, large amounts of fossil energy and environmentally unfriendly co-reagents are used to incorporate *e.g.* ammonia by means of ammoxidation.[33] In this way, fossil energy is invested to convert simple hydrocarbons (naphtha) to nitrogen functionalized compounds with (substantially) lower calorific values (represented by the height of the horizontal bars in Figure 1.4).[34]

If an amino acid would be used, which already has nitrogen incorporated *via* nature's solar powered anabolic processes, far less fossil energy input would be required to produce the same nitrogen functionalized chemical (dotted curves represent process energy barriers in Figure 1.4).[35]

Take for example the production of 1,2-diaminobutane (Figure 1.5). Currently, 1,2-diaminoethane is produced from fossil based ethylene, which is oxidized to its epoxide followed by amination to ethanolamine and finally to 1,2-diaminoethane. Per ton product approximately 22 GJ in the form of the ethylene raw material

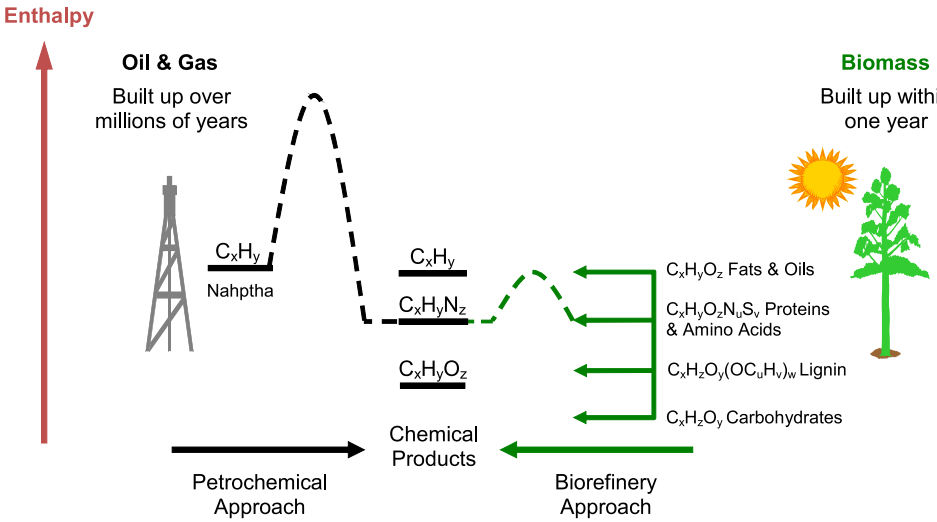
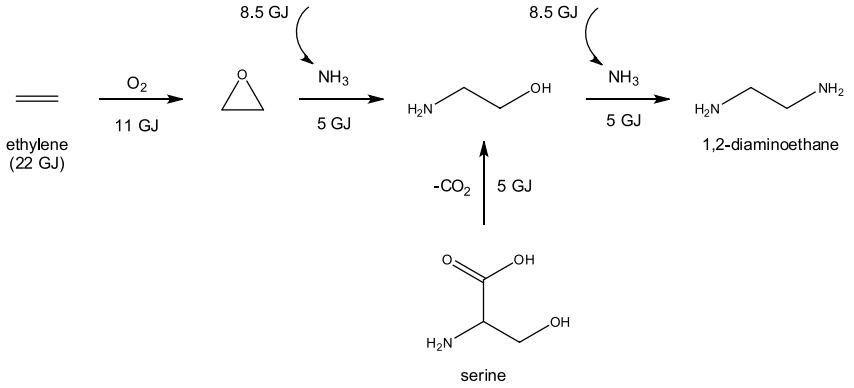


Figure 1.4. Scheme representing the energy benefits, both in terms of calorific value and process energy input, of amino acid based production of nitrogen containing chemicals as opposed to naphtha based production. Adapted from Sanders *et al.*[34]

and 38 GJ in the form of various process energies (including 17 GJ for ammonia production) is invested.

Starting from serine, which already has one nitrogen atom incorporated *via* nature's solar powered anabolic processes, the fossil energy input required for incorporation of nitrogen functionality is strongly reduced. In this way, the use of serine as a feedstock for 1,2-diaminoethane potentially saves 41.5 GJ of fossil energy (22 GJ in form of the calorific value of ethylene plus about 19.5 GJ in the form of process energies). In practice, this saving may be lower, as the production of serine from biomass has not been taken into account.

Brehmer *et al.*[36] estimated that the fossil energy savings in the production of first generation US corn ethanol could be increased from 16 GJ ha^{-1} to 70 GJ ha^{-1} if in addition to starch from corn grains also lignocellulose present in corn stover, after pretreatment and hydrolysis (second generation technology), would be converted to ethanol. If in addition 57% of the total corn protein content would be used for chemicals (biorefinery approach), this would result in an additional 47 GJ ha^{-1} in terms of fossil energy savings.



Overview of fossil energy inputs per route:

	Petrochemical	-	Biobased	=	Potential fossil energy savings
Calorific value of feedstock	22 GJ		0 GJ		
Process energy	38 GJ +		18.5 GJ +		
Total fossil energy input	**60 GJ**	-	**18.5 GJ**	=	**41.5 GJ**

Figure 1.5. Energy input for the biobased and petrochemical production of 1 ton 1,2-diaminoethane following Scott *et al.*[35]

Improved economics

As explained in the previous section, considerable amounts of fossil energy and thus energy costs could be saved when starting from biomass that already contains the functionality desired in the product. Additional to energy costs, also process costs will be lower in the application of biomass, since fewer steps will be required, and thus less process equipment, to convert functionalized biomass compounds into functionalized chemicals.

In Table 1.1 the raw material costs and integral cost prices are given for the most common applications of fossil feedstocks, expressed in euros per GJ end product. The discrepancy between the raw material cost price and the integral cost price can partly be explained by the conversion yields. While the yield in the production of heat and transport fuels is nearly 100%, the yield in the production of electricity is approximately 50% and in case of functionalized chemical this can be significantly lower. Additionally, the energy and process costs in the production of functionalized chemicals are substantial.[35]

Table 1.1. Raw material costs and integral costs per GJ end product for different applications of fossil feedstocks.[35]

Application	Raw material costs (fossil energy) (€ per GJ end product)	Integral cost prices (€ per GJ end product)
Heat	3 (coal)	4
Transport fuels	8 (oil)	10
Electricity	6 (coal)	22
Average functionalized chemical	30 (oil)	75

The high energy and process costs in the oil based production of functionalized chemicals leave room for improvement and thus opportunities for the profitable application of functionalized biomass. On the other hand, production of heat and power leaves less room for improvement in terms of energy and process costs and therefore these applications have lower potential for the profitable application of functionalized biomass.[35]

When 25% of the Dutch agricultural acreage would be devoted to the production of biofuels, electricity and chemicals, this would include 250,000 ha of meadows and 200,000 ha of fields with an average yield of 22 ton ha^{-1}. When all harvested biomass would be used for a mixture of electricity and biofuels, a value of 1,330 € ha^{-1} would be created (Figure 1.6). Since cultivation of wheat on the same area would generate a value of 1700 € ha^{-1}, the production of only electricity and biofuels would not be economically attractive. However, when 10% of the biomass would be applied for the production of functionalized chemicals using a biorefinery, the value would increase significantly to 2,310 € ha^{-1}. When 20% of the biomass would be devoted to the production of functionalized chemicals, the value of the same hectare would be more than doubled (3,290 € ha^{-1}) compared to the initial situation.[37]

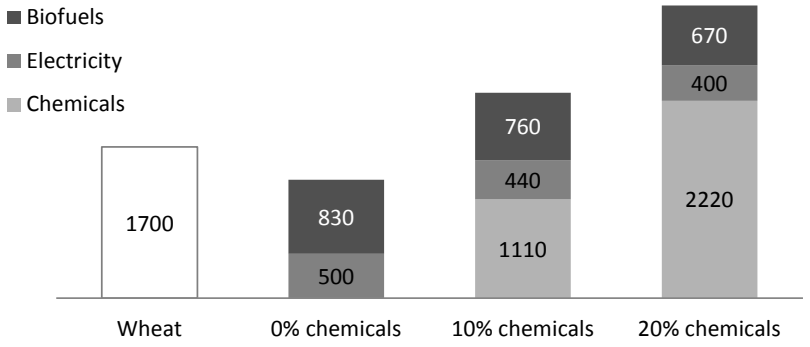


Figure 1.6. Value created (€ ha^{-1}) in three scenarios based on 25% of Dutch agricultural acreage (250,000 ha of meadows and 200,000 ha of fields with an average yield of 22 ton ha^{-1}) devoted to production of biofuels, electricity and chemicals. Adapted from Sanders *et al.*[37]

Food versus fuel

Over the recent years, the expanding biofuel production has raised concerns about the possible divergence of valuable cropland from producing food needed to feed people to producing biomass for application in biofuels.[38] This debate is known as the 'food versus fuel debate'. When applied *via* the biorefinery approach, biomass could serve as a feedstock for chemicals without threatening food security. A biorefinery would be able to process biomass from different sources, including non-food biomass.

Considering only the US, there is a potential of $1.96 \cdot 10^9$ tons of residual, non-food plant biomass available per annum ($7.0 \cdot 10^8$ tons forest residues, $6.0 \cdot 10^8$ tons biomass from idle US cropland and $6.6 \cdot 10^8$ tons crop residues).[39] Additionally, the application of current by-product streams could be assessed for their most optimal application and subsequently be improved. For example, de-oiled press-cakes from soya and rapeseed are currently applied as animal feed. Once fed to animals, their digestion system only removes the required nutrients from the cake and the rest is excreted as manure.[32] If proteins were hydrolyzed in a biorefinery, and poultry and pigs were fed with the right proportion of essential amino acids, the remaining amino acids could be used for the production of nitrogen containing chemicals.

1.2.3 Application of amino acids as feedstock for functionalized chemicals

Previously, it was explained how the application of amino acids, obtained from biomass residues, as feedstock for the production of nitrogen containing chemicals could be a favorable alternative for fossil feedstocks, both energetically and economically.

In nature, amino acids are generally contained in the form of protein. In order to replace at least part of the oil based chemicals with amino acid based chemicals, considerable amounts of protein need to be available. Additionally, methods are required to isolate and hydrolyze proteins and to isolate the desired amino acids. To give a general idea, these topics will be shortly addressed in the following paragraphs.

Protein sources

Protein fractions will be an abundant by-product from the boost in production of biofuels. Assuming that biofuels will have a share of 10% in the transportation fuel market by 2020, 100 million tons of protein will be produced per annum as a side stream. This is about four times the protein requirement of the world's human population.[34] Based on this number, each type of amino acid would become available at a volume of approximately 5 million tons per annum. Considering bulk chemicals such as 1,4-diaminobutane (> 10,000 tons $year^{-1}$ in Europe) and acrylamide (globally 3-5 million tons $year^{-1}$), sufficient amounts of the required amino acid should be available to allow a large proportion of the production to be from biobased feedstocks.[34]

Alternatively, proteins can also be obtained from herbaceous crops such as switchgrass and alfalfa. Protein production in these crops is comparable to that of crops such as corn and soybean, approximately 0.6 tons ha^{-1} $year^{-1}$.[40] These non-food crops are attractive in view of the ongoing 'food versus fuel' debate and due to several favorable characteristics: high crop yields, low soil erosion, low water, fertilizer and pesticide requirements and their ability to sequester carbon.[41,42]

However, to be able to successfully utilize herbaceous crops as a source of protein, all of their biomass compounds should be utilized following the biorefinery concept. For example, the abundantly present lignocellulose could be used for the production of biofuel.[43]

Protein recovery

In general, proteins first need to be isolated and subsequently hydrolyzed before their amino acid content can be utilized as feedstock for functionalized chemicals. Several techniques have been developed for the isolation of protein from biomass residues, mainly based on aqueous extraction using acid, alkaline or ethanol, solutions combined with protease or cellulase aided solubilization.[40,44-46]

A promising technology for protein extraction is based on the ammonia fiber expansion (AFEX) pretreatment technology.[47] With AFEX, biomass is incubated in an ammonia solution (0.65–3.5 MPa, 70–150°C, 5–15 min), which enables ammonia to permeate in the fiber structures and to solubilize protein. Upon a rapid drop in pressure, cellulosic structures such as cell walls are disrupted, resulting in the liberation of various biomass components.[48] Using this technology, protein is extracted and simultaneously cellulose is processed to be more susceptible to cellulase driven hydrolysis to yield fermentable sugars. In a two-step protein extraction with AFEX pretreatment in between, approximately 80% of the total protein content could be extracted.[40]

Protein hydrolysis

Once extracted, proteins need to be hydrolyzed to liberate the amino acids. In the food industry it is general practice to obtain hydrolyzed vegetable proteins upon exposure to 6 M hydrogen chloride at elevated temperatures over a prolonged period of time. A major drawback of this method is the considerable amount of base required to neutralize the hydrolysate, which will lead to undesirably high concentrations of inorganic salt in the hydrolysate. These inorganic salts need to be separated from the amino acid mixture as it may interfere with downstream processing.[32] As well as this a number of amino acids are destroyed during the

hydrolysis process.

An alternative method for protein hydrolysis, which does not involve high salt concentrations and elevated temperatures, is based on the use of proteases. Using mixtures of proteases, hydrolysis yields up to 90% can be achieved.[49] The major drawback in the application of proteases for the hydrolysis of proteins lies in the high enzyme costs. In this respect, enabling reuse of proteases by means of immobilization might be promising. An interesting immobilization method would be the formation of cross-linked enzyme aggregates, also known as CLEAs.[50,51] In this way protease would not only be immobilized in an economically feasible way, but it would also be protected against autolysis.

A more recent development is the application of supercritical water in the simultaneous extraction and hydrolysis of proteins from biomass residues.[52-55] At supercritical conditions (T > 374.2 °C, P > 22.05 MPa) water exhibits a lower dielectric constant and an ion product which is about 1000 times the value of water under normal temperature and pressure. This results in a higher H_3O^+ and OH^- ion concentration in supercritical water, meaning that both acid and base catalyzed reactions can be performed without actual addition of acid or base. However, reported hydrolysis yields are limited and addition of low concentrations of acid or base still appears to be necessary. Therefore application of supercritical water in the hydrolysis of proteins can be seen as a measure to limit the use of mineral acid and base and their related problems. A drawback of supercritical water is its corrosiveness and the high energy input needed for its formation.

Isolation of desired amino acids

Finally, methods are required which enable the selective isolation of desired amino acids for their application in the production of nitrogen functionalized chemicals. In the case of simple mixtures of amino acids which differ enough with respect to their isoelectric point and polarity, selective precipitation can be achieved based on pH adjustment or addition of a water-miscible organic solvent, or both, in combination with cooling and concentration.[56]

Another option in case of a limited amino acid population would be to selectively

isolate the desired amino acid by means of reactive extraction. *Via* the pH of the solution and thus the protonation state, it can be determined which amino acid will react with the extractant, *e.g.* di-(2-ethylhexyl)phosphoric acid, by means of an interfacial chemical reaction of the ion-exchange type and thereby allowing its extraction from the aqueous phase (Figure 6.9).[57]

However, protein hydrolysates are often complex mixtures and therefore more advanced separation techniques such as ion-exchange chromatography (IEC) will be required. IEC is effective in the separation of complex mixtures of amino acids, but for large scale applications costs are anticipated to be high due to elution times and the need for regeneration.[32]

In search of a method that cannot only isolate specific amino acids from complex mixtures with high selectivity, but at the same time can do so at acceptably low costs, our attention was drawn towards amino acid isolation by means of cyanophycin production.

1.3 Cyanophycin as an intermediate in the amino acid based production of nitrogen containing chemicals

1.3.1 Cyanophycin

CGP was first discovered in 1887 by Borzi[58] during microscopic studies of cyanobacteria. It appeared to be a nitrogen storage polymer that is naturally produced in all groups of cyanobacteria.[59] CGP is built-up from an α-amino-α-carboxy linked poly-L-aspartic acid backbone with L-arginine side chains bound in equimolar amounts via the β-carboxylic groups of the backbone (Figure 1.7, left).[60] CGP production occurs via a non-ribosomal pathway and is regulated through the cyanophycin synthetase A gene *(CphA*).[61]

Since CGP is insoluble under physiological conditions (Figure 1.7, right), it can be easily isolated,[62-64] which together with its defined composition and its

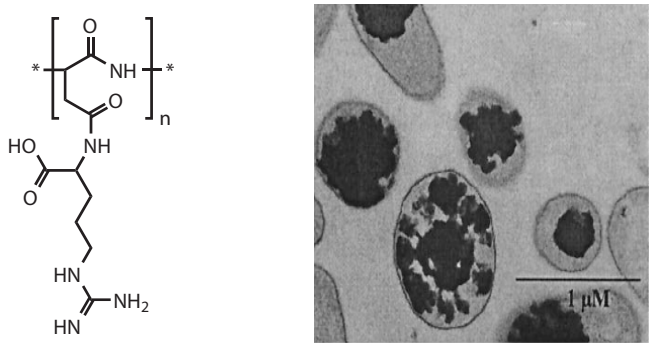


Figure 1.7. Left. Chemical structure of cyanophycin, which consists of a poly-L-aspartic acid backbone with L-arginine branches in equimolar amounts. In cyanobacteria, the length of the backbone *n* was found to be between 73 and 290.[60] **Right.** Microscopic image of insoluble cyanophycin granules in cells of *Acinetobacter calcoaceticus* as published by Elbahloul *et al.*[75]

high nitrogen content make CGP formation a suitable method to selectively isolate L-aspartic acid and L-arginine from amino acid rich residues of the agro-industries.[65] In this way it is possible to 'channel' a dilute stream of twenty different amino acids and many other functionalized compounds to an easy recoverable product stream of just two amino acids.

Heterologous expression of *CphA* in industrially relevant bacteria has opened up the possibility to produce CGP in considerable amounts.[66-70] Even more interestingly, recent work on *Saccharomyces cerevisiae* gives rise to possibilities for the simultaneous production of CGP and ethanol.[71,72] A proof of principle has already been delivered with the pilot-scale production of cyanophycin using protamylasse, an amino acid rich residue from the potato starch processing industries.[65,73,74]

1.3.2 N-ergy project: integrated production of bioethanol and nitrogen containing chemicals

To investigate the possibilities of the integrated production of bioethanol and nitrogen containing chemicals from amino acid rich residues, the N-ergy project was founded. N-ergy involved three PhD research projects hosted by Wageningen

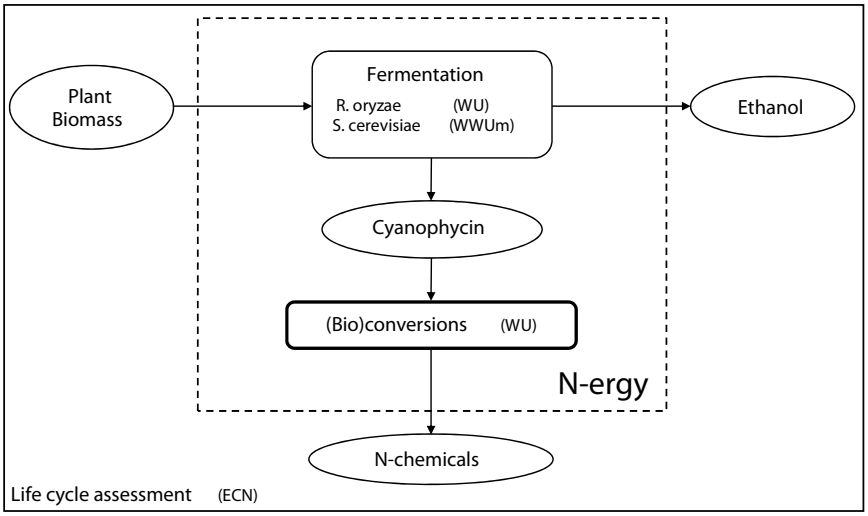


Figure 1.8. Schematic overview of the N-ergy project. WU = Wageningen University, WWUm = Westfälische Wilhelms-Universität Münster, ECN = Energy research Centre of the Netherlands.

University and the Westfälische Wilhelms-Universität Münster. The N-ergy project as a whole was subjected to a life cycle assessment study conducted by the Energy research Centre of the Netherlands (Figure 1.8).

Two PhD research projects focused on the development of microorganisms with the ability to selectively isolate L-aspartic acid and L-arginine from amino acid rich biomass residues by formation of insoluble cyanophycin, while simultaneously producing ethanol. For this purpose *Rhizopus oryzae* was studied at Wageningen University and *Saccharomyces cerevisiae* at the Westfälische Wilhelms-Universität Münster. The third PhD project, which is the basis for this PhD thesis, was conducted at Wageningen University and focused on the (bio)conversion of cyanophycin to nitrogen containing chemicals (N-chemicals).

The N-ergy project was 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.

1.3.3 From cyanophycin towards nitrogen containing chemicals

The envisioned route from cyanophycin towards nitrogen containing chemicals is depicted in Figure 1.9.

In the first step cyanophycin is selectively or completely hydrolyzed, yielding (poly-)L-aspartic acid and L-arginine. Because poly-L-aspartic acid is water soluble and biodegradable, it has been studied as a replacement for polycarboxylate components, which are mainly applied for their functionality as scale inhibitor or dispersing agent.[76,77] The potential market for this polymer could be as high as US$ 450 million per year.[78]

L-aspartic acid is subsequently converted to β-alanine via α-decarboxylation. It is envisioned that β-alanine would subsequently serve as an intermediate in the production of acrylamide (3-5·10^6 tons $year^{-1}$ (worldwide), 3,000 € ton^{-1}).[35] The major application of acrylamide is the manufacture of polyacrylamide, which is mainly used in the treatment of wastewater. Nowadays, acrylamide is produced via the hydrolysis of acrylonitrile, which is produced by the energy intensive ammoxidation of propene.[33]

L-arginine is hydrolyzed to L-ornithine and urea. Urea could be directly applied as fertilizer (1·10^7 tons $year^{-1}$ (Europe and US combined), 210-230 € ton^{-1}) and L-ornithine can be decarboxylated to yield 1,4-diaminobutane (1·10^4 tons $year^{-1}$ (Europe), > 1,600 € ton^{-1}), one of the monomers in nylon-4,6 which is commercially available under the name Stanyl®.[35,79,80] Nowadays, production of 1,4-diaminobutane relies mainly on chemical synthesis by hydrogenation of succinonitrile, which is produced by the addition of hydrogen cyanide to acrylonitrile.[34]

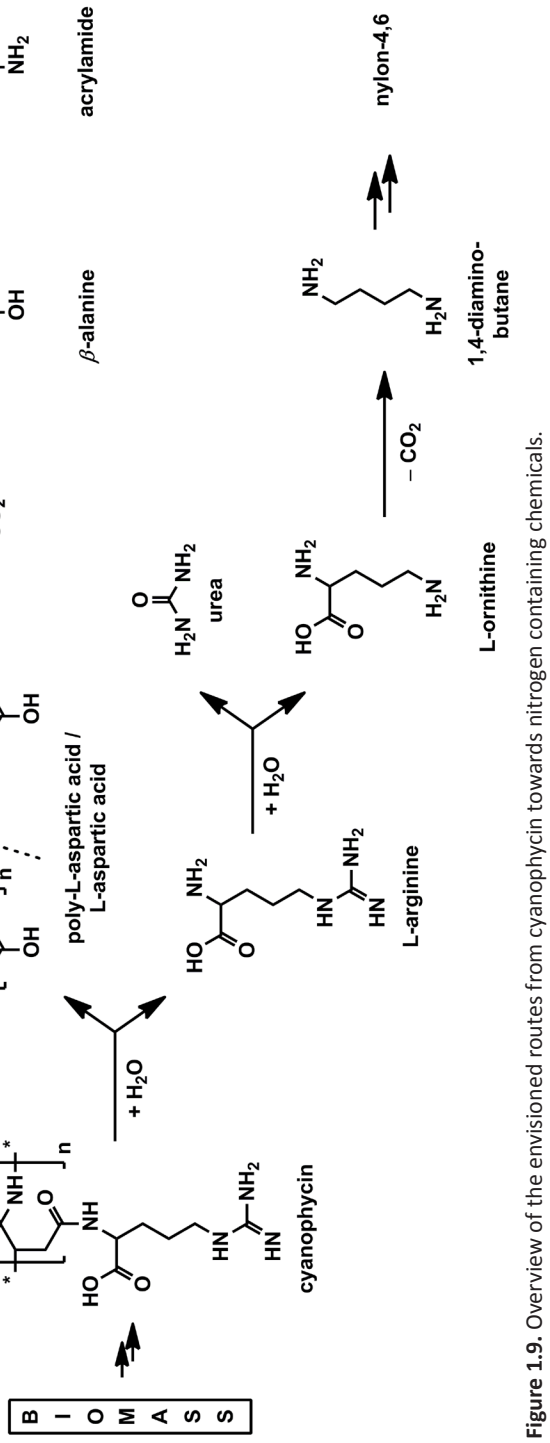


Figure 1.9. Overview of the envisioned routes from cyanophycin towards nitrogen containing chemicals.

1.4 Research aim

The aim of the research presented in this thesis was to explore and optimize the different (bio)conversion steps involved in the envisioned route from cyanophycin towards nitrogen containing chemicals. More specifically, for each conversion that we investigated we aimed to determine the crucial process parameters enabling us to improve productivity or at least identify the bottlenecks of the envisioned route.

1.5 Thesis outline

Chapter 2: Cyanophycin hydrolysis. In this chapter a study is presented on the acid and base catalyzed hydrolysis of cyanophycin (CGP). While using different concentrations of acid and base at 90 and 100°C, the hydrolysis yields were determined in the course of time. Using this information, the rate constants for the liberation of L-aspartic acid and L-arginine related compounds were determined. Finally, the optimal conditions for either complete or selective hydrolysis of CGP were determined. The first leads to the individual amino acids, the second to an industrially interesting polymer with reduced L-arginine content.

Chapter 3: L-aspartic acid α-decarboxylation. In this chapter the results of a study on the applicability of *Escherichia coli* L-aspartate α-decarboxylase (ADC) for the production of β-alanine from L-aspartic acid are presented. For the soluble enzyme the temperature and pH dependent activity and stability and the effect of product inhibition were investigated. ADC was immobilized on two different types of Sepabeads supports: the relatively hydrophobic epoxy support Sepabeads EC-EP and the relatively hydrophilic amino-epoxy support Sepabeads EC-HFA. The thermal and operational stability of the immobilized and soluble enzyme were compared.

Chapter 4: L-arginine hydrolysis. Here the results of a study on stabilization and immobilization of *Bacillus subtilis* arginase are presented for the production of L-ornithine and urea. For the soluble enzyme the conditions for optimal operational stability were determined using a continuously stirred membrane

reactor system. Furthermore, *B. subtilis* arginase was immobilized on three different epoxy functionalized supports: Sepabeads EC-EP and EC-HFA, and Eupergit C 250L. The thermal and operational stability of the immobilized and soluble enzyme were compared.

Chapter 5: L-ornithine decarboxylation. In this chapter a study on the applicability of *Trypanosoma brucei* ornithine decarboxylase (TbODC) in the production of 1,4-diaminobutane is presented. For soluble TbODC the effects of different types of additives, pH, temperature and ionic strength on activity and stability were investigated. Furthermore, TbODC was immobilized on three different epoxy-activated supports: Sepabeads EC-EP and EC-HFA, and Immobead-150. The thermal and operational stability of soluble and immobilized TbODC were compared.

Chapter 6: General discussion. Finally, the impacts of the results obtained in Chapter 2, 3, 4 and 5 on the envisioned conversion of CGP to nitrogen containing chemicals are discussed. The chapter is concluded with suggestions for further research.

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Chapter 2

Acid and base catalyzed hydrolysis of cyanophycin for the biobased production of nitrogen containing chemicals

This chapter is based on

Paul M. Könst, Elinor L. Scott, Maurice C.R. Franssen and Johan P.M. Sanders,

Journal of Biobased Materials and Bioenergy, 2011, **5**, 102-108.

Abstract

While growing on side-streams of the agro-industries, engineered microorganisms can produce ethanol and simultaneously bind L-aspartic acid and L-arginine in equimolar amounts in the polyamino acid cyanophycin. In this way, widely available amino acids can be isolated and utilized as an alternative feedstock for the production of nitrogen containing chemicals which are nowadays based on naphtha. The first step in the envisioned route from cyanophycin towards nitrogen containing chemicals is the complete or selective hydrolysis of cyanophycin. In this study, we investigated the acid and base catalyzed hydrolysis of cyanophycin. Acid catalyzed hydrolysis of cyanophycin yields both L-aspartic acid and L-arginine at comparable rates and is therefore suitable for complete hydrolysis. Upon base catalyzed hydrolysis, the rate of L-arginine liberation is overall significantly higher than that of L-aspartic acid, which enables selective hydrolysis of cyanophycin to yield a residue with commercially interesting polyaspartic acid functionality. To conclude this study, a cyanophycin residue with reduced L-arginine content was prepared and isolated.

2.1 Introduction

Many nitrogen containing bulk chemicals are produced from naphtha. In these processes large amounts of energy and ammonia are used, *e.g.* in the ammoxidation of olefins.[1] Global warming, depletion of fossil resources and geopolitical instability are forcing us to make the transition towards CO_2 neutral, sustainable and economically stable alternatives for the naphtha based production of functionalized bulk chemicals. In this respect, biomass has great potential.

Following the biorefinery concept, it would be more energy efficient and eventually more economical to start from biobased, functionalized compounds that already have nitrogen incorporated, such as amino acids, instead of starting from naphtha's plain hydrocarbons.[2]

Together with other functionalized compounds (*e.g.* organic acids and lignins), enormous amounts of amino acids are generated as side streams of biofuel production and of the agro-industries in general.[3] As a result, the bottleneck in the application of amino acids in the biobased production of nitrogen containing bulk chemicals lies not in the available quantity, but in the isolation of the desired amino acids from a mixture of available functionalized compounds, *e.g.* protein hydrolysate. Bearing this in mind, our attention has been drawn to the polypeptide cyanophycin (CGP).

CGP consists of a poly-L-aspartic acid backbone with L-arginine branches in equimolar amounts and is naturally produced in cyanobacteria as a nitrogen storage polymer.[4] Heterologous expression in industrially relevant bacteria has opened up the possibility to produce CGP in considerable amounts.[5-8] Even more interestingly, recent work on *Saccharomyces cerevisiae*[9,10] gives rise to possibilities for the simultaneous production of CGP and ethanol from amino acid rich waste streams from the agro-industries.[11-13]

Since CGP is insoluble under physiological conditions, it can be easily isolated which, together with its defined composition, its integrated production and its high nitrogen content, makes CGP an interesting feedstock for the production of

nitrogen containing bulk chemicals.[13-16]

In view of this, we have investigated the conversion of CGP's hydrolysate, consisting of L-aspartic acid and L-arginine (described in subsequent chapters). The application of the enzymes aspartate α-decarboxylase (EC 4.1.1.15), arginase (EC 3.5.3.1) and ornithine decarboxylase (EC 4.1.1.17) in the novel routes towards nitrogen containing bulk chemicals like acrylamide, urea and 1,4-diaminobutane was studied (Figure 2.1).[17-19]

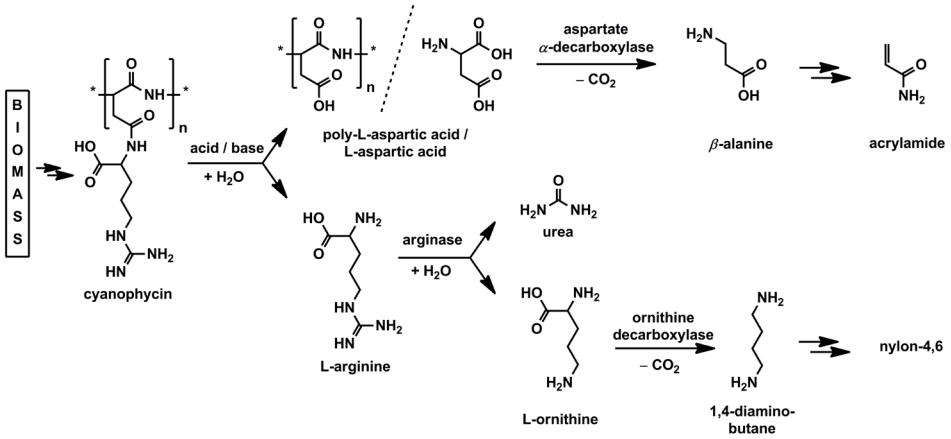

Figure 2.1. Envisioned route from CGP towards nitrogen containing chemicals. We investigated the conversion of the CGP building blocks L-aspartic acid and L-arginine, using the enzymes aspartate α-decarboxylase, arginase and ornithine decarboxylase (described in subsequent chapters).[17-19]

Complete hydrolysis of polyamino acids such as CGP is readily achieved upon reaction with high concentrations of a strong acid at elevated temperatures, *e.g.* 6 M hydrochloric acid for 16 hours at 90°C, which is part of the standard procedure for the determination of the amino acid content of proteins in general.[15,20,21] Although this method of protein hydrolysis is widely applied at the lab scale, complete hydrolysis of CGP needs to be further investigated in advance of large scale application.

An alternative route to the complete hydrolysis of CGP followed by the conversion of L-aspartic acid and L-arginine towards nitrogen containing bulk chemicals, is

the selective hydrolysis of the L-arginine branches yielding a residue with reduced L-arginine content which can be applied as polyaspartic acid (PASP). Because PASP is water soluble and biodegradable, it has been studied as a replacement for polycarboxylate components, which are mainly applied for their functionality as a scale inhibitor or dispersing agent.[22,23] PASP can be obtained by chemical synthesis, *e.g.* by either the thermal polymerization of aspartic acid or maleic acid anhydride in the presence of ammonia to polysuccinimide followed by alkaline hydrolysis.[23,24]

Selective hydrolysis of CGP with the purpose to obtain a residue with PASP properties is often mentioned in literature.[7,11,13,15,25,26] These publications refer to either a patent by Joentgen *et al.*[27] or a more recent patent by Elbahloul *et al.*[28] Both claim that selective hydrolysis can be achieved with both acid and base, but preferably under basic conditions since CGP's poly-L-aspartic acid backbone is more stable under these conditions. Nonetheless, neither patent is very specific in the required conditions and both present only one practical example of the selective hydrolysis of CGP to PASP: Elbahloul *et al.* suggest 0.07-0.55 M of either acid or base at 75-90°C for 15 hours to achieve selective hydrolysis, while Joentgen *et al.* used 0.375 M sodium hydroxide at 90°C for 12 hours.

Altogether, more details about the required conditions for either complete or selective hydrolysis of CGP are desired to get better insight into the possible application of CGP as a feedstock for the production of nitrogen containing chemicals. In this paper, we present a study on the acid and base catalyzed hydrolysis of CGP.

2.2 Results and discussion

Before the effect of base or acid catalyzed hydrolysis of CGP was investigated, the amino acid content of CGP was determined after complete hydrolysis (6 M hydrochloric acid, 90°C, 16 hours) using UHPLC and ^{1}H-NMR analysis. The CGP used in this study consisted of 50% L-aspartic acid, 45% L-arginine and 5% L-lysine. For the sake of clarity, since L-lysine entails only a small percentage, we assumed

that the CGP used in this study consisted of only L-aspartic acid and L-arginine in a 50:45 ratio and we did not pay attention to the formation of L-lysine or any products derived thereof.

The acid and base catalyzed hydrolysis was investigated by subjecting aliquots of CGP to different concentrations of hydrochloric acid or sodium hydroxide (0.01, 0.10, 0.50 and 1.00 M) at 90 and 110°C. Over time the concentrations of the amino compounds in the hydrolysate were determined using UHPLC analysis.

2.2.1 Acid catalyzed hydrolysis

Using hydrochloric acid for the hydrolysis of CGP, only L-aspartic acid and L-arginine were found in the hydrolysate. Additionally, HPLC based organic acid analysis revealed that organic acids such as maleic and fumaric acid were absent in the hydrolysate. These observations are in agreement with previous reports in literature stating that degradation of L-aspartic acid and L-arginine is minimal at the conditions applied in this study.[29-31]

Assuming first-order kinetics, the rate constants for amino acid liberation k_{lib} were determined by plotting $-\ln(1-[AA_{free}]_t/[AA_{CGP}]_0)$ against time for each amino acid and each condition tested, where $[AA_{free}]_t$ is the concentration of free amino acid in the hydrolysate after t hours and $[AA_{CGP}]_0$ is the amount of amino acid initially bound in CGP. Indeed linear relations were obtained in the form of Equation 1.1, allowing calculation of the rate constants for amino acid liberation k_{lib} for every reaction condition and each amino acid.

$$-\ln\left(1-\frac{[AA_{free}]_t}{[AA_{CGP}]_0}\right) = k_{lib}\cdot t \qquad (1.1)$$

The obtained rate constants were plotted against the applied hydrochloric acid concentrations and a linear relation was observed (Figure 2.2a). The rate constants for the liberation of L-aspartic acid and L-arginine are both significantly higher at 110°C when compared to 90°C. At 90°C the rate constants for the liberation

of L-aspartic acid and L-arginine do not differ significantly. At 110 °C the rate constants for L-arginine liberation are overall slightly higher than those determined for L-aspartic acid.

Using the appropriate equation from Figure 2.2a, the concentration of liberated amino acid can be predicted at any given time *t* using Equation 1.2 (derived from Equation 1.1).

$$[AA_{free}]_t = [AA_{CGP}]_0 \cdot (1 - \exp(-k_{lib} \cdot t)) \quad (1.2)$$

It has been mentioned in literature that acid catalyzed hydrolysis is suitable for the selective hydrolysis of CGP yielding a residue with PASP properties.[27,28] Nonetheless, due to the low selectivity between the liberation of L-aspartic acid and L-arginine upon acid catalyzed hydrolysis of CGP, as observed in this study, acid catalyzed hydrolysis does not seem to be a suitable method for the selective hydrolysis of CGP. For the same reason, acid is a suitable catalyst for the complete hydrolysis of CGP yielding a mixture of only L-aspartic acid and L-arginine.

2.2.2 Base catalyzed hydrolysis

Rate constants

Due to subsequent hydrolysis of L-arginine (Figure 2.4), base catalyzed hydrolysis of CGP resulted in a more complex product mixture than acid catalyzed hydrolysis. Following the procedure described in section 2.2.1, the rate constants for the liberation of L-aspartic acid and L-arginine were determined for each tested condition. For the calculation of the rate constants for L-arginine liberation, the sum of the L-arginine related hydrolysis products was used. The rate constants were plotted against the applied sodium hydroxide concentrations, grouped by temperature (Figure 2.2b).

The rate constant for L-aspartic acid liberation has a positive linear relation with the applied concentration of sodium hydroxide. For comparison, the rate constants for sodium hydroxide catalyzed L-aspartic acid liberation are overall six to eight

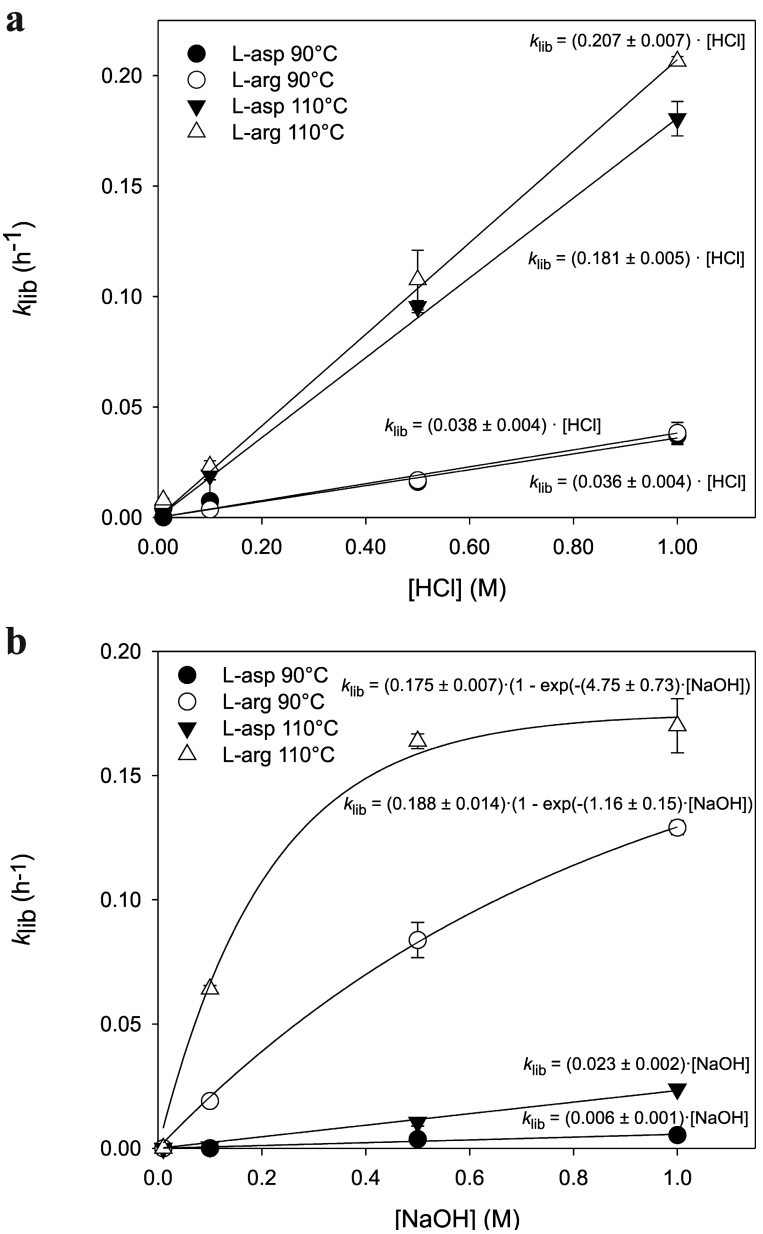

Figure 2.2. Rate constants for the liberation of amino acid plotted against concentration of catalyst, grouped by temperature. The equations present linear and non-linear relations between rate constant and catalyst concentration. **a.** Rate constants obtained upon acid catalyzed hydrolysis. **b.** Rate constants obtained upon base catalyzed hydrolysis. The rate constants for the liberation of L-arginine are obtained using the sum of L-arginine and related compounds, namely L-ornithine, L-citrulline and 3-aminopiperidine-2-one.

times smaller than observed with hydrochloric acid catalyzed liberation. This is reasonable as acid catalyzed hydrolysis enables the protonation of the amino group, turning it into a better leaving group.

The rate constant for the liberation of L-arginine and related compounds rises nonlinearly with the sodium hydroxide concentration, leveling off at high base concentration. The maximum rate constant is approximately 0.18 h^{-1} at both 90 and 110°C.

Similar to the acid catalyzed hydrolysis of CGP, the obtained equations for the rate constants for base catalyzed liberation of L-aspartic acid and L-arginine related compounds (Figure 2.2b) can be used in Equation 1.2 to determine the concentration of liberated amino acid at any given time *t*.

Overall, the rate of liberation for L-aspartic acid was significantly smaller than that observed for L-arginine and related compounds. From this observation can be concluded that base catalyzed hydrolysis of CGP occurs with a preference for the L-arginine branches. This selectivity gives opportunities for the production of PASP from CGP.

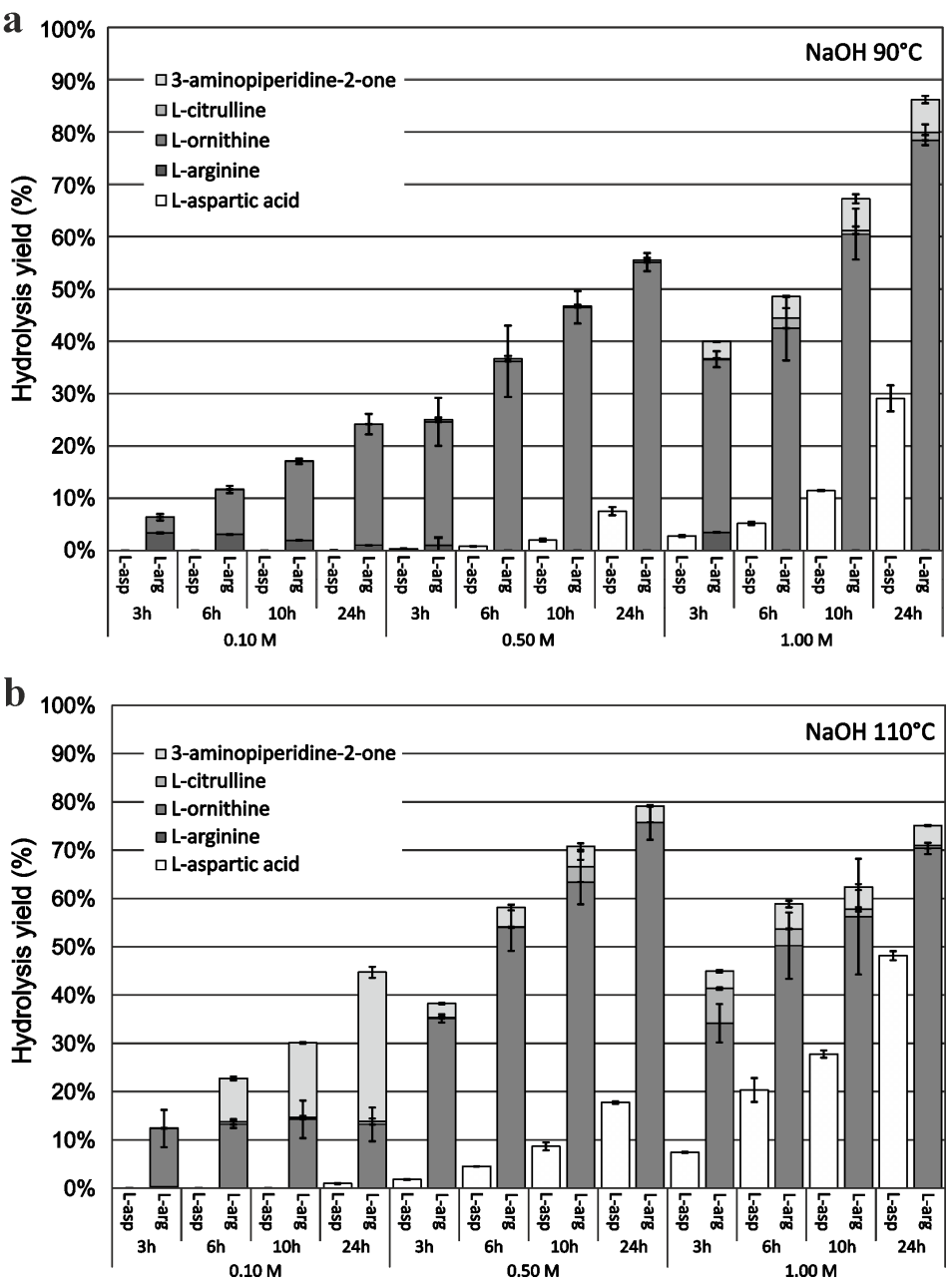

Figure 2.3. Hydrolysis yields upon reaction with different concentrations of sodium hydroxide, presented as the amounts of liberated amino acid or related amino compound relative to the amount of their origin, either L-aspartic acid or L-arginine, initially bound in CGP. **a.** Results obtained upon reaction at 90°C. **b.** Results obtained upon reaction at 110°C.

Hydrolysis yields

The hydrolysis yields are presented in Figure 2.3 to give an overview of the hydrolysis products over time. The hydrolysis yields are determined as the amount of liberated amino compound relative to the amount of their origin, either L-aspartic acid or L-arginine, initially bound in CGP. As low yields were obtained with 0.01 M sodium hydroxide, it was decided to omit these results from Figure 2.3 for the sake of clarity.

Only small quantities of L-arginine could be observed at 90°C and in particular at 0.10 M sodium hydroxide, while at higher base concentrations and 110°C L-arginine was absent in the hydrolysate. As mentioned before, in the course of the base catalyzed hydrolysis of CGP L-arginine was further hydrolyzed yielding L-ornithine, L-citrulline and 3-aminopiperidine-2-one (Figure 2.4). The formation of these amino compounds upon base catalyzed hydrolysis of free L-arginine under similar conditions has been reported in literature.[29,32]

The possibility to reduce the L-arginine content of CGP and to form L-ornithine in one simple step might be advantageous for the envisioned application of CGP (Figure 2.1). In this way the anticipated enzymatic hydrolysis of liberated L-arginine would not be required. To improve the L-ornithine yields, the formation of L-citrulline and 3-aminopiperidine-2-one could be minimized by choosing the most optimal conditions.

However, base catalyzed hydrolysis of L-arginine does not only yield L-ornithine, but also D-ornithine due to racemization. For example, 10 hours of reaction of L-arginine at 110°C in 0.5 M sodium hydroxide results in a mixture of D/L-ornithine in a ratio of respectively 17/83 as determined by ^{1}H-NMR following Hisayuki *et al.*[33] Formation of D-ornithine is problematic since chemical methods for the decarboxylation of ornithine to 1,4-diaminobutane are rather limited and the preferred enzyme ornithine decarboxylase has a high preference for L-ornithine.[18] As a result additional steps would be needed to convert D-ornithine to L-ornithine to make decarboxylation possible.

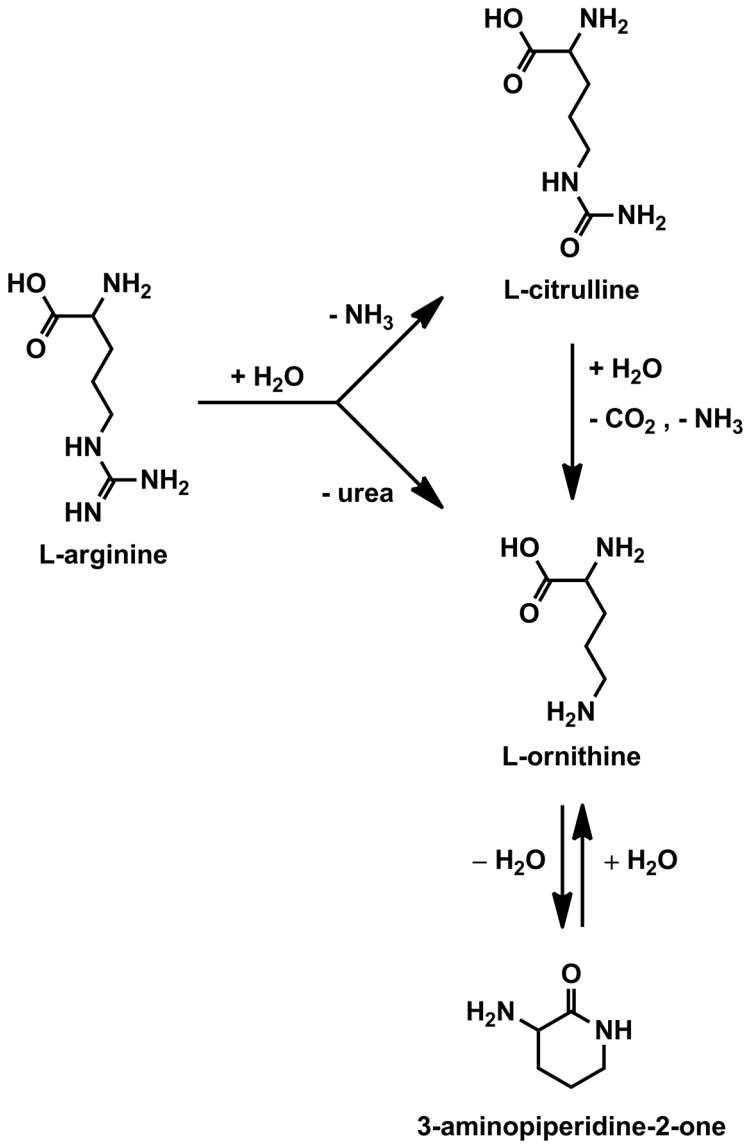

Figure 2.4. Schematic overview of the products involved in the base catalyzed hydrolysis of L-arginine based on the work of Warner[29] and Murray *et al.*[32]

PASP backbone degradation

Earlier we deduced from the rate constants (Figure. 2.2b) that base catalyzed hydrolysis of CGP occurs with a preference for CGP's L-arginine branches. Nonetheless, over time and at higher sodium hydroxide concentrations, the concentration of L-aspartic acid will increase to considerable amounts (Figure 2.3). Liberation of L-aspartic acid from CGP implies that its polyaspartic acid backbone is hydrolyzed. To get insight in the degree of hydrolysis, the average molecular weight of the CGP residues was determined over time using gel permeation chromatography (GPC) (Figure 2.5).

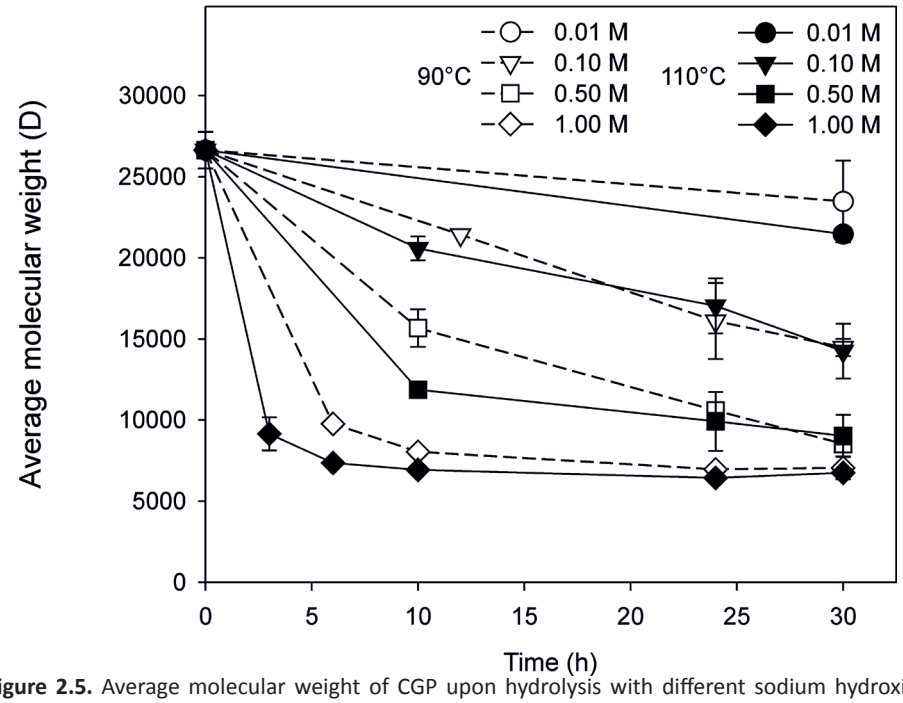

Figure 2.5. Average molecular weight of CGP upon hydrolysis with different sodium hydroxide concentrations at 90 and 110°C in time. The average molecular weights were determined using GPC analysis with a polystyrene sulfonic acid standard.

There is a clear relation between the increase in yield of liberated L-aspartic acid (Figure 2.3) and the decrease in the average molecular weight of the CGP residue (Figure 2.5). Accordingly, the rate of CGP degradation increases with higher sodium

hydroxide concentrations and a higher temperature as does the rate of L-aspartic acid liberation. In summary, the occurrence of L-aspartic acid in the hydrolysate is an indication for the degree of hydrolysis of CGP's polyaspartic acid backbone. Since L-aspartic acid liberation will occur over time, a choice needs to be made between the degree of PASP functionality and the polymer length of the CGP residue.

Partial CGP hydrolysis: Proof of principle

To demonstrate the proof of principle, CGP with a reduced L-arginine content was prepared. A quantity of 500 mg CGP was reacted in 0.5 M sodium hydroxide for 3 hours at 110°C. After reaction, the pH of the solution was adjusted to 7.0 and the precipitate of unreacted CGP was separated by means of filtration. Joentgen *et al.*[27] stated that unreacted CGP would precipitate together with the liberated L-arginine at alkaline conditions and therefore pH adjustment would not be necessary. However, CGP is known to be soluble below pH 4 and above pH 9[26] and following the results presented in this study (Figure 2.3) L-arginine is mainly liberated in the form of L-ornithine, which has a high solubility and therefore remains in solution.

After filtration, the CGP residue with reduced L-arginine content was precipitated using methanol. The precipitate was isolated by centrifugation and dried *in vacuo*. The CGP residue with reduced L-arginine content was analyzed by ^{1}H-NMR (Figure 2.6).

Using the peak areas in the obtained ^{1}H-NMR spectrum, we calculated that the L-arginine fraction was reduced by 36%, which is in agreement with the amount of liberated amino compounds observed in Figure 2.3. The ^{1}H-NMR spectrum also points out that 0.2 equivalents of free L-ornithine co-precipitated upon methanol precipitation. Although currently an isolated yield of 53% was obtained, this experiment highlights the principle for the production of a CGP residue with reduced L-arginine content by means of base catalyzed hydrolysis.

$$X_{PASP} = \left(1 - \frac{A_{\text{L-arg/orn branch C}\alpha\text{H}}}{A_{\text{L-asp backbone C}\alpha\text{H}}}\right) \cdot 100\% = 36\%$$

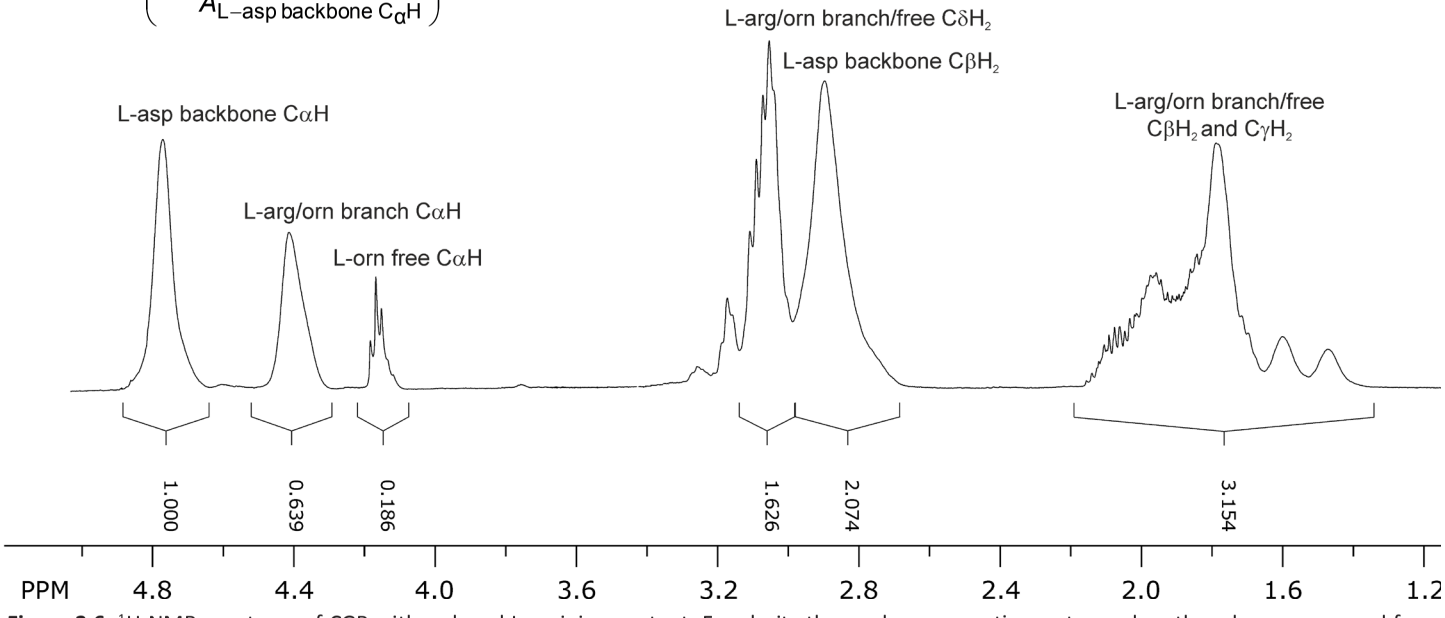

Figure 2.6. ^{1}H-NMR spectrum of CGP with reduced L-arginine content. For clarity the peaks representing water and methanol were removed from this spectrum.

2.3 Conclusions

Acid catalyzed hydrolysis of CGP yields both L-aspartic acid and L-arginine at comparable rates and is therefore suitable for complete hydrolysis. Upon base catalyzed hydrolysis of CGP, the rate of L-arginine liberation is overall significantly higher than that of L-aspartic acid, which enables selective hydrolysis of CGP. Over time L-aspartic acid liberation and thus hydrolysis of the polyaspartic acid backbone of CGP will occur and therefore a choice needs to be made between the degree of PASP functionality and the polymer length of the CGP residue.

2.4 Materials and Methods

2.4.1 Materials

The insoluble CGP used in this study was produced by recombinant *Escherichia coli* grown on protamylasse and was kindly provided by Prof. Alexander Steinbüchel from the Westfälische Wilhelms-Universität Münster.[11,15]

All solutions and buffers used in this study were prepared with MilliQ water. All chemicals used in this study were of analytical grade or better and used as received.

2.4.2 Methods

Acid and base catalyzed hydrolysis

Reaction mixtures were prepared by adding 0.6 mL hydrogen chloride or sodium hydroxide solution of different concentrations (0.01, 0.10, 0.50, 1.00 M) to aliquots of 15 mg cyanophycin in glass screw-cap vials (12 x 32 mm, Grace Alltech). After sealing the vials using screw caps with PTFE/silicone septa, the vials were submerged in a preheated oil bath at the desired temperature. At given time intervals, complete vials were taken from the oil bath and stored at 4°C. In advance of the analyses using UHPLC and GPC, the reaction mixtures were neutralized by adding 0.6 mL of the appropriate solution, *e.g.* 0.1 M hydrogen chloride was added

to neutralize the 0.1 M sodium hydroxide solution.

UHPLC analyses

The UHPLC analyses were performed on a Dionex Ultimate 3000 UHPLC system, mounted with a Dionex Acclaim 120 C18 reversed phase column (2.1x100 mm, 2.2 µm), operated at 40°C. After derivatization with o-phthaldialdehyde (OPA), the amino compounds were separated using a gradient at 0.722 mL min^{-1} of solution A (2 mM NaN_3 in 10 mM sodium phosphate buffer pH 6.5) and solution B (45 % methanol and 45% acetonitrile in water): 0 min, 5.5% B; 0.21 min, 5.5% B; 7.216 min, 58.3% B; 9.416 min, 100.0% B; 10 min, 100.0% B, 10.12 min, 5.5% B, until 20 min. The eluted compounds were detected by means of UV absorption at 338 nm.

The samples were prepared and derivatized as follows. An aliquot of 500 µL of 100 times diluted, neutralized sample was combined with 100 µL internal standard solution (L-norleucine) and 400 µL methanol. After filtration (0.20 µm) the sample mixture was placed in the sample carousel at 10°C.

The samples were derivatized in the syringe of the injector combining, respectively, 5 µL derivatization solution (40 mM OPA and 225 mM mercaptopropionic acid in 0.1 M borate buffer pH 9.0), 50 µL water, 0.5 µL sample, 1 µL 2.5 mg mL^{-1} fluorenylmethyloxycarbonyl chloride in acetonitrile and 3 µL 3% phosphoric acid.

GPC analyses

A 25 µL aliquot of neutralized reaction mixture was 20 times diluted with 475 µL 0.5 M sodium hydroxide solution, filtered (0.45 mm) and analyzed as such. The GPC (gel permeation chromatography) system used was based on two columns in series packed with Toyopearl gels (Tosoh bioscience GmbH), one with HW–55F and the other with HW-75F. The system was operated isocraticly using 0.5 M sodium hydroxide at a flow rate of 1.0 mL min^{-1} at 30°C (Waters Breeze System). The samples were injected at a volume of 50 µL (Waters 717 Autosampler) and the compounds were detected by means of UV absorption at 230 nm (Waters 2487 Dual Absorbance UV/vis Detector). The results were analyzed using a set of

polystyrene sulfonic acid standards.

Preparation of CGP with reduced L-arginine content

The desired amount of CGP was divided in 30 mg aliquots and hydrolyzed as previously described in the section "Partial CGP hydrolysis: Proof of principle". After hydrolysis the aliquots were combined and the pH was adjusted to pH 7 using concentrated hydrogen chloride.

In order to collect insoluble residue, the combined fractions were filtered by centrifugation (21,380 x *g*, 5 min) using Costar Spin-X centrifuge tube filters (cellulose acetate membrane, 0.22 µm pores). The filtrate was added dropwise to five equivalents of cold methanol, followed by vigorously vortexing and cooling for one hour in the fridge (5°C). The precipitate was collected by filtration using the previously described centrifuge tube filters.

The precipitates were washed by adding 1 mL methanol, followed by vortexing and centrifugation (19.000 rpm, 5 min), after which the supernatant was removed. The residue was further washed using 1 mL and two times 0.5 mL 80% methanol in water. The washed residues were dried overnight *in vacuo* at room temperature.

^{1}H-NMR analyses

The samples were dissolved in deuterium oxide of which the pH was adjusted by addition of deuterized sulfuric acid. All samples were supplemented with 5 mg trimethylsilylpropanoate for reference. Analyses were performed using a Bruker Avance III 400MHz NMR spectrometer.

Acknowledgements

We would like to thank Prof. Alexander Steinbüchel from the Westfälische Wilhelms-Universität Münster for being so kind to supply us with cyanophycin. Furthermore, we would like thank 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.

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Chapter 3

A study on the applicability of L-aspartate α-decarboxylase in the biobased production of nitrogen containing chemicals

This chapter is based on

Paul M. Könst, Maurice C.R. Franssen, Elinor L. Scott and Johan P.M. Sanders

Green Chemistry, 2009, **11**, 1646-1652.

Abstract

β-Alanine could serve as an intermediate in the biobased production of nitrogen containing chemicals from L-aspartic acid. Following the biorefinery concept, L-aspartic acid could become widely available from biomass waste streams via the nitrogen storage polypeptide cyanophycin. Since *α*-decarboxylation of L-aspartic acid is difficult to perform chemically, the applicability of *Escherichia coli* L-aspartate *α*-decarboxylase (EC 4.1.1.11) (ADC) for the production of *β*-alanine was studied. With an increasing activity up to 90°C and maintaining its activity upon storage for 24 hours at 60°C, ADC showed a remarkably high thermostability. ADC has an optimum at pH 7.5 and starts to lose activity upon storage below pH 6. An inhibiting effect by *β*-alanine was not observed. Immobilization on Sepabeads EC-EP and EC-HFA epoxy supports did not result in an increased thermostability, but did improve operational stability. Nonetheless, enzyme inactivation occurs during catalysis, probably caused by irreversible transamination of the catalytically essential pyruvoyl group.

3.1 Introduction

Global warming, depletion of fossil resources and geopolitical instability are forcing us to make the transition towards CO_2 neutral, sustainable and economically stable alternatives for our naphtha based society. In this respect, biomass has great potential. At the moment of writing, the emphasis is on the application of biomass as a feedstock for energy and transport fuels. However, the application of biomass as a feedstock for functionalized bulk chemicals receives far less attention. This is remarkable considering the enormous amounts of functionalized compounds (*e.g.* amino acids, organic acids, and lignin) that are generated as side streams of the continuously growing biofuel production.[1]

Nowadays, most nitrogen containing bulk chemicals (*e.g.* 1,4-diaminobutane and acrylamide) are produced from naphtha. In these processes large amounts of energy and ammonia are used for the ammoxidation of olefins.[2] Following the biorefinery concept, it would be more energy efficient to start from functionalized compounds that already have nitrogen incorporated, such as amino acids, instead of starting from naphtha's plain hydrocarbons.[3] Bearing this in mind our attention has been drawn to the polypeptide cyanophycin (CGP).

CGP consists of a poly-L-aspartic acid backbone with L-arginine branches in equimolar amounts and is naturally produced in cyanobacteria as a nitrogen storage polymer.[4] Heterologous expression in industrially relevant bacteria[5-8] opened up the possibility to produce CGP in considerable amounts. Even more interestingly, recent work on *Saccharomyces cerevisiae*[9,10] has opened possibilities for the simultaneous production of CGP and ethanol from amino acid rich waste streams from the agro-industries.[11-13]

Since CGP is insoluble under physiological conditions, it can be easily isolated[14,15] which together with its defined composition, its integrated production and its high nitrogen content make CGP a potential feedstock for the production of nitrogen containing bulk chemicals.[13]

Our research is focused on the development of integrated processes for the

conversion of CGP's hydrolysate, L-aspartic acid and L-arginine, towards existing nitrogen containing bulk chemicals. Starting from L-aspartic acid, we envision *β*-alanine (3-aminopropionic acid) as intermediate in the production of nitrogen containing bulk chemicals such as acrylamide and acrylonitrile (Figure 3.1).

In this route L-aspartic acid is decarboxylated at the *α*-position, yielding *β*-alanine and carbon dioxide. Examples of non-enzymatic methods for the *α*-decarboxylation of L-aspartic acid and analogues such as L-glutamic acid are rather limited.[16,17] For this reason we focus on the applicability of the enzymatic *α*-decarboxylation of L-aspartic acid by *E. coli* L-aspartate *α*-decarboxylase (EC 4.1.1.11) (ADC) leading to *β*-alanine as the sole product.[18] The only study known to the authors that investigated the applicability of ADC concerns a patent on the conversion of D/L-aspartic acid to D-aspartic acid and *β*-alanine using whole cell biocatalysis.[19]

When considering the application of an enzyme, the temperature and pH dependent activity and stability are crucial. Previously, the optimal pH (pH 6.5-7.5) and temperature (55°C) for ADC were determined.[18,20] However, this information is not very detailed and apart from the conditions for optimal activity, also information about the temperature and pH dependent stability of ADC is required.

On an industrial scale immobilization of enzymes is desirable, because this enables reuse and overcomes problems with product recovery. Additionally, immobilization might enhance the enzyme's stability towards the stressed conditions of an industrial process.[21] Due to their ease of operation and their robustness under physical stress, epoxy activated Sepabeads are suitable supports for immobilization on an industrial scale.[22,23]

In this chapter, we present the results of a study on the applicability of *Escherichia coli* L-aspartate *α*-decarboxylase for the production of *β*-alanine from L-aspartic acid. For the soluble enzyme the temperature and pH dependent activity and stability and the effect of product inhibition were investigated. ADC was immobilized on two different types of Sepabeads epoxy supports: EC-EP and EC-HFA. The thermal and operational stability of the immobilized and soluble enzyme were compared.

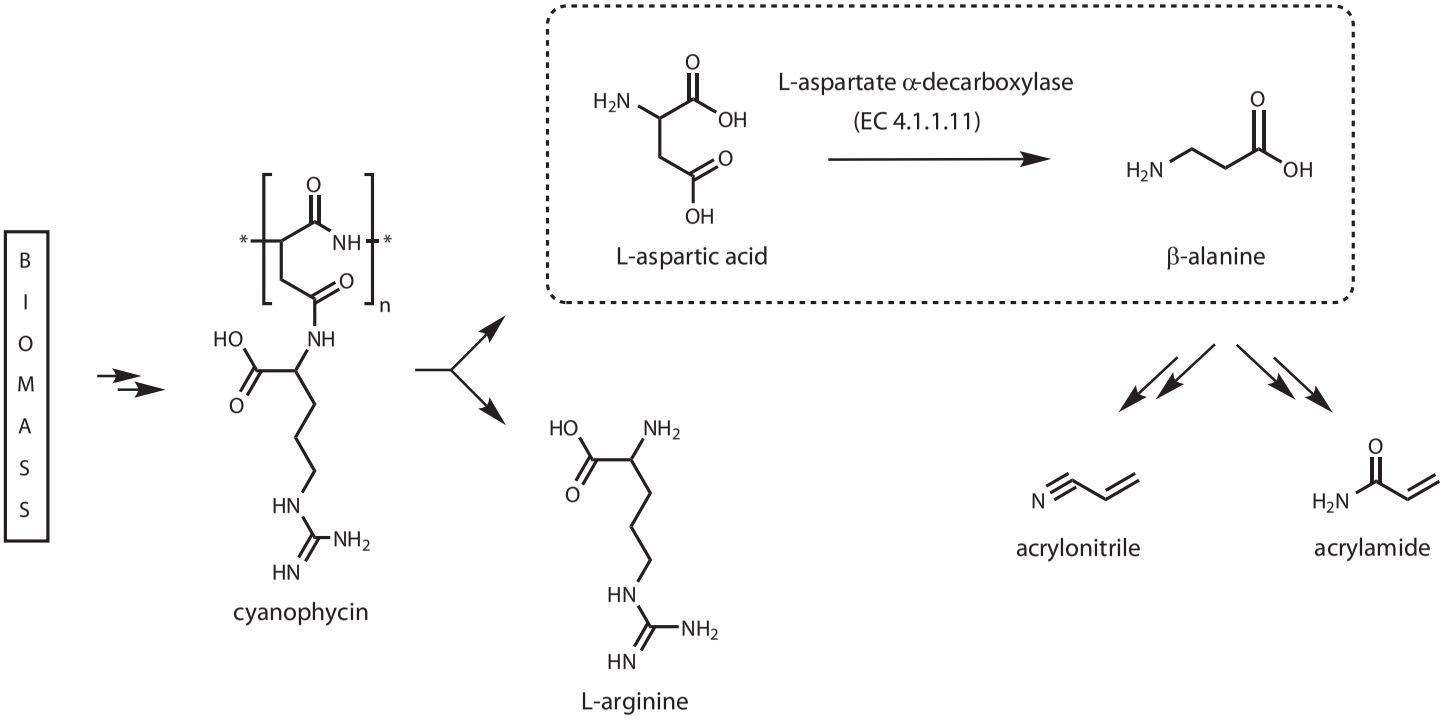

Figure 3.1. Route from cyanophycin towards nitrogen containing bulk chemicals with the enzymatic α-decarboxylation of L-aspartic as an intermediate step.

3.2 Results and Discussion

3.2.1 Product inhibition

Williamson and Brown[18] did not observe product inhibition for ADC, based on an assay with 1 mM L-aspartic acid and 0.250 mM *β*-alanine. In the current study the inhibiting effect of *β*-alanine was investigated within a range of different combinations of *β*-alanine and L-aspartic acid concentrations. Using SigmaPlot 10.0 (Systat Software, Inc.) in combination with Enzyme Kinetics Module 1.3 (Systat Software, Inc.), a Michaelis-Menten competitive inhibition model was fitted to the obtained specific activities.

A first observation of the results clearly revealed that *β*-alanine does not have an inhibiting effect in the observed concentration range (results not shown). Subsequently, the Michaelis-Menten competitive inhibition model returned an extremely high and insignificant product inhibition constant. Although an inhibiting effect at higher concentrations of *β*-alanine cannot be ruled out, it can safely be assumed that with conversions at higher concentrations of L-aspartic acid inhibition by *β*-alanine can be neglected.

Fitting a Michaelis-Menten model without inhibition returned a Michaelis-Menten constant (K_M) of 0.240 ± 0.011 mM and a maximum activity (V_{max}) of 640 ± 8 mol min^{-1} mg^{-1}.

3.2.2 Temperature dependent activity and stability

The results of the temperature dependent activity and stability study are remarkable. Instead of a temperature optimum, ADC showed an increasing specific activity from 224 nmol min^{-1} mg^{-1} at 25°C to 4390 nmol min^{-1} mg^{-1} at 90°C (Figure 3.2, 0 h incubation). The absence of an optimum contradicts previous findings that report a temperature optimum at 55°C with half-maximal activities at 26°C and 78°C.[18,20]

The temperature dependent stability of ADC was determined by performing activity assays at different temperatures after one and 24 hours of incubation at

the same temperature. ADC was stable for one hour up to 70°C (Figure 3.2, 1 h incubation) and for 24 hours up to 60°C (Figure 3.2, 24 h incubation).

Although pyruvoyl-dependent L-aspartate α-decarboxylases from other sources have been isolated and studied, the thermostability was not determined in these cases.[24,25] It is difficult to make a justified comparison with other pyruvoyl-dependent decarboxylases, because although they are all activated via autocatalytic serinolysis and share similar decarboxylation mechanisms, they share little structural similarity.[26,27] It is, however, notable that the thermostability of *E. coli* ADC is comparable to that of pyruvoyl-dependent *S*-adenosylmethionine decarboxylase from *Sulfolobus solfataricus* (stable at 50°C for 16 hours)[28] and L-arginine decarboxylase from *Methanococcus jannachii* (50% activity after 20 minutes at 121°C).[29]

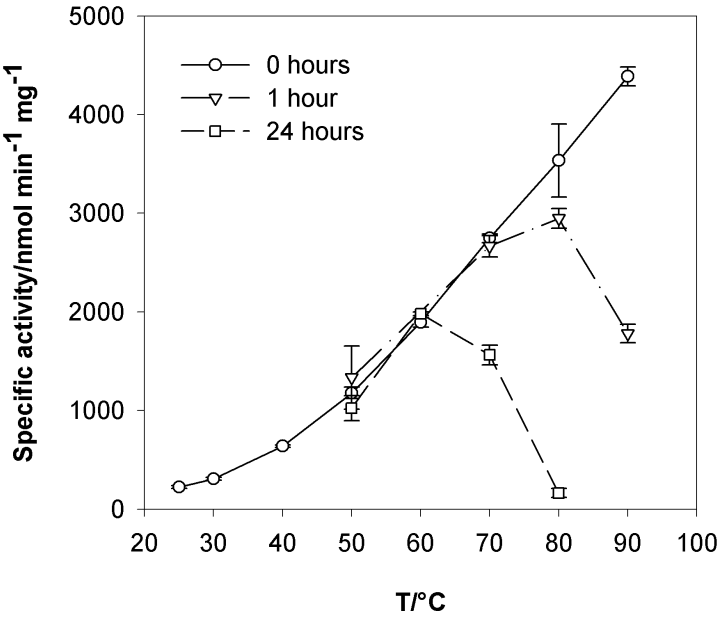

Figure 3.2. Temperature dependent activity and stability of ADC. ADC solutions were incubated in 50 mM sodium phosphate buffer pH 7.0 for 0, 1, and 24 hours at the given temperatures. Directly after incubation L-aspartic acid was added up to 1.5 mM and the specific decarboxylase activity was determined at the same temperature. The error bars represent standard deviation; the depicted lines are a guide to the eye.

3.2.3 pH Dependent activity and stability

The pH optimum for ADC was found at pH 7.5 (Figure 3.3, 0 h incubation). This observation is in agreement with the results of Williamson and Brown.[18] Twenty-four hours of incubation revealed that ADC was stable under alkaline conditions, but lost activity below pH 6 (Figure 3.3, 24 h incubation).

The optimum at pH 7.5 and the instability at acidic conditions have their impacts on the application of ADC in the production of β-alanine. It would be beneficial if ADC could be applied in a buffer free process, since addition of buffer agents introduces salts to the reaction mixture that most probably need to be removed before further conversion of β-alanine.

A buffer-free solution of 50 mM L-aspartic acid (close to saturation) will have a pH of approximately 3, which will increase when L-aspartic acid is converted

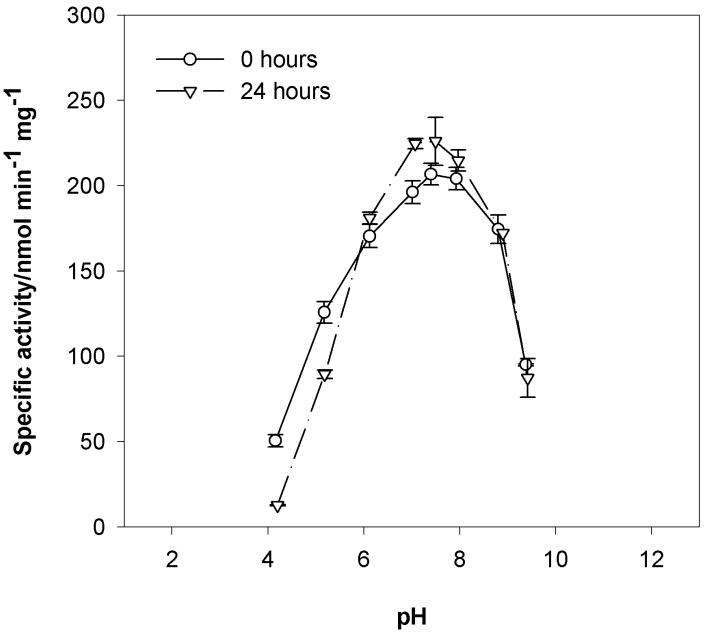

Figure 3.3. pH Dependent activity and stability of ADC. Directly or after 24 hours of incubation at 30°C, the specific activity of ADC solutions with different pH values was determined after addition of L-aspartic acid up to 1.5 mM. To maintain a constant ionic strength, a three component buffer was used.[30] The error bars represent standard deviation; the depicted lines are a guide to the eye.

to the less acidic β-alanine. However, assuming that the released carbon dioxide will dissipate, the pH of the reaction mixture will only start to rise above pH 6 at 95% conversion.[31] Furthermore, buffering the reaction mixture using continuous or fed-batch process designs, in which the presence of previously formed, more basic β-alanine results in a less acidic environment, will never lead to the optimum of pH 7.5. Therefore addition of a buffer agent to the reaction mixture will be necessary to achieve and maintain the highest activity for ADC.

3.2.4 Immobilization of L-aspartate α-decarboxylase

It is proposed that protein immobilization on epoxy supports follows a two-step mechanism: (i) first the enzyme is adsorbed to the surface of the support and (ii) subsequently the protein's nucleophilic groups (amino, thiol, or hydroxyl groups) react with the epoxy groups on the support to form covalent bonds.[32] Following this mechanism, the hydrophobicity of the support plays a major role in the binding performance of the support. For this reason it was decided to compare the immobilization of ADC on the relatively hydrophobic epoxy support EC-EP and the relatively hydrophilic amino-epoxy support EC-HFA.[33,34] To facilitate adsorption, the recommended buffer concentrations were used: 1 M for EC-EP and 5 mM for EC-HFA.[35]

The residual specific activity of ADC in the supernatant was followed during immobilization. In case of both supports the specific activity of ADC in the supernatant disappeared within the hour (results not shown). Comparisons between the two supports made with other enzymes under similar conditions, show a much slower adsorption to EC-EP supports even though four times lower protein concentrations were used (0.9 mg protein g^{-1} support).[33,35] Possibly the size of the enzymes plays a role in the rate of adsorption, since ADC in its tetrameric form of 55 kD[36] is notably smaller than the other enzymes studied (90 to 270 kD)[33,35,37].

After immobilization, both ADC preparations were washed and the amount of enzyme lost through leaching from the supports was determined using Bradford

protein assays (results not shown). Using these data, the immobilization yields were calculated and it appeared that ADC was to a greater extent covalently bound to EC-EP (94.8%) than to EC-HFA (88.0%).

Aliquots of EC-EP and EC-HFA were incubated in 3 M glycine at pH 8 to block the remaining epoxy groups in the supports. Since both the substrate and product are amino acids, their amino groups might react with remaining epoxy groups. However more importantly, these epoxy groups might interact with enzyme residues during catalytic operation, leading to deactivation.[38] Blocking of remaining epoxy groups by incubation in an excess of glycine has become a common step in protocols for protein immobilization on epoxy supports.[39]

After immobilization, the recovered activity (the activity relative to the expected activity) for all ADC preparations was determined (Table 3.1). The recovered activity was higher for EC-HFA (89%) than for EC-EP (80%). Taking into account the immobilization yields, the difference can be explained by the fact that covalent binding of ADC to EC-HFA occurred to a lesser extent and thus led to less deactivation during immobilization. Blocking of the ADC preparations with glycine appeared to have a negative effect on the recovered activity (Table 3.1).

Overall, when compared to studies with EC-EP and EC-HFA applied with other enzymes, the recovered activities for immobilized ADC are quite good.[33,40-42]

Table 3.1. The recovered activity, the activity relative to the expected activity, of different ADC preparations.

ADC preparations	Recovered activity (%)
EC-EP	80 ± 0.5
EC-HFA	89 ± 1.6
EC-EP blocked	76 ± 5.4
EC-HFA blocked	72 ± 0.9

3.2.5 Thermostability of L-aspartate α-decarboxylase preparations

Since ADC appears to have a high thermostability, the thermostability of immobilized and soluble ADC was compared by following their residual activity in time at a relatively high temperature of 80°C to be able to observe deactivation

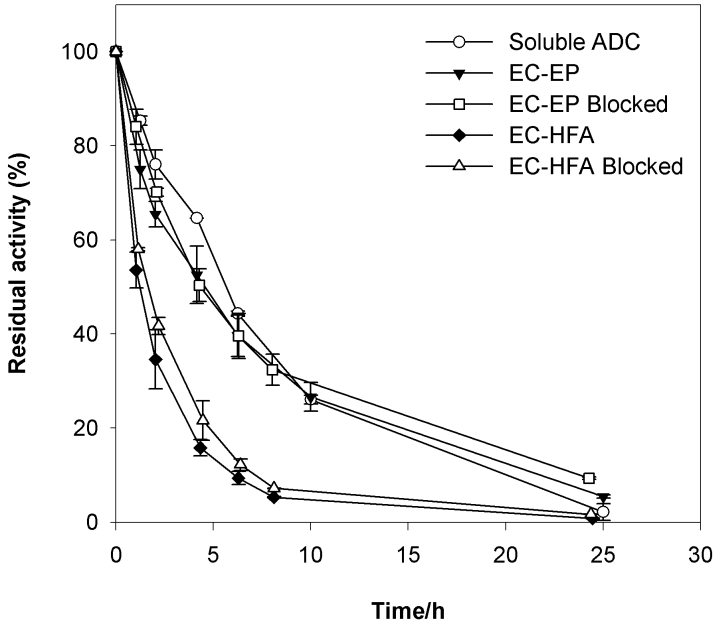

Figure 3.4. Thermostability of immobilized ADC compared to soluble ADC. Aliquots of ADC preparations were incubated in 50 mM sodium phosphate buffer pH 7.0 at 80°C and in course of time the residual activity was determined upon addition of L-aspartic acid up to 1.5 mM. The error bars represent standard deviation; the depicted lines are a guide to the eye.

(Figure 3.4). The thermostability of EC-HFA preparations is much lower than that of EC-EP preparations and that of soluble enzyme. It is difficult to give a direct explanation for this observation, but fact is that covalently bound to ADC, EC-EP and EC-HFA only differ in the structure of their spacer arms.

When covalently bound, EC-EP has relatively short (two bond lengths), apolar spacer arms, while EC-HFA has relatively long (fifteen bond lengths), polar spacer arms. It has been suggested by Mateo *et al.*[43] and more recently by Magner *et al.*[44]

that short spacer arms (two to three bond lengths) are beneficial for the conformational stability of the covalently bound enzyme. Binding through multiple short spacer arms provides that immobilized enzyme residues are likely to preserve their relative positions in the event of heat induced conformational change. However, the stabilizing effect of covalent immobilization through the short spacer arms of EC-EP does not seem to add up to the already remarkable thermostability of soluble ADC (Figure 3.4).

Accordingly EC-HFA's significantly longer spacer arms provide a higher degree of conformational freedom and therefore the enzyme is more susceptible to heat induced conformational changes leading to deactivation. This could explain why ADC immobilized on EC-HFA even shows a lower thermostability than soluble ADC (Figure 3.4).

Although immobilization on EC-HFA has a negative effect on the thermostability of ADC, it must be stated that there are examples of enzymes that show an increased thermostability upon immobilization on EC-HFA[35,41] and that compared to EC-HFA the relatively short EC-EP spacer arms are not always beneficial.[33,42,45]

The results in Figure 3.4 show that blocking with 3 M glycine at pH 8, did not have a significant effect on the thermostability of the ADC preparations. While in some studies blocking of remaining epoxy groups under similar conditions was applied as a standard procedure,[33,40,42,46] the results in Figure 3.4 stress that not every aspect of an immobilization procedure can be generalized for each and every enzyme and some steps could be left out or should be optimized.

Although immobilization on Sepabeads did not lead to an increased thermostability, it was successful in case of EC-EP in the respect that ADC was converted into a heterogeneous catalyst while retaining its high thermostability. This opens possibilities for application of ADC in continuous processes.

3.2.6 Operational stability of L-aspartate α-decarboxylase preparations

In order to test operational stability, the reusability of immobilized ADC was tested at 30°C in 30 minutes batch cycles. After each cycle the supernatant of the reaction mixtures was discarded and replaced by fresh buffer and the next cycle was started by adding substrate. After four cycles all ADC preparations showed a decrease in activity of approximately 30% (results not shown).

Activity and Bradford protein assays applied on the discarded supernatant ruled out the possibility of ADC leaching from the supports. Additional to the standard applied filter-sterilization, addition of sodium azide or protease inhibitor (Complete, Roche), degassing of the reaction mixture before and after each cycle to diminish the effect of CO_2 release or different methods and speeds of mixing had no effect on the strong deactivation over the cycles.

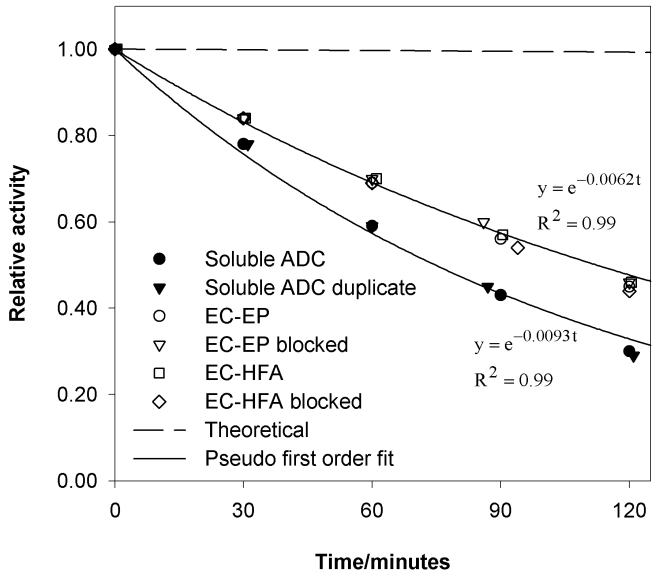

Figure 3.5. Comparison of operational stability of different ADC preparations. Using the data obtained from batch experiments (3 mM L-aspartic acid in 50 mM sodium phosphate buffer pH 7.5 at 30°C), the amount of β-alanine produced was plotted against the amount of ADC spent, grouped by soluble and immobilized ADC. Linear curves were fitted through the data points giving the total turnover numbers (TTN).

Based on these results, it was decided to compare the operational stability of immobilized ADC with that of soluble ADC using batch experiments For all ADC preparations the amount of *β*-alanine produced was plotted against the amount of ADC spent, giving the total turnover number (TTN) (Figure 3.5).

The linearity of the curves in Figure 3.5 together with the observation that ADC is stable when stored under the applied conditions (30°C, pH 7.5) clearly shows that the inactivation of the enzyme is turnover dependent. Although covalent immobilization on Sepabeads, independent of the type, has an overall positive effect on the operational stability, this improvement is relatively small in relation to the total decrease in activity. The resulting total turnover numbers (TTNs) of $2.39 \cdot 10^3 \pm 0.04 \cdot 10^3$ and $3.73 \cdot 10^3 \pm 0.06 \cdot 10^3$ respectively, are relatively low for enzymatic reactions.

It has been suggested that deactivation of ADC in the presence of substrate occurs via decarboxylation-dependent transamination of the catalytically essential pyruvoyl group, resulting in a catalytically inactive alanine residue at the *N*-terminus (Figure 6.2).[26,47] This suggestion has been based on observations by Smith[48], who observed an irreversible deactivation of ADC in the presence of L-aspartic acid in combination with the transamination of the pyruvoyl group, as determined by *N*-terminal sequencing. The mechanism behind this 'suicidal' transamination is proposed to be comparable to that observed in several pyridoxal-`5-phosphate-dependent enzymes[49,50] and has been confirmed by mechanistical studies for *S*-adenosylmethionine decarboxylase, another pyruvoyl-dependent enzyme.[51,52]

Since deactivation caused by transamination of the catalytically essential pyruvoyl group is irreversible, the solution to this problem has to be found in the field of protein engineering. A study in which protein engineering is applied to overcome substrate deactivation in pyruvoyl-dependent enzymes is to date not known to the authors. Nonetheless, McElroy and Robertus[53] showed us that at least the opposite is possible; with help of site-directed mutagenesis they were able to induce substrate deactivation in pyruvoyl-dependent *Lactobacillus* 30a histidine

decarboxylase, where it was not present before.

3.3 Conclusions

In this chapter, we have investigated the possibilities for the application of the enzyme L-aspartate α-decarboxylase from *E. coli* (ADC) in the biobased production of nitrogen containing chemicals as an alternative to the petrochemical route. ADC has several advantages, such as its perfect selectivity, ease of production, lack of product inhibition and high thermostability under storage conditions. In addition, covalent immobilization of ADC on Sepabeads EC-EP epoxy supports is easy and enables its reuse and application in continuous processes. However, ADC's low operational stability, probably caused by irreversible transamination of its catalytically essential pyruvoyl group, needs to be addressed before large scale applications become feasible. Although operational stability was improved by covalent immobilization on Sepabeads epoxy supports, protein engineering is the most likely solution to this problem.

3.4 Experimental

pRSETA plasmids containing one copy of the *panD* gene with a *N*-terminal histidine tag, constructed following Schmitzberger *et al.*[54], were kindly provided by Prof. Alison Smith from the Department of Plant Sciences, Cambridge University, UK.

Z-competent *E. coli* XL1-blue and One Shot chemically competent *E. coli* BL21star(DE3) were obtained from respectively Zymo Research and Invitrogen.

The Sepabeads EC-EP (epoxide) and EC-HFA (epoxide on a an amino spacer) used in this study are epoxy activated methacrylic beads with pore sizes in the range of 30-40 nm and diameters in the range of 150-300 µm.[55] Both types were kindly donated by Dr. Paolo Caimi from Resindion S.R.L. (Mitsubishi Chemical, Milan, Italy).

All buffers and solutions used for procedures involving ADC were prepared

with MilliQ water and filter sterilized (0.20 µm) before use. Instant low salt LB agar (Duchefa Biochemie) and instant high salt LB broth (Sigma) were prepared according to protocol, autoclaved and ampicillin stock solution was added up to a concentration of 100 µg mL^{-1}.

Bradford protein assays were performed using Bio-Rad Protein Assay Kit II with BSA standard. All chemicals used in this study were of analytical grade or better and used as received.

3.4.1 Plasmid amplification

Z-competent *E. coli* XL1-blue cells were transformed with the *pRSETA* plasmid according to protocol. After selection on low salt LB agar (16 h, 37°C), the transformants were grown in high salt LB medium (16 h, 37°C, 250 rpm). The plasmids were isolated from the cultures using a GenElute HP Plasmid Kit (Sigma) according to protocol and stored at 20°C.

3.4.2 L-Aspartate *α*-decarboxylase overexpression

Chemically competent *E. coli* BL21star(DE3) was transformed with *pRSETA*, according to protocol. After selection on low salt LB agar, transformed cells were grown in high salt LB medium (25°C, 200 rpm). After 16 hours incubation (OD_{600} = 0.90), the cultures were used to prepare a 80% glycerol stocks, which was stored at -80°C.

The glycerol stock was used to inoculate 10 mL high salt LB medium. After 4 hours incubation (37°C, 250 rpm), the culture (OD_{600} = 0.30) was used to inoculate 0.5 L high salt LB medium. After 4 hours incubation (37°C, 250 rpm), the culture (OD_{600} = 0.30) was induced with IPTG (1 mM) and incubated for another 16 hours (25°C, 250 rpm). The cells were harvested in three subsequent centrifugation steps (3000 rpm, 4°C, 25 min) and stored at 80°C.

3.4.3 L-Aspartate *α*-decarboxylase isolation and activation

The pellet with IPTG induced cells was thawed on ice and suspended in 5 mL binding buffer (20 mM imidazole, 500 mM NaCl in 10 mM sodium phosphate

buffer, pH 7.4). 5 mg Egg white lysozyme was added and the suspension was kept at room temperature for 15 minutes, while carefully vortexing from time to time. After sonication on ice, 3 times 30 seconds with one minute in between, the suspension was centrifuged (12000 rpm, 4°C, 10 min). The supernatant was passed through a syringe filter (0.45 µm) and stored on ice.

A 5 mL HisTrap HP column (GE Healthcare) was operated on an ÄKTA Explorer system. After equilibration (25 mL binding buffer), 2 mL cell extract was applied to the column. Subsequent to washing (25 mL binding buffer), histidine tagged ADC was eluted with elution buffer (500 mM imidazole and 500 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). The fractions were collected upon UV detection at 280 nm and stored at 4°C. The purity and protein content were determined with respectively SDS-PAGE analyses and Bradford protein assays.

To activate ADC, fractions were filter-sterilized (0.20 µm) and incubated for 16 hours at 50°C. Finally, the buffer was exchanged with reaction buffer (section 3.4.4) using a Millipore Centriprep centrifugal filter (10 kD cutoff). The activated ADC was stored at 4°C.

3.4.4 L-Aspartate α-decarboxylase activity assay

A solution of 25 µg ADC in 970 µL reaction buffer (1 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0) was incubated for 5 minutes at 30°C using an Eppendorf Thermomixer (1200 rpm). The activity assay was started by adding 30 µL 50 mM L-aspartic acid solution. At given time intervals 50 µL samples were taken and quenched with 5 µL of 1 M NaOH and stored at -20°C.

β-Alanine concentrations were determined using the fluorescamine derivatization method described by Udenfriend *et al.*[56] The samples were thawed at room temperature and to 5 µL of each sample 75 µl of 100 mM borate buffer (pH 9.0) was added. The diluted sample was derivatized by adding 20 µl 1 mg mL^{-1} fluorescamine in acetonitrile, directly followed by vortexing.

The HPLC system used for quantification was based on separation on a NovaPak C18-column (60 Å, 4 µm, 3.9 x 150 mm) at 30°C, isocratic elution with 1 mL min^{-1}

20% acetonitrile in 20 mM sodium acetate buffer (pH 4.50) and detection with a Jasco 820-FP fluorescence detector (λ_{ex} = 390 nm, λ_{em} = 460 nm).

3.4.5 Product inhibition

Assays were performed with 0.050, 0.100, 0.250, 0.600, 1.000, 1.400 and 1.800 mM L-aspartic acid in combination with 0.000, 0.500, 1.000 or 1.500 mM *β*-alanine. Solutions of 25 µg ADC in 800 µL reaction buffer were incubated for 5 minutes at 30°C. The activity assays were started by addition of 200 µL *β*-alanine/ L-aspartic acid solution with the desired concentration. Sampling and HPLC analysis were performed as previously described.

3.4.6 Temperature dependent activity and stability

Solutions of 25 µg ADC in 485 µL reaction buffer were prepared and incubated for 0, 1 and 24 hours in a water bath at different temperatures. After incubation, the solutions were transferred to an Eppendorf Thermomixer (1200 rpm) and incubated for 5 minutes at the same temperature as the water bath. The activity assays were started by adding 15 µL of 50 mM L-aspartic acid. Sampling and HPLC analysis were performed as previously described.

3.4.7 pH Dependent activity and stability

To maintain a constant ionic strength, a three-component buffer composed of acetic acid (100 mM), MES (100 mM) and triethanolamine (200 mM) was used following Ellis *et al.*[30] A 30 mM L-aspartic acid stock was prepared using the three-component buffer. Aliquots of both the three-component buffer and the L-aspartic acid stock were adjusted to the desired pH values (4, 5, 6, 7, 7.5, 8, 9 and 10) with 37% HCl or 10 M NaOH.

To determine the pH dependent activity 25 µg ADC in 475 µL of buffer was incubated for 5 minutes at 30°C in an Eppendorf Thermomixer (1200 rpm). Activity assays were started by adding 25 µL of 30 mM L-aspartic acid stock with a pH similar to that of the respective buffer. To determine the pH dependent stability, the activity assays were preceded by 24 hours incubation in a 30°C water bath.

Apart from using 5 M NaOH for enzyme quenching, sampling and analyses were performed as previously described.

3.4.8 Immobilization of L-aspartate α-decarboxylase

Two different 500 µg mL^{-1} ADC solutions were prepared, one in 5 mM and another in 1.0 M sodium phosphate buffer (pH 7.0). From each type of Sepabeads 1.000 gram dry weight was transferred to a 10 mL screw-cap tube. To EC-EP 8.0 mL of 1.0 M sodium phosphate ADC solution and to EC-HFA 8.0 mL of 5 mM sodium phosphate ADC solution was added. Of each ADC solution 1.5 mL was transferred to a 2 mL Eppendorf tube to serve as blanks.

All tubes were mounted on a rotator (Cole-Palmer RotoTorque Heavy Duty Rotator) and incubated for 24 hours at room temperature. In time 80 µL samples were taken of which 25 µL was directly used in an activity assay and the remainder was stored at 4°C. The 25 µL was added to 460 µL reaction buffer and after 5 minutes incubation at 30°C, the activity assay was started by adding 15 µL 50 mM L-aspartic acid solution. Sampling and analyses were conducted as previously described.

After 24 hours of incubation the supports were filtered over a Büchner-filter, rinsed with 8 mL reaction buffer, resuspended in 8 mL reaction buffer and incubated for another hour at room temperature. After this washing step the supports were once more rinsed with 8 mL reaction buffer. After weighing the filter-dry supports, 500 mg of each preparation was transferred to a fresh 10 mL screw-cap tube for blocking and the remainder was stored at 4°C.

The 500 mg aliquots were blocked by incubation on the rotator in 5 mL 3 M glycine (pH 8.0) for 16 hours at room temperature. After incubation the blocked supports were washed as described above, weighed and stored at 4°C.

The protein content in the supernatant during immobilization and washing was determined with Bradford protein assays. Residual activities of the enzyme preparations were determined with 30 mg immobilized ADC in the standard activity assay.

3.4.9 Thermostability of L-aspartate *α*-decarboxylase preparations

Aliquots of soluble and immobilized ADC in 970 µL reaction buffer were incubated in a 80°C water bath. At various time intervals the aliquots were transferred to an Eppendorf Thermomixer (80°C, 1200 rpm). After 5 minutes incubation activity assays were started by adding 30 µL 50 mM L-aspartic acid. Sampling and analyses were performed as previously described.

3.4.10 Operational stability of L-aspartate *α*-decarboxylase preparations

Reusability

Reaction mixtures with 25 mg immobilized ADC were prepared in 970 µL reaction buffer. After 5 minutes of incubation in an Eppendorf Thermomixer (30°C, 1200 rpm), activity assays were performed as described earlier. After the activity assay, the reaction mixtures were left for in total 30 minutes. The supernatants were removed and replaced by fresh reaction buffer and the initial reaction rate was determined for three additional cycles in a similar way.

Batch reactions

Reaction mixtures with approximately 25 µg soluble and 25 mg immobilized ADC were prepared in 4.700 mL 50 mM phosphate buffer (pH 7.5) with 0.05% sodium azide. After incubation for 10 minutes in a stirred 30°C water bath, the activity assays were started by adding 300 µL 50 mM L-aspartic acid. Sampling and analyses were performed as previously described. The total turnover number (TTN) was calculated by dividing the moles of *β*-alanine produced by the moles of ADC spent.

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Chapter 4

Stabilized and immobilized *Bacillus subtilis* arginase for the biobased production of nitrogen containing chemicals

This chapter is based on

Paul M. Könst, Pedro M.C.C.D. Turras, Maurice C.R. Franssen, Elinor L. Scott and Johan P.M. Sanders, *Advanced Synthesis and Catalysis*, 2010, **352**, 1493-1502.

Abstract

L-ornithine could serve as an intermediate in the biobased production of 1,4-diaminobutane from L-arginine. Using the concept of biorefinery, L-arginine could become widely available from biomass waste streams via the nitrogen storage polypeptide cyanophycin. Selective hydrolysis of L-arginine to L-ornithine is difficult to perform chemically, therefore the stabilization and immobilization of *Bacillus subtilis* arginase (EC 3.5.3.1) was studied in a continuously stirred membrane reactor system. Initial pH of the substrate solution, addition of L-aspartic acid and reducing agents all appeared to have an effect on the operational stability of *B. subtilis* arginase. A remarkably good operational stability (TTN = $1.13 \cdot 10^8$) at the pH of arginine free base (pH 11.0) was observed, which was further improved with the addition of sodium dithionite to the substrate solution (TTN > $1 \cdot 10^9$). *B. subtilis* arginase was successfully immobilized on three commercially available epoxy-activated supports. Immobilization on Sepabeads EC-EP was most promising, resulting in a recovered activity of 75% and enhanced thermostability. In conclusion, the stabilization and immobilization of *B. subtilis* arginase has opened up possibilities for its application in the biobased production of nitrogen containing chemicals as an alternative to the petrochemical production.

4.1 Introduction

Global warming, depletion of fossil resources and geopolitical instability are forcing us to make the transition towards CO_2 neutral, sustainable and economically stable alternatives for the production of chemicals and transportation fuels. In this respect, biomass has great potential. Currently the emphasis is on the application of biomass as a feedstock for energy and transport fuels. However, its application as a feedstock for functionalized bulk chemicals receives far less attention. This is remarkable considering the enormous amounts of functionalized compounds (*e.g.* amino acids, organic acids and lignin) that are generated as side streams of the biofuel production.[1]

Nowadays, most nitrogen containing bulk chemicals are produced from naphtha. In these processes large amounts of energy and ammonia are used *e.g.* in the ammoxidation of olefins.[2] Following the biorefinery concept, it would be more energy efficient and eventually more economic to start from functionalized compounds that already have nitrogen incorporated, such as amino acids, instead of starting from naphtha's plain hydrocarbons.[3] Bearing this in mind, our attention has been drawn to the polypeptide cyanophycin (CGP).

CGP consists of a poly-L-aspartic acid backbone with L-arginine side chains in equimolar amounts and is naturally produced in cyanobacteria as a nitrogen storage polymer.[4] Heterologous expression in industrially relevant bacteria[5-8] has opened up the possibility to produce CGP in considerable amounts. Even more interestingly, recent work on *Saccharomyces cerevisiae*[9,10] gives rise to possibilities for the simultaneous production of CGP and ethanol from amino acid rich waste streams from the agroindustries.[11-13]

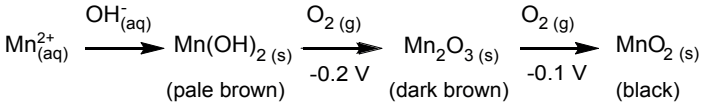
Since CGP is insoluble under physiological conditions, it can be easily isolated[14-16] which together with its defined composition, its integrated production and its high nitrogen content make CGP an interesting feedstock for the production of nitrogen containing bulk chemicals.[13]

Our research is focused on the development of integrated processes for the

conversion of CGP's hydrolysate, L-aspartic acid and L-arginine, towards existing nitrogen containing bulk chemicals. Earlier we investigated the applicability of L-aspartate α-decarboxylase (EC 4.1.1.11) in the conversion of L-aspartic acid to β-alanine (3-aminopropionic acid), a possible intermediate in the production of acrylamide and acrylonitrile (Chapter 3).[17] Starting from L-arginine, we envision a route towards 1,4-diaminobutane, one of the monomers in nylon-4,6 which is commercially available under the name Stanyl® (Figure 4.1).[18,19]

In this route L-arginine is hydrolyzed yielding L-ornithine and urea. Methods for the non-enzymatic hydrolysis of L-arginine have limitations due to either slow reaction or to the production of a number of side products (Chapter 2).[20-23] On the other hand, the enzyme arginase (L-arginine amidinohydrolase, EC 3.5.3.1) from the bacterium *Bacillus subtilis* is capable of selectively hydrolyzing L-arginine to L-ornithine and urea under mild conditions.[24]

For its catalytic activity arginase is dependent on two manganese ions (Mn^{2+}), which form a binuclear cluster in the active site in order to ionize the water molecule that attacks the guanidinium carbon of L-arginine in the catalytic cycle.[25-27] However, at the pH optimum (pH 9-10) of *B. subtilis* arginase and many other arginases,[24,28] Mn^{2+} is readily oxidized by air (Scheme 4.1).[29]



Scheme 4.1. Base induced oxidation of Mn^{2+} towards $MnO_{2(s)}$.

Mn^{2+} oxidation poses problems for the application of arginase, since formation of precipitates may cause operational problems, but most of all it withdraws Mn^{2+} from the reaction solution which might result in deactivation.

Most examples of studies on the application of arginase are focused on analytical purposes,[30-34] while only a few investigate its application for the production of L-ornithine.[35-37] One of the more elaborate studies on the enzymatic L-ornithine production is by Bommarius *et al.*[37] and investigates the operational stability of calf

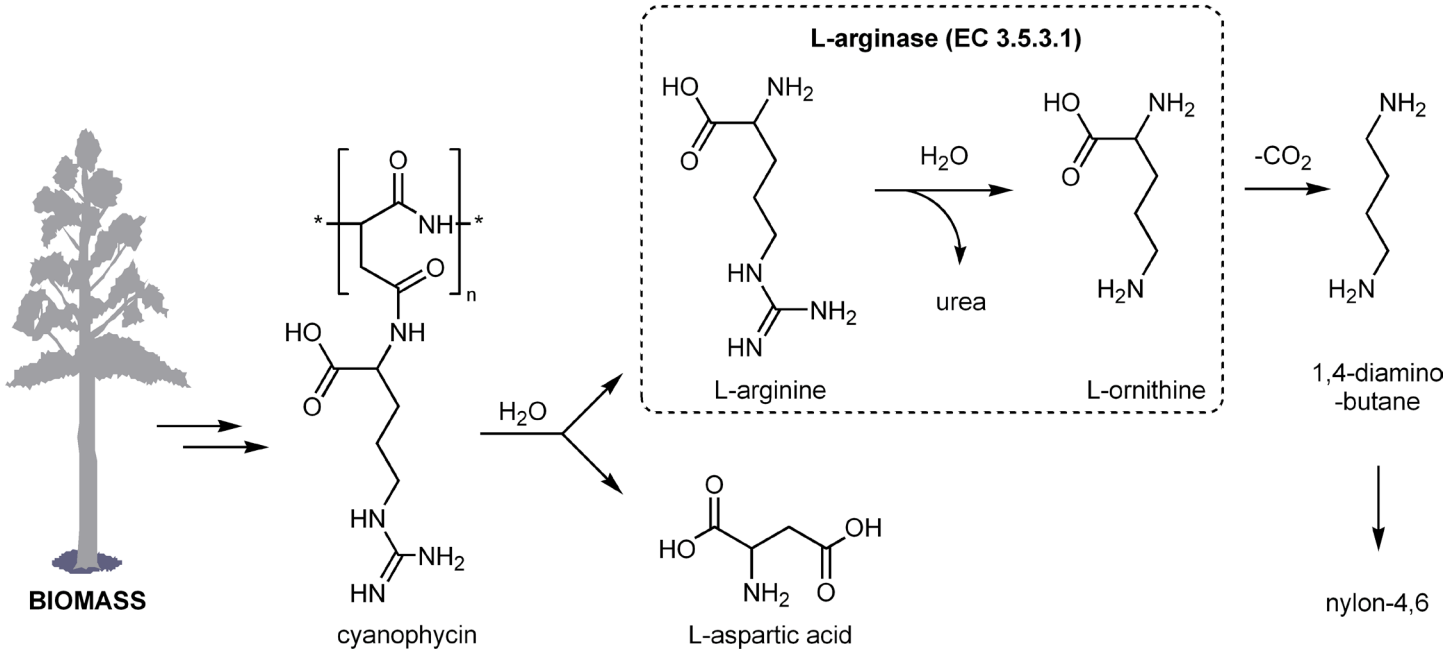


Figure 4.1. Route from cyanophycin towards nitrogen containing bulk chemicals such as 1,4-diaminobutane with the enzymatic hydrolysis of L-arginine as an intermediate step.

liver arginase in membrane reactors. They observed a good stabilization of arginase upon addition of L-ascorbic acid, which is attributed to preventing the loss of Mn^{2+} as a result of oxidation. Deactivation as result of mechanical stress by stirring, however, remained a problem.

Immobilization might enhance the enzyme's stability towards conditions that may be encountered in an industrial process.[38] On an industrial scale immobilization of enzymes is desirable, as this enables reuse and overcomes problems with product recovery. There are several example of arginase immobilization, either by entrapment in a matrix[32,35] or covalent binding to a support[30,31,34,36]. However, immobilization of arginase on commercially available and industrially relevant epoxy-activated supports was not tested until now. A suitable support would be Eupergit, which was one of the first epoxy-activated supports introduced on the market.[39] Another suitable epoxy-activated support would be Sepabeads, which due to their ease of operation and their robustness under physical stress are very suitable for enzyme immobilization for industrial purposes.[40,41]

In this chapter, we present the results of a study on stabilization and immobilization of *Bacillus subtilis* arginase for the production of L-ornithine as an intermediate for biobased, nitrogen containing, bulk chemicals. For the soluble enzyme the conditions for optimal operational stability were determined using a continuously stirred membrane reactor system. Furthermore, *B. subtilis* arginase was immobilized on three different epoxy functionalized supports: Sepabeads EC-EP and EC-HFA, and Eupergit C 250L. The thermal and operational stability of the immobilized and soluble enzyme were compared.

4.2 Results and Discussion

4.2.1 Operational stability of soluble arginase

The influence of pH, L-aspartic acid and reducing agents on the operational stability of *B. subtilis* arginase was investigated, since they affect the enzyme as

such and the availability of Mn^{2+} for the enzyme. The operational stabilities were determined using a continuously stirred membrane reactor system, which was fed through a nitrogen gas pressurized substrate solution reservoir. The outflow of the reactor was collected in tubes over set time intervals. After a steady state was reached the specific reaction rate, r (μmol min^{-1} mg^{-1}), in the course of time was calculated from the flow rate Q (mL min^{-1}), the product concentration C_p (μmol mL^{-1}) and the amount of enzyme applied X_{enz} (mg) (Equation 4.1).[42]

$$r = \frac{C_p \cdot Q}{X_{enz}} \qquad (\mu mol\ min^{-1}\ mg^{-1}) \qquad (4.1)$$

Table 4.1. Summary of results obtained with soluble *B. subtilis* arginase using a continuously stirred membrane reactor system. The values are the result of duplicate experiments ± S.E.

pH of substrate solution (-)	Additional to 0.5 mM $MnCl_2$ [a]	r_{init} (μmol min^{-1} mg^{-1})[b]	k_{deact} (h^{-1})[b]	TTN (mol mol^{-1})[c]
not adjusted (11.0)	-	462 ± 9	2.50 (± 0.26) ·10^{-2}	1.13 (± 0.13) ·10^{8}
	0.50 mM DTN	341± 9	stable[d]	stable
9.5	without $MnCl_2$	215 ± 12	4.50 (± 0.43) ·10^{-1} 3.58 (± 0.69) ·10^{-2}	n.d.
	-	283 ± 4	5.88 (± 0.22) ·10^{-2}	3.49 (± 0.09) ·10^{7}
	25 mM L-asp	410 ± 7	3.30 (± 0.22) ·10^{-2}	9.11 (± 0.89) ·10^{7}
	0.06 mM L-asc	243 ± 6	3.65 (± 0.25) ·10^{-2}	4.80 (± 0.24) ·10^{7}
	0.12 mM L-asc	162 ± 4	1.70 (± 0.24) ·10^{-2}	6.22 (± 0.57) ·10^{7}
	0.25 mM L-asc	52.9 ± 0.1	6.70 (± 2.10) ·10^{-3}	6.82 (± 0.63) ·10^{7}
	0.25 mM DTN	336 ± 3	stable	stable
	0.50 mM DTN	383 ± 4	stable	stable
	1.00 mM DTN	303 ± 12	stable	stable
8.5	-	289 ± 7	3.26 (± 0.31) ·10^{-2}	6.68 (± 0.62) ·10^{7}
	25 mM L-asp	326 ± 3	2.75 (± 0.13) ·10^{-2}	8.76 (± 0.40) ·10^{7}
	0.50 mM DTN	329 ± 5	stable	stable

[a] L-asp = L-aspartic acid, DTN = sodium dithionite, L-asc = L-ascorbic acid. [b] The initial reaction rate r_{init} and deactivation constant k_{deact} were determined from fitted first order deactivation models unless stated otherwise. [c] The total turnover number TTN was determined from the linear relation between the amount of L-ornithine formed and the amount of arginase spent as determined from the residual activity. [d] stable: no significant deactivation was observed, at least 1·10^{9} mol L-ornithine was formed per mol arginase spent.

From the data obtained, the extrapolated initial reaction rate r_{init} (µmol min^{-1} mg^{-1}) and the deactivation constant k_{deact} (h^{-1}) were determined using a single exponential deactivation model unless stated otherwise. Furthermore, the total turnover number (TTN) (mol L-ornithine mol^{-1} arginase) was determined from the linear relation between the amount of L-ornithine formed and the amount of arginase spent. The results are summarized in Table 4.1 and discussed below.

Effect of pH

The operational stability of arginase was tested with substrate solutions at three different pH values: pH of non-adjusted substrate solution (pH 11.0), optimum pH as previously determined for *B. subtilis* arginase (pH 9.5)[24] and a pH at which Mn^{2+} oxidation is negligible (pH 8.5). Additionally a run was included with substrate solution at pH 9.5, but without additional Mn^{2+}. The results are depicted in Figure 4.2.

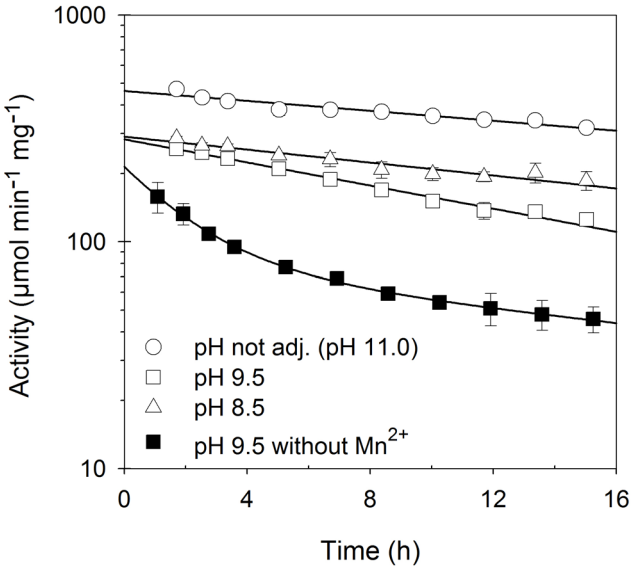


Figure 4.2. Effect of initial pH on arginase stability. The pH of 0.25 M L-arginine solutions was adjusted and as such applied with soluble arginase in a continuously stirred membrane reactor at 30°C. The specific reaction rates were determined using Equation 4.1. The error bars represent standard deviation; the depicted lines represent fitted first order deactivation models unless stated otherwise.

Comparing the results of the experiments with initial pH 9.5 with and without additional Mn^{2+}, it becomes clear that the addition of Mn^{2+} is crucial for both activity and stability. While arginase shows a single exponential deactivation in the presence of Mn^{2+} (k_{deact} = 5.88·10^{-2} h^{-1}), deactivation in the absence of Mn^{2+} occurs much faster following a model involving two deactivation constants ($k_{deact,1}$ = 4.51·10^{-1} h^{-1}, $k_{deact,2}$ = 0.36·10^{-2} h^{-1}).

Although no signs of Mn^{2+} oxidation were observed, the use of substrate solution with initial pH 8.5 still led to arginase deactivation (k_{deact} = 3.26·10^{-2} h^{-1}). On the other hand, application of the non-adjusted substrate solution (pH 11.0), which almost instantly turned brown upon preparation as a result of Mn^{2+} oxidation, resulted in a deactivation constant slightly lower than that observed with initial pH 8.5 (k_{deact} = 2.50·10^{-2} h^{-1}). From these results can be concluded that the observed arginase deactivation is not only due to loss of Mn^{2+} through oxidation, but moreover appears to be the result of another phenomenon.

In addition to the higher stability observed, the initial reaction rate as determined for initial pH 11.0 (462 µmol min^{-1} mg^{-1}) is significantly higher than that observed with initial pH 9.5 (283 µmol min^{-1} mg^{-1}), the previously reported pH optimum for *B. subtilis* at 37°C.[24]

A similar effect of initial pH on both activity and stability is described in an article on the application of calf liver arginase.[43] While it was previously determined that calf liver arginase has an optimum at pH 9.5,[44] a significant higher activity and stability was observed with initial pH 11.5 (0.75 M arginine) upon application in a membrane reactor system.

Effect of L-aspartic acid

Patchett *et al.*[45] reported that addition of a chelating agent such as L-aspartic acid increases the stability of *B. caldovelox* arginase. They suggest that this effect might be the result of complexation of Mn^{2+} with L-aspartic acid. As an L-aspartic acid-manganese complex has a much higher dissociation constant (2.5 mM) compared to the arginase-manganese complex (45 µM), manganese will always be available to arginase.

In the envisioned process from cyanophycin towards nitrogen containing chemicals, L-aspartic acid will be available in equimolar amounts to L-arginine upon complete hydrolysis (Figure 4.1 and Chapter 2). The potentially stabilizing effect of L-aspartic acid on *B. subtilis* arginase could simply be utilized by postponing the isolation of L-aspartic acid until the conversion of L-arginine has been performed. Various methods for the separation of aspartic acid from arginine/ornithine are available,[46-49] all based on the different charges of the two amino acids at different pH values. Nonetheless, it has to be taken into account that as a result of the acidic character of L-aspartic acid adjustment of the substrate solution's pH will be required, which will lead to increased process costs.

The stabilizing effect of L-aspartic acid on the operational stability of *B. subtilis* arginase was tested at the previously reported optimum pH (pH 9.5)[24] and at the pH at which Mn^{2+} oxidation is negligible (pH 8.5). The results are presented in Table 4.1. Addition of L-aspartic acid to the substrate solution has an overall positive effect on the initial reaction rate, especially at pH 9.5. Concerning arginase's stability, the addition of L-aspartic acid at pH 9.5 resulted in an almost two times smaller deactivation constant (k_{deact} = $3.30 \cdot 10^{-2}$ h^{-1}), which is in agreement with the observations of Patchet *et al.*[45] As described earlier, this stabilizing effect is assumed to be the result of Mn^{2+} complexation with L-aspartic acid, which reduces the susceptibility to oxidation.

At pH 8.5, arginase was only slightly stabilized upon addition of L-aspartic acid (k_{deact} = $2.75 \cdot 10^{-2}$ h^{-1}). This is not surprising since Mn^{2+} oxidation is negligible at pH 8.5 and therefore the stabilizing effect of complexation would be small.

Effect of reducing agents

Bommarius *et al.*[37] showed that L-ascorbic acid has a stabilizing effect on calf liver arginase. Initially L-ascorbic acid was applied with the perception that as a reducing agent it functions by lowering the reaction mixture's redox potential and thus prevents the oxidation and concurrent removal of Mn^{2+}.

The stabilizing effect of L-ascorbic acid on the operational stability of *B. subtilis* arginase was tested by adding different amounts to a substrate solution with initial

pH 9.5. In addition, the stabilizing effect of the more economic reductant sodium dithionite was tested. The results are summarized in Table 4.1.

First, as suggested by Bommarius *et al.*[37], 0.25 mM L-ascorbic acid in combination with 0.5 mM Mn^{2+} was tested. Oxidation of Mn^{2+} was not observed and *B. subtilis* arginase was stabilized, but nonetheless at a very low initial reaction rate of 52.9 µmol min^{-1} mg^{-1}. Lowering the L-ascorbic acid concentration led to increased initial reaction rates, but at the expense of the stability.

At a concentration of 0.25 mM, sodium dithionite showed a similar stabilizing effect as L-ascorbic acid, but at a much higher initial reaction rate of 336 µmol min^{-1} mg^{-1}. The initial reaction rate increased upon increasing the sodium dithionite concentration to 0.50 mM (383 µmol min^{-1} mg^{-1}) and decreased again with 1.0 mM (303 µmol min^{-1} mg^{-1}), while maintaining the stabilizing effect. At all three tested concentrations of sodium dithionite arginase showed a good stabilization, although even 1.0 mM sodium dithionite was not sufficient to prevent Mn^{2+} from oxidizing.

The observed decrease in initial reaction rate with increased L-ascorbic acid concentration is possibly the result of enzyme deactivation. To investigate if this decrease in initial activity is reversible, for example due to Mn^{2+} chelation, the following experiment was performed.

Immobilized *B. subtilis* arginase (*vide infra*) was incubated in buffer with 0.5 mM Mn^{2+} and 2 mM L-ascorbic acid for 15 minutes. Subsequently, the L-ascorbic acid was removed by extensively washing in buffer containing 0.5 mM Mn^{2+}, after which the residual activity was determined in absence of L-ascorbic acid. The same experiment was performed with 2 mM sodium dithionite and the results were compared with a blank experiment in which reducing agents were omitted.

While 15 minutes of incubation with sodium dithionite had no negative effect on the initial activity, incubation with L-ascorbic acid resulted in an irreversible decrease in initial activity of 74%.

It is not clear how L-ascorbic acid lowers the initial activity of *B. subtilis* arginase, but it is more likely due to a structural effect than a reductive effect, since

arginases from the genus *Bacillus* appear to be remarkably stable towards reducing agents.[50] Arginases from the genus *Bacillus* have a low cysteine content, which diminishes the chance of deactivation due to changes in the secondary structure of the protein as a result of disulfide bond reduction. *B. subtilis* arginase, for example, contains only one cysteine (UniProtKB accession code P39138 aligned in NCBI-BLAST).[51]

Effect of sodium dithionite at different initial pH values

Although 0.5 mM sodium dithionite could not prevent oxidation of Mn^{2+}, it did result in an excellent stabilization of *B. subtilis* arginase when applied in a substrate solution with initial pH 9.5. To further investigate its stabilizing ability, sodium dithionite was tested in substrate solutions with the pH adjusted to 8.5 and with the pH of arginine free base (pH 11.0).

Although the initial reaction rate at pH 11.0 was significantly reduced, addition of sodium dithionite, both at initial pH 8.5 and pH 11.0, did result in stabilization comparable to that found with initial pH 9.5 (Table 4.1). However, as observed at pH 9.5, addition of 0.5 mM dithionite could not prevent oxidation of Mn^{2+} at pH 11.0. Emphasizing the results obtained studying the effect of initial pH, the increased stability of arginase appears not only to be related to the availability of free Mn^{2+}. It might be speculated that not Mn^{2+} availability, but the reduced redox potential that results from dithionite addition has its effect on the operational stability of *B. subtilis* arginase.

Conditions for optimal operational stability

The lowest operational stability determined for *B. subtilis* arginase in this study (initial pH 9.5 and 0.5 mM Mn^{2+}, $3.49 \cdot 10^7$ mol mol^{-1}) is already considered to be in the range of good biocatalyst performance for large scale processing (TTN > 10^6 mol mol^{-1})[52]. However, when not adjusting the pH of the substrate solution the TTN already increased to a very high $1.13 \cdot 10^8$ mol mol^{-1}. This high TTN was not exceeded upon addition of L-aspartic acid, although this had a positive effect at initial pH's 8.5 and 9.5.

Addition of sodium dithionite to the substrate solution at any initial pH led to an impressive stabilization. Over the observed time period no significant deactivation could be observed upon addition of sodium dithionite, so the TTNs are estimated to be larger than $1 \cdot 10^9$ mol mol^{-1}.

Since addition of sodium dithionite resulted in stabilization at all three pH values tested, the choice whether to adjust the pH of the substrate solution depends on the robustness of the process design towards precipitates formed upon Mn^{2+} oxidation. When *B. subtilis* arginase is to be applied in a membrane reactor it might be beneficial to work at pH 8.5 to avoid membrane fouling, but in a CSTR with immobilized enzyme it might be possible to simply work at the pH of arginine free base.

4.2.2 Covalent immobilization of arginase

B. subtilis arginase was covalently immobilized on the commercially available, epoxy-activated supports Sepabeads EC-EP and EC-HFA, and Eupergit C 250L. The immobilization was performed by simply incubating the enzyme together with the support in a triethanolamine-acetate buffer with 0.5 mM Mn^{2+}.

After immobilization the arginase preparations were washed and the immobilization yields were determined (Table 4.2). In all cases leaching appeared to be minimal. Furthermore, the recovered activity (the activity relative to the expected activity) was determined (Table 4.2). Compared to studies with other enzymes, the recovered activities of arginase immobilized on EC-HFA (84%) and EC-EP (75%) are good.[53-56] Compared to the values obtained with Sepabeads the recovered activity in case of Eupergit C 250L (44%) is considerably lower.

Table 4.2. Immobilization yield and recovered activities, relative to the expected activity, of the arginase preparations using a loading of 2.6 mg arginase per g dry support.

Arginase preparations	Immobilization yield (%)	Recovered activity (%)
EC-EP	100 ± 0.3	75 ± 1.1
EC-HFA	98 ± 0.1	84 ± 2.6
Eupergit C 250L	99 ± 0.5	44 ± 2.1

Since the immobilization yield is accounted for, the loss in recovered activity appears to be directly related to deactivation due to covalent binding. The considerable loss in activity upon immobilization on Eupergit C 250L could be related to its higher density of epoxy groups relative to Sepabeads EC-EP and EC-HFA (functional group densities of 0.9%, 0.4% and 0.2%, respectively, as determined for wet support by Resindion S.R.L.). This would result in a very intensive covalent multipoint attachment of the enzyme to the support, hence altering the enzyme conformation and distorting its active site.[57]

4.2.3 Thermostability of arginase preparations

The thermostabilities of covalently immobilized and soluble arginase were compared by following their residual activity in time at a relatively high temperature of 60°C (Figure 4.3).

Immobilization of *B. subtilis* arginase on Eupergit C 250L, and to a higher extent on Sepabeads EC-EP, resulted in an enhanced thermostability. On the other hand, arginase immobilized on Sepabeads EC-HFA showed a thermostability comparable to that of soluble arginase. Incubation of aliquots of covalently immobilized arginase in an excess of glycine in order to block unreacted epoxy groups, did not result in an improved thermostability (results not shown). While this blocking step has become a common procedure for immobilization on epoxy supports, it has been shown here and in Chapter 3 that it is not always effective.[17]

When covalently bound, Sepabeads EC-EP and Eupergit C 250L have relatively short (two bond lengths) spacer arms, while Sepabeads EC-HFA has relatively long (fifteen bond lengths) spacer arms. It has been suggested by Mateo *et al.*[58] and more recently by Magner *et al.*[59] that short spacer arms (two to three bond lengths) are beneficial for the conformational stability of the covalently bound enzyme.

Binding through multiple short spacer arms provides that immobilized enzyme residues are likely to preserve their relative positions in the event of heat induced conformational change. Accordingly Sepabeads EC-HFA's significantly longer

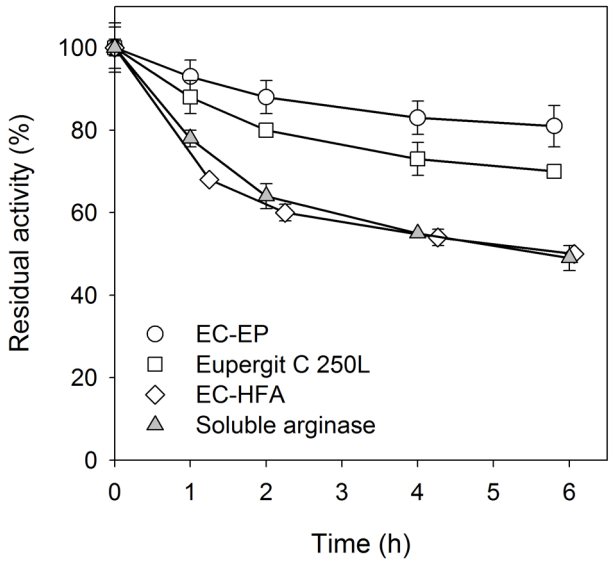


Figure 4.3. Thermostability of covalently immobilized and soluble arginase compared. Aliquots of *B. subtilis* arginase preparations were incubated in 50 mM triethanolamine-acetate buffer pH 8.0 with 0.5 mM Mn^{2+} at 60°C and in course of time the residual activity was determined upon addition of L-arginine up to 100 mM. The error bars represent standard deviation; the depicted lines are a guide to the eye.

spacer arms provide a higher degree of conformational freedom and therefore the enzyme is more susceptible to heat induced conformational changes leading to deactivation. It must however be stated that there are examples of enzymes that show an increased thermostability upon immobilization on Sepabeads EC-HFA[54,60] and that relatively short spacer arms are not always beneficial.[53,55,61]

Considering the recovered activity and the enhanced thermostability, immobilization of *B. subtilis* arginase on Sepabeads EC-EP was most successful.

4.2.4 Operational stability of soluble and covalently immobilized arginase

Because of its high operational stability, recycling of arginase would be more efficient as opposed to discarding it after single use.[52] In this study two methods of enzyme retention were employed: entrapment of soluble arginase in a membrane

reactor and covalent binding on epoxy supports. Besides the purpose of recycling, enzyme retention also facilitates product recovery and allows continuous process operation.[62]

The operational stabilities of soluble arginase (entrapment) and arginase immobilized on Sepabeads EC-EP (covalent binding) were compared by applying them in the previously described continuously stirred membrane reactor system. The reactions were carried out over a 72 hours period at 30°C using a substrate solution with 250 mM L-arginine adjusted to pH 9.5, 0.5 mM Mn^{2+} and 0.5 mM dithionite.

After 72 hours, neither soluble nor covalently immobilized arginase showed significant deactivation. *B. subtilis* arginase appears to be a robust enzyme, since it was not affected by the shear stress caused by stirring with 250 rpm and the applied temperature of 30°C. Application of calf liver arginase under similar conditions (pH 9.5, 200 rpm, 25°C) and stabilized by 0.25 mM ascorbic acid, resulted in a deactivation constant of $3.8 \cdot 10^{-3}$ h^{-1}.[37]

Covalent immobilization of arginase on Sepabeads EC-EP does not seem to add up to the already high operational stability of *B. subtilis* arginase. This result together with the observed loss in activity (Table 4.2) and the additional costs upon covalent immobilization favors the use of soluble arginase, entrapped in a membrane reactor. On the other hand, the capital costs of a membrane reactor are rather high and covalent immobilization on spherical beads allows the application of a packed-bed reactor in which higher degrees of conversion or higher space-time yields can be achieved than in a CSTR.[52]

The final choice whether to use entrapment or covalent immobilization will heavily depend on the expertise and the equipment available to the manufacturer and should be subject of studies on the pilot-scale. Nonetheless, the overall high operational stability achieved in this study shows the potential of *B. subtilis* arginase in the large scale conversion of L-arginine to L-ornithine and urea.

4.3 Conclusions

In our pursuit to make *B. subtilis* arginase suitable for application in the biobased production of nitrogen containing chemicals as an alternative to petrochemical production, we were successful in its stabilization and immobilization. While the pH of the substrate solution and addition of L-aspartic acid have their effect on the operational stability of *B. subtilis* arginase, addition of sodium dithionite to the substrate solution resulted in very good stabilization. Furthermore *B. subtilis* arginase was successfully immobilized on three commercially available epoxy-activated supports. Immobilization on Sepabeads EC-EP was most successful resulting in a recovered activity of 75% and enhanced thermostability. The stabilization and immobilization of *B. subtilis* arginase has opened up possibilities for its application in the biobased production of nitrogen containing chemicals as an alternative to the petrochemical production.

4.4 Experimental Section

The Bacillus subtilis arginase stock solution used in this study was obtained from an L-arginine/urea/ammonia assay kit (K-LARGE) from Megazyme International Ireland Ltd. and was used as received. SDS-PAGE analysis revealed that the arginase stock was of high purity and the enzyme consisted of 38 kD subunits.

All buffers and solutions used were prepared with MilliQ water and sterilized (0.20 μm) before use. Bradford protein assays were performed using Bio-Rad Protein Assay Kit II with BSA standard. L-arginine free base, used as the substrate, and other chemicals used in this study were of analytical grade or better and used as received.

Sepabeads EC-EP (epoxide) and EC-HFA (epoxide on an amino spacer) used in this study are epoxy activated methacrylic beads with pore sizes in the range of 30-40 nm and diameters in the range of 150-300 μm.[63] Both were kindly donated by Dr. Paolo Caimi from Resindion S.R.L. (Mitsubishi Chemical, Milan, Italy). Eupergit C 250L (epoxide) are epoxy activated acrylic beads with an average pore

size of 100 nm and diameter of 250 µm and was obtained through Sigma-Aldrich B.V., the Netherlands.

4.4.1 Arginase activity assay

To 595 µL activation buffer (0.5 mM $MnCl_2$ in 50 mM triethanolamine-acetate buffer pH 8.0), 5 µL arginase stock solution was added. This solution was incubated in an Eppendorf Thermomixer (37°C, 1200 rpm). After 5 minutes the assay was started by addition of 400 µL 250 mM L-arginine solution of which the pH was adjusted to 9.5 using acetic acid. 10 µL samples were drawn after 2, 4, 6 and 8 minutes and immediately quenched with 150 µL 1 M acetic acid. The samples were diluted by adding 700 µL MilliQ water.

4.4.2 HPLC analysis

L-arginine and L-ornithine concentrations were determined using the fluorescamine derivatization method described by Udenfriend *et al.*[64] To 5 µL of sample 75 µL 100 mM borate buffer pH 9.0 was added and both substrate and product were derivatized by addition of 20 µL 5 mg mL^{-1} fluorescamine in acetonitrile followed by vigorously vortexing.

The HPLC system used for quantification was based on separation on a NovaPak C18-column (60 Å, 4 µm, 3.9 x 150 mm) at 30°C, isocratic elution with 1 mL min^{-1} 50% methanol, 50% 20 mM sodium acetate buffer (pH 4.50) and detection with a Jasco 820-FP fluorescence detector (λ_{ex} = 390 nm, λ_{em} = 460 nm).

4.4.3 Operational stability experiments

The stability experiments were performed in a stirred cell (Model 8010, Millipore) connected (CDS20 Selector Valve, Millipore) to a N_2 pressurized reservoir (RC800 Mini-Reservoir, Millipore). A standard substrate solution of L-arginine and 0.05% NaN_3 in MilliQ water was prepared. After adjusting the pH with glacial acetic acid, Dithionite, L-ascorbic acid, L-aspartic acid and manganese chloride were added or omitted to obtain the desired solution.

The freshly prepared substrate solution was transferred to the reservoir and

subsequently the headspace was flushed with N_2 gas for 10 minutes. Meanwhile, the cell was mounted with a 25 mm diameter regenerated cellulose ultrafiltration membrane with a 10,000 D cut-off (PLGC02510, Millipore). The cell was filled with 8 mL substrate solution and upon addition of 100 µL 1.2 mg mL^{-1} arginase stock, the cell was closed and the pressure was built up to 2.0 bar, allowing a flow of 0.25 mL min^{-1}. The cell was placed in a isothermal waterbath and stirred at 250 rpm with the by default applied stirrer. The cell's outflow was collected in fractions of 10 minutes using a fraction collector (LKB Bromma, 2211 Superrac). Upon dilution of 5 µL with 800 µL MilliQ water, the fractions were analyzed with the previously described HPLC analysis.

4.4.4 Effect of pre-incubation with reducing agents

Approximately 20 mg of *B. subtilis* arginase immobilized on Sepabeads EC-EP was incubated for 15 minutes in 600 µL 50 mM triethanolamine-acetate buffer pH 8.0 with 0.5 mM Mn^{2+} and 2 mM of reducing agent in an Eppendorf Thermomixer at 37°C and 1200 rpm. Subsequently, the supernatant was removed and upon addition of 1 mL buffer without reducing agent the supports were suspended by gently vortexing for 20 seconds. This rinsing step was repeated once more and finally the supernatant was removed and replaced with 600 µL buffer without reducing agent. After 5 minutes of incubation (37°C, 1200 rpm), 400 µL 250 mM L-arginine solution was added and the activity was determined as previously described. The blank experiment was performed in a similar way, but with the reducing agent omitted.

4.4.5 Covalent immobilization of arginase

Two different 0.325 mg mL^{-1} arginase solutions were prepared, one without and another with 2 M NaCl added to 0.5 mM $MnCl_2$ in 50 mM triethanolamine-acetate buffer pH 8.0. Of each epoxy support duplicates of 1.000 gram dry weight was transferred to a 10 mL screw-cap tube. To Sepabeads EC-EP and Eupergit C 250L 8.0 mL of the 2 M NaCl arginase solution was added and to Sepabeads EC-HFA

8.0 mL of the arginase solution without NaCl was added. All tubes were mounted on a rotator (Cole-Palmer RotoTorque Heavy Duty Rotator) and incubated for 24 hours at room temperature. In time 80 µL samples were taken from the supernatant which were stored at 4°C.

After 24 hours of incubation the supports were filtered over a sintered glass filter with reduced pressure, twice rinsed with 8 mL wash buffer (80 mM NaCl and 0.5 mM $MnCl_2$ in 50 mM triethanolamine-acetate buffer pH 8.0), resuspended in another 8 mL of wash buffer and incubated for another hour at room temperature. After washing the supports were rinsed twice with 8 mL activation buffer (0.5 mM $MnCl_2$ in 50 mM triethanolamine-acetate buffer pH 8.0) and finally filter-dried. The filter-dry supports were weighed and stored at 4°C.

500 mg aliquots of each filter-dried support was transferred to 10 mL screw-cap tube and blocked by incubation on the rotator in 5 mL 3 M glycine in activation buffer (pH 8.0) for 18.5 hours at room temperature. After incubation the blocked supports were washed as described above, weighed and stored at 4°C.

The protein content in the supernatant during immobilization and washing was determined with Bradford protein assays. Residual activities of the enzyme preparations were determined with 30 mg immobilized arginase in the standard activity assay.

4.4.6 Thermostability of arginase preparations

Aliquots of soluble and immobilized arginase in 600 µL activation buffer were incubated in a 60°C water bath. At various time intervals the aliquots were transferred to an Eppendorf Thermomixer (60°C, 1200 rpm). After an incubation of 5 minutes, activity assays were started by adding 400 µL 250 mM L-arginine (pH 9.5). Sampling and analyses were performed as previously described.

4.4.7 Operational stability of soluble and immobilized arginase

The reactor was setup and operated as previously described using a substrate solution with 250 mM L-arginine adjusted to an initial pH of 9.5, 0.5 mM Mn^{2+} and 0.5 mM sodium dithionite. Aliquots of 100 mg immobilized arginase on Sepabeads EC-EP or 25 µL 1.2 mg mL^{-1} soluble arginase stock were used. To be able to perform a run of 72 hours without having to refill the substrate reservoir, the pressure was adjusted to 0.75 bar, allowing a flow of 0.10 mL min^{-1}. The fraction interval was increased to 15 min. The collected fractions were analyzed as previously described.

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Chapter 5

Stabilization and immobilization of *Trypanosoma brucei* ornithine decarboxylase for the biobased production of 1,4-diaminobutane

This chapter is based on

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Abstract

Using the biorefinery concept, L-arginine could become widely available from residual biomass streams via the nitrogen storage polypeptide cyanophycin. In our pursuit to develop a route from biobased L-arginine to 1,4-diaminobutane, one of the monomers in nylon-4,6, we were previously successful in the stabilization and immobilization of *Bacillus subtilis* arginase (Chapter 4). In the present study, we investigated the stabilization and immobilization of *Trypanosoma brucei* ornithine decarboxylase (EC 4.1.1.17) (TbODC) for its application in the decarboxylation of L-ornithine, the final step in the envisioned route towards 1,4-diaminobutane. The stability of TbODC was substantially improved upon addition of dithiothreitol (DTT), which not only has a stabilizing, but also an activating effect. For optimal TbODC performance, the pH should be controlled at pH 8 and the ionic strength should be kept to a minimum. The temperature for optimal productivity is 40°C. Immobilization of TbODC on Sepabeads EC-HFA was most successful, leading to an almost three-fold improvement in operational stability as compared to the soluble enzyme. Overall, we demonstrated that by optimization of reaction conditions and covalent immobilization the productivity of TbODC was vastly improved, opening up possibilities for its application in the biobased production of 1,4-diaminobutane.

5.1 Introduction

Many nitrogen containing bulk chemicals are produced from naphtha. In these processes large amounts of energy and ammonia are used, *e.g.* in the ammoxidation of olefins.[1] Global warming, depletion of fossil resources and geopolitical instability are forcing us to make the transition towards CO_2 neutral, sustainable and economically stable alternatives for the naphtha-based production of functionalized bulk chemicals.

Following the biorefinery concept, it would be more energy efficient and eventually more economic to start from biobased, functionalized compounds that already have nitrogen incorporated, such as amino acids.[2] The bottleneck in the application of amino acids for this purpose lies not in their availability,[3] but in their isolation from mixtures of available functionalized compounds, *e.g.* protein hydrolysate. Bearing this in mind, our attention has been drawn to the polypeptide cyanophycin (CGP).

CGP is a nitrogen storage polymer that is naturally produced in cyanobacteria and consists of a poly-L-aspartic acid backbone with L-arginine side chains in equimolar amounts.[4] Since CGP is insoluble under physiological conditions, it can be easily isolated.[5-7] Additionally, heterologous expression in industrially relevant bacteria has opened up the possibility to produce CGP in considerable amounts.[8-11] These properties make CGP formation a suitable method to selectively isolate L-aspartic acid and L-arginine from side streams of the agro-industries.[12]

Our research is focused on the conversion of CGP's hydrolysate, L-aspartic acid and L-arginine, towards existing nitrogen containing bulk chemicals. Starting from L-arginine, we envision a route towards 1,4-diaminobutane, one of the monomers in nylon-4,6 which is commercially available under the name Stanyl® (Figure 5.1).[13,14]

Recently Qian *et al.* demonstrated that significant levels of 1,4-diaminobutane could be produced by means of fermentation with an engineered *Escherichia coli* strain.[15] This glucose based process offers the possibility to reduce the use of

fossil carbon resources, but still requires a nitrogen source which is produced with large amounts of (fossil) energy. By using engineered microorganisms as a means to store nitrogen that is available as amino acids from side streams of the agro-industries in the form of CGP, the use of fossil energy in the production of 1,4-diaminobutane could be further reduced.

Earlier we successfully stabilized and immobilized *Bacillus subtilis* arginase (EC 3.5.3.1) and thereby proved its feasibility in the enzymatic hydrolysis of L-arginine to L-ornithine and urea (Chapter 4).[16] Urea can be directly applied as a fertilizer and L-ornithine can be decarboxylated to yield 1,4-diaminobutane (Figure 5.1). Examples of non-enzymatic L-ornithine decarboxylation are limited and proceed at rather extreme reaction conditions with low selectivity.[17,18] For this reason we focus on the application of ornithine decarboxylase (EC 4.1.1.17).

Because a vector containing its gene was made available to us, ornithine decarboxylase from Trypanosoma brucei (TbODC) was used in this study. TbODC is a homodimeric pyridoxal phosphate (PLP) dependent enzyme that decarboxylates L-ornithine with very high selectivity to 1,4-diaminobutane.[19]

ODC catalyzes the first step in the *de novo* polyamine synthesis in higher animals and as a result its intracellular level indirectly controls normal and neoplastic cell growth.[20,21] Therefore, in pursuit of *e.g.* cancer drugs, most ODC studies up till now have been focused on the therapeutic effects of its inhibition.[22] The number of publications concerning the industrial application of ODC is however rather limited and only recently a patent was issued describing the production of 1,4-diaminobutane using resting cells with elevated ODC levels.[23]

Although most catalytic properties have been determined for TbODC (V_{max} = 53 µmol min^{-1} mg^{-1}, k_{cat} = 7.4 s^{-1}, K_M = 0.18 mM, K_I = 0.35 mM),[24,25] there are several topics that need to be investigated concerning its industrial application. First of all, L-ornithine decarboxylases are generally known for their low stability *in vitro*,[26-28] which will also pose a problem in the application of TbODC. Possibly this problem can be solved with a suitable additive. Furthermore, the effect of essential parameters such as pH, temperature and ionic strength on activity and

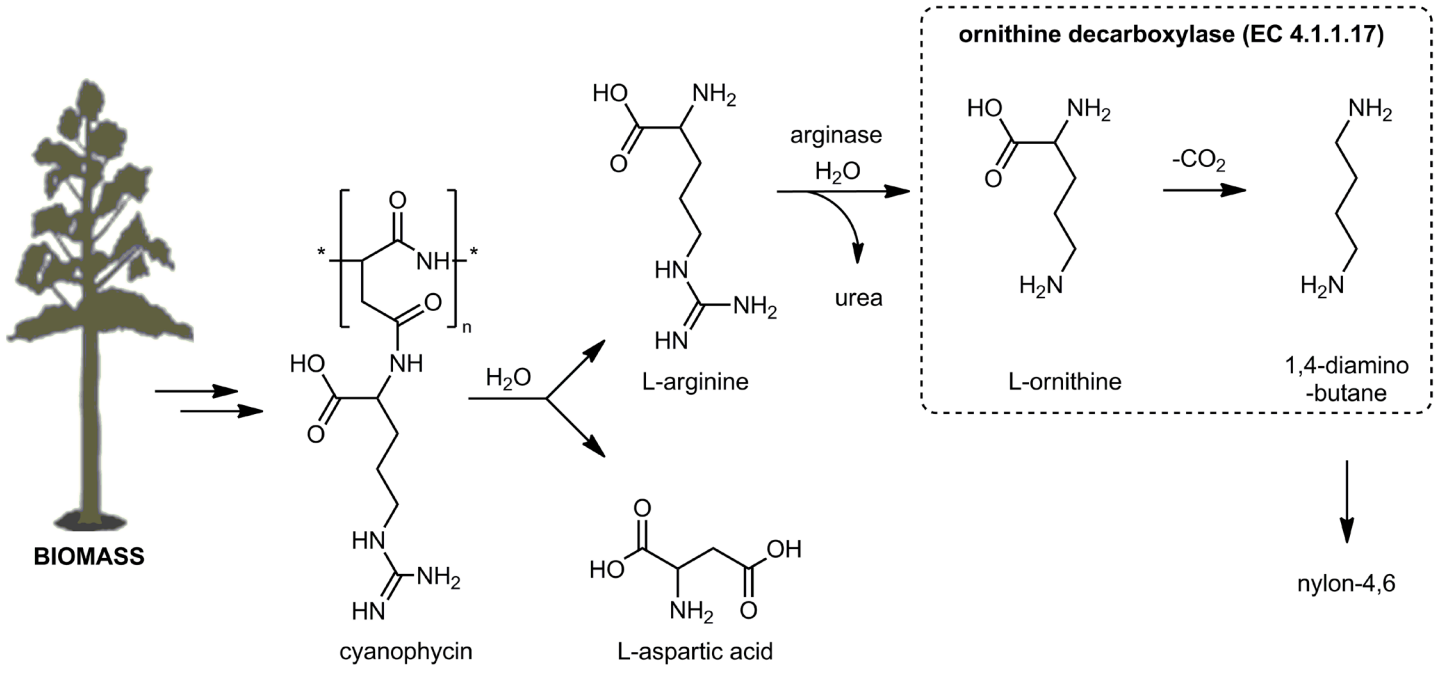

Figure 5.1. Route from cyanophycin towards 1,4-diaminobutane with the enzymatic hydrolysis of L-ornithine as the final step.

stability is not well studied for TbODC. Finally, examples of TbODC immobilization on commercially relevant supports are not known to the authors. On an industrial scale immobilization of enzymes is desirable, as this enables reuse and overcomes problems with product recovery. In addition immobilization might enhance the enzyme's stability towards conditions that may be encountered in an industrial process.[29]

In this chapter, we present a study on the stabilization and immobilization of TbODC for its application in the biobased production of 1,4-diaminobutane. For soluble TbODC the effects of different types of additives, pH, temperature and ionic strength on activity and stability were investigated. Furthermore, TbODC was immobilized on three different epoxy-activated supports: Sepabeads EC-EP and EC-HFA, and Immobead-150. The thermal and operational stability of soluble and immobilized TbODC were compared.

5.2 Results and discussion

5.2.1 Activity and stability

Effect of additives

In the past, the low storage stability of mammalian ODC posed practical problems with its characterization and therefore several publications were devoted to finding additives which could enhance its stability.[26,30-34] Reducing agents that contain thiol groups such as dithiothreitol (DTT) and β-mercaptoethanol (BME), non-ionic surfactants such as polyethylene glycol dodecyl ether (Brij 35P) and bovine albumin serum (BSA) appeared to be most effective in enhancing the storage stability of mammalian ODC.

Since a strict conservation is observed between the active sites of *T. brucei* and mammalian ODC (human and mouse),[35] it was decided to test the effect of a selection of similar additives on the activity and storage stability of TbODC. The results are summarized in Table 5.1.

Table 5.1. Influence of a selection of additives on TbODC stability. The activity ± standard deviation was determined after 0 and 4 hours of incubation in reaction buffer (0.5 mM PLP, pH 7.5) with the indicated additive at 30°C.

Additive	Initial activity relative to blank (%)	Residual activity after 4 hours (%)
Blank	100 ± 13	23 ± 6
0.1% Brij 35 P	131 ± 13	38 ± 12
0.1% BSA	151 ± 8	64 ± 9
0.25 mM sodium dithionite	80 ± 1	5 ± 2
0.25 mM L-ascorbic acid	134 ± 8	21 ± 6
5 mM L-cysteine	113 ± 3	31 ± 1
5 mM dithiothreitol	209 ± 5	126 ± 2
5 mM β-mercaptoethanol	127 ± 4	166 ± 3
10 mM β-mercaptoethanol	154 ± 1	197 ± 7

When incubated in a buffered solution with only PLP, TbODC lost 77% of its initial activity in 4 hours of incubation. Addition of Brij 35P or BSA enhanced the enzyme's performance by not only improving its stability, but also by increasing its activity. Kitani *et al.*[26] obtained similar results with rat liver ODC and concluded that these additives are active in both stabilizing and renaturing ODC.

The reducing agents that contain thiol groups, DTT and BME, appeared to have a similar activating and an even stronger stabilizing effect on TbODC. Since sodium dithionite and L-ascorbic acid, used in concentrations effective with other enzymes (Chapter 4),[16,36] and the weak reductant L-cysteine were not very successful in enhancing TbODC's performance, it appears that the combination of a reducing agent that contains thiol groups is essential.

Several studies with rat liver ODC pointed out that addition of DTT can reverse or prevent inactivation of ODC, which is attributed to DTT's ability to, respectively, restore or keep critical cysteine thiol groups in their reduced form.[30,33,37-39] In TbODC, the side chain of Cys-360, which is assumed to act as a general acid in the protonation of the Cα carbon of the product intermediate after decarboxylation,[40] would be such a critical thiol-group. The importance of Cys-360 is underlined by the fact that its mutation to either alanine or serine converts TbODC into a

decarboxylation-dependent transaminase.[19]

Since DTT and BME are most effective in enhancing TbODC's performance, their long-term effects were further investigated (Figure 5.2).

In time, both DTT (after 7.5 h) and BME (after 4 h) activated TbODC to a level of approximately 300% of the initial activity of the blank. After reaching a maximum in activity, TbODC deactivated following a single exponential model ($r(t) = r_{max} \cdot \exp(-k_{deact} \cdot t)$): blank (k_{deact} = 0.36 ± 0.04 h^{-1}), BME (k_{deact} = 0.056 ± 0.012 h^{-1}) and DTT (k_{deact} = 0.024 ± 0.001 h^{-1}). DTT and BME stabilized TbODC by six- and fifteen-fold, respectively, which combined with the increased activities boosts TbODC's overall performance.

To further investigate the action of the two thiol containing reductants, aliquots of TbODC incubated with DTT or BME were replenished with an amount equal to the initially applied amount after 16 hours of incubation. Subsequently, the residual activities were determined 2 and 8 hours after replenishment (Figure 5.2, dashed

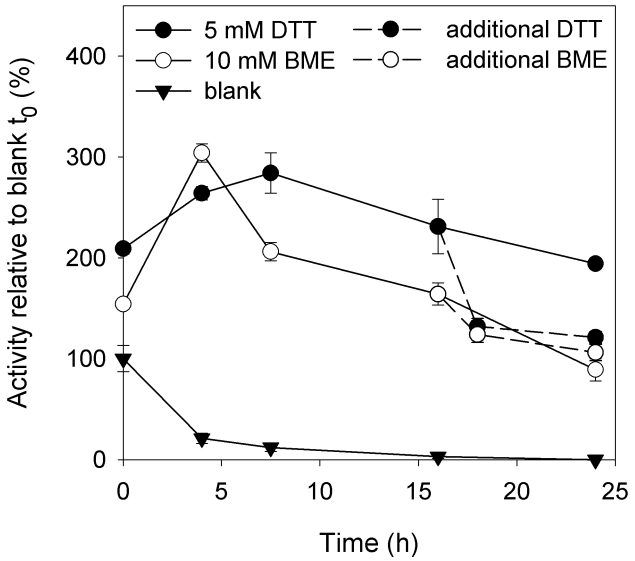

Figure 5.2. Long-term effect of DTT and BME on TbODC stability. In the course of time, the residual activities of TbODC incubated at 30°C with 5 mM DTT, 10 mM BME or without additive were determined. After 18 hours, the effect of DTT and BME replenishment was tested. The error bars represent standard deviation; the depicted lines are a guide to the eye.

lines). It appeared that the deactivation of TbODC, after reaching a maximum, cannot be stopped by replenishment of the thiol containing reductant and in case of DTT even caused faster deactivation.

These observations imply that the change in TbODC activity is the sum of the restoring and conserving effect of the thiol containing reducing agents on one hand, and the effect of thermally induced deactivation on the other. Following this scenario, the activity increases upon restoration of critical thiol groups until all are restored upon reaching the observed maximum activity. After this point, thermally induced deactivation prevails and TbODC starts to deactivate.

Effect of pH

The pH dependent activity and stability of TbODC were determined by assaying its activity after 0 and 24 hours of incubation in buffered solutions with different pH values (Figure 5.3).

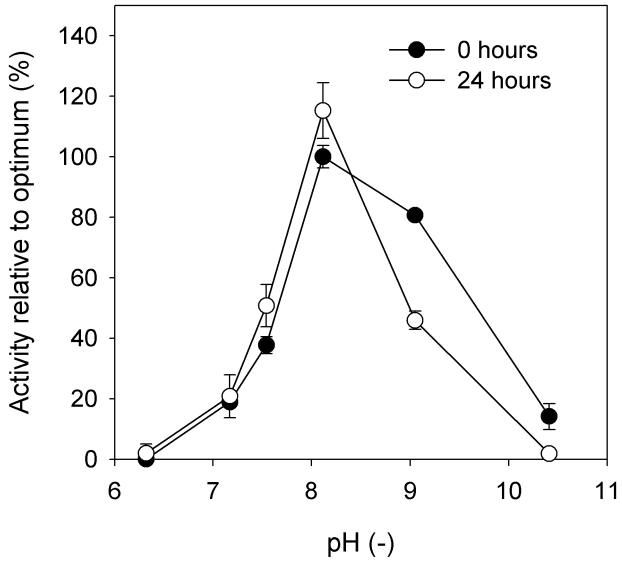

Figure 5.3. Effect of pH on activity and stability of TbODC. The residual activity of TbODC was determined after 0 and 24 hours of incubation at 30°C in a three-component buffer with 0.5 mM PLP and 5 mM DTT. The error bars represent standard deviation; the depicted lines are a guide to the eye.

Purified TbODC appears to have a pH optimum at pH 8 which is somewhat higher than previously determined by Garofalo *et al.*[41] for a cell free extract (pH 7), but it is not exceptional when compared with ODC from other sources.[42]

Concerning its stability, TbODC showed a good stability up to pH 8, but in more alkaline environments it showed a lower stability. Taking into account that a non-buffered feed stream of L-ornithine will have a pH of approximately 9.7 (estimated using CurTiPot)[43], it will be necessary to adjust it to pH 8 in favor of TbODC's activity and stability. When L-ornithine is converted to 1,4-diaminobutane, the pH will increase to approximately pH 10.5 upon complete conversion (estimated using CurTiPot)[43] and as a consequence the pH of the reaction needs to be continuously corrected.

Effect of temperature

The effect of temperature dependent activity and stability was investigated by incubating aliquots of TbODC at different temperatures and assaying its residual activity after 0, 4 and 24 hours of incubation at the same temperature as applied during the incubation. The results are presented relative to the activity observed at 30°C after 0 hours of incubation (Figure 5.4).

After 0 hours of incubation at different temperatures, the highest activity was observed at 50°C (Figure 5.4, 0 hours), which is remarkably comparable with the temperature optimum of the hyperthermophile *C. thermohydrosulfuricum*.[44] However, at prolonged incubation TbODC appeared to be rather unstable at 50°C (Figure 5.4, 4 and 24 hours). In terms of overall activity, TbODC performed best at a temperature of 40°C with an activity which is approximately three-fold higher as observed at 30°C.

Effect of ionic strength

It has been observed that an increase in ionic strength has a negative effect on the stability of rat liver ODC.[45,46] Supported by observations during gel filtration chromatography, these studies suggest that the normal form of ODC is a homodimer that dissociates into inactive monomers upon addition of high

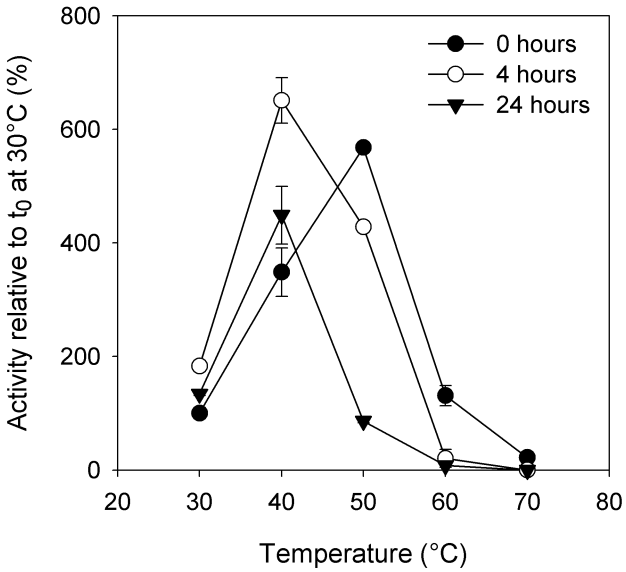

Figure 5.4. Effect of temperature on TbODC activity and stability. Aliquots of TbODC were incubated in reaction buffer (5 mM DTT, 0.5 mM PLP, pH 7.5) at different temperatures and after 0, 4 and 24 hours the residual activity was determined at the same temperature as applied during incubation. The activities are presented relative to the activity determined at 30°C after 0 hours of incubation under similar conditions. The error bars represent standard deviation; the depicted lines are a guide to the eye.

concentrations of salt.

The effect of ionic strength on TbODC activity and stability was investigated by determining the activity after 0 and 2 hours of incubation in solutions with different sodium chloride concentrations.

Although ionic strength had no significant effect on the initial activity (results not shown), the stability of TbODC was clearly negatively affected by increased salt concentrations. If deactivation of TbODC is the result of an electrostatic effect and thus related to the ion activity coefficient of the reaction solution, the residual activity after 2 hours of incubation should obey the following modified Debye-Hückel equation:[47]

$$\log(A) = B \cdot \mu^{0.5} + \log(A_0)$$

where A_0 and A are the residual activities after 2 hours of incubation at ionic strengths of 0 and μ mol L^{-1}, respectively, B is an empirical parameter, which is dependent on solvent and temperature, and μ is the ionic strength of the reaction solution in mol L^{-1}.

Indeed a linear relation was observed between the logarithm of the residual activity and the square root of the corresponding ionic strength (Figure 5.5), which confirms that the observed deactivation is the result of an electrostatic effect. This observation and the ODC dissociation with increasing ionic strength observed in other studies suggest that an increased ionic activity interferes with the subunit association, which is driven by many small energetic contributions throughout the interface,[48] leading to dissociation of the active TbODC dimer.

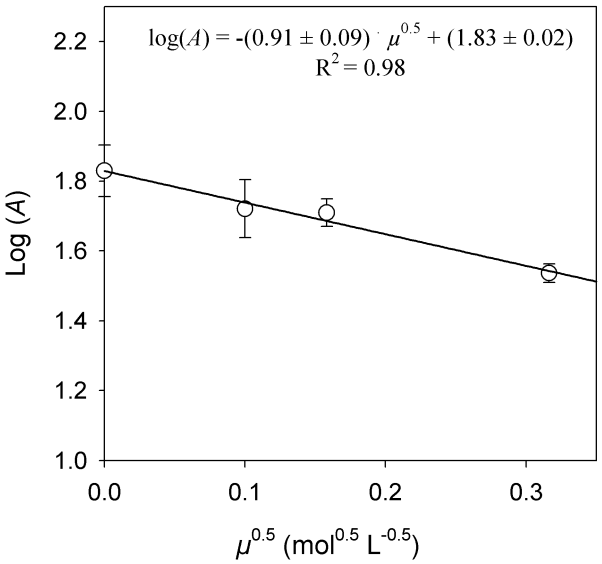

Figure 5.5. Plot with the logarithm of the residual activity after 2 hours of incubation in reaction buffer (5 mM DTT, 0.5 mM PLP, pH 7.5) with different sodium chloride concentrations (A) plotted against the square root of the corresponding ionic strength (μ). The error bars represent standard deviation; the depicted line represents the linear relation as described by the modified Debye-Hückel equation.

5.2.2 Covalent immobilization

TbODC was immobilized on three different epoxy-supports, Sepabeads EC-EP (EC-EP) and EC-HFA (EC-HFA), and Immobead-150 (IB-150). Due to their ease of operation and their robustness under physical stress, Sepabeads are very suitable for enzyme immobilization for industrial purposes.[49,50] Although lipases from different sources immobilized on IB-150 are readily available (Sigma Aldrich B.V., the Netherlands), this relatively new epoxy-support is not well described in literature.

It is proposed that protein immobilization on epoxy supports follows a two-step mechanism: (i) first the enzyme is adsorbed to the surface of the support and (ii) subsequently the protein's nucleophilic groups (amino, thiol, or hydroxyl groups) react with the epoxy groups on the support to form covalent bonds.[51] Following this mechanism, immobilization on supports with relatively hydrophobic surfaces, such as EC-EP and IB-150, are ideally performed in buffer solutions with higher ionic strengths. Such environment would allow the enzyme to get in close vicinity with the support's surface, facilitating subsequent covalent binding.[52] Nevertheless, because of the observed instability at higher ionic strengths, immobilization of TbODC was initially performed in a relatively low concentration sodium phosphate buffer (10 mM).

After 15 hours of incubation, the applied TbODC was completely adsorbed in case of all three epoxy-supports and in the subsequent washing steps no leaching was observed as determined by Bradford protein assays. For all preparations the recovered activity (the activity relative to the expected activity) was determined (Table 5.2).

With a maximum of 17% recovered activity for EC-HFA, the recovered activities can be considered to be low.[16,53] Since the low recovered activities are not due to incomplete immobilization or leaching during washing, the observed loss in activity is assumed to be the result of physical stress, as observed for the Blank, and the process of covalent binding.

In an attempt to improve the recovered activity, the immobilizations with EC-EP

and IB-150 were repeated using a 250 mM sodium phosphate buffer. Although the result obtained for the control clearly shows the effect of the higher buffer concentration on stability, the recovered activities were considerably improved (Table 5.2). This improvement can be attributed to the previously described effect of increased ionic strength.

Table 5.2. Recovered activity (activity relative to the expected activity) ± standard deviation of all TbODC preparations using an enzyme loading of 1.0 mg TbODC per g dry support.

Support	Recovered activities (%)	
	10 mM Buffer	250 mM buffer
Blank	88 ± 7	47 ± 2
EC-HFA	17 ± 1	n.d.
EC-EP	12 ± 1	35 ± 8
Immobead-150	9 ± 2	20 ± 2

For each type of support the preparation with the highest recovered activity was selected and its thermostability was compared with soluble TbODC by determining its residual activity after 4 hours of incubation at 50°C (Table 5.3).

The thermostabilities of the EC-EP and IB-150 preparations are significantly lower than that of the EC-HFA preparation, which has a thermostability comparable to that of soluble TbODC. The cause of these differences in thermostability are probably related to the type of spacer arm applied in the epoxy support as

Table 5.3. Thermostability of TbODC preparations. For immobilized TbODC the preparations with the highest recoveries were selected for each type of support. Aliquots of TbODC preparations were incubated in reaction buffer (5 mM DTT, 0.5 mM PLP, pH 7.5) at 50°C and after 4 hours the residual activity ± standard deviation was determined.

TbODC preparation	Residual activity after 4 hours at 50°C (%)
Soluble	75 ± 1
EC-HFA	71 ± 6
EC-EP	29 ± 1
Immobead-150	0

extensively discussed in literature (Chapter 3 and 4).[54-56] While the surfaces of EC-EP and IB-150 are relatively hydrophobic due to their relatively short (two bond lengths), apolar spacer arms, the surface of EC-HFA is more hydrophilic due to its relatively long (fifteen bond lengths), polar spacer arms.

5.2.3 Operational stability

The operational stability of TbODC at different conditions was compared by means of the estimated total turnover numbers (TTN) (mol 1,4-diaminobutane mol^{-1} TbODC), which were calculated using a method described by Rogers and Bommarius:[57]

$$TTN = k_{cat,obs} / k_{deact,obs}$$
$$k_{cat,obs} = V_{spec,init} \cdot Mw_{ODCsubunit}$$
$$k_{deact,obs} = \ln(2) / (t_{½})$$

The initial specific activity $V_{spec,initial}$ in mol s^{-1} g^{-1} and the half-life $t_{½}$ in s were both derived from isothermic batch reactions in which [S] >> KM and [S] >> [P]. The resulting estimated TTNs are depicted in Table 5.4.

Omission of either PLP or DTT from the reaction mixture led to a decrease in TTN by a factor of ten, showing that both cofactor and thiol containing reducing agent are crucial for TbODC stability. Furthermore, an optimum in TTN was observed at 40°C over the three reaction temperatures tested (30, 40 and 50°C). This is in agreement with the previous finding that TbODC shows an optimal performance at 40°C in terms of thermostability (Figure 5.4).

In the envisioned route from CGP to 1,4-diaminobutane (Figure 5.1), decarboxylation of L-ornithine by TbODC will be preceded by the previously studied L-arginine hydrolysis by *Bacillus subtilis* arginase (Chapter 4).[16] For this reason the effects of compounds involved in this conversion were tested: co-product urea (10 mM), traces of unconverted L-arginine (2 mM) and essential cofactor Mn^{2+} (0.5 mM). While addition of Mn^{2+} had no effect, addition of

L-arginine and urea had only a slightly negative effect.

Finally, the operational stability of TbODC immobilized on EC-HFA was determined. It appeared that upon covalent immobilization on EC-HFA, the operational stability was improved almost three-fold. Possibly immobilization on EC-HFA protects TbODC against physical stress encountered by mechanical stirring.

In the choice whether to covalently immobilize TbODC, the disadvantages of the observed loss in activity (Table 5.2) and the additional catalyst costs need to be weighed against the advantages that arise. In addition to the benefit of improved operational stability, immobilization on EC-HFA also converts TbODC into a heterogeneous catalyst, which provides advantages for its application on an industrial scale. Immobilization on spherical beads not only facilitates reuse and downstream processing, it also allows the application of a packed-bed reactor in which higher degrees of conversion or higher space-time yields can be achieved than in a CSTR.[58]

Table 5.4. Estimated total turnover numbers ± standard deviation at different reaction conditions. Using the data obtained from batch experiments (10 mM L-ornithine with 0.5 mM PLP and 5 mM DTT in 20 mM sodium phosphate buffer pH 7.5) the TTNs were estimated using a method described by Rogers and Bommarius.[57]

Additional conditions	Temperature (°C)	TTN (mol mol^{-1})
-	30	$4.61 (\pm 0.09) \cdot 10^4$
without PLP		$5.44 (\pm 0.04) \cdot 10^3$
without DTT		$6.83 (\pm 0.05) \cdot 10^3$
-	40	$6.90 (\pm 0.13) \cdot 10^4$
with 0.5 mM Mn^{2+}		$6.67 (\pm 0.36) \cdot 10^4$
with 2.0 mM L-arginine		$6.17 (\pm 0.31) \cdot 10^4$
with 10 mM urea		$5.86 (\pm 0.77) \cdot 10^4$
-	50	$4.82 (\pm 0.35) \cdot 10^4$
Immobilized on EC-HFA	40	$1.95 (\pm 0.13) \cdot 10^5$

5.3 Conclusions

In this study, we have investigated the stabilization and immobilization of the enzyme *Trypanosoma brucei* ornithine decarboxylase (TbODC) for its application in the biobased production of 1,4-diaminobutane. The stability observed for TbODC in vitro was substantially improved upon addition of dithiothreitol (DTT), which not only has a stabilizing, but also an activating effect. In addition, the pH should be controlled at pH 8 and the ionic strength should be kept to a minimum for optimal TbODC performance. Furthermore, TbODC has an optimum in productivity at 40°C with respect to its temperature dependent activity and stability. Immobilization of TbODC on Sepabeads EC-HFA was most successful, leading to an almost three-fold improvement in operational stability. Overall, we demonstrated that by optimization of reaction conditions and covalent immobilization the productivity of TbODC was vastly improved, opening up possibilities for its application in the biobased production of 1,4-diaminobutane.

5.4 Experimental section

The *pODC29* plasmid, constructed following Osterman *et al.*[25] by cloning the *tbODC* gene into a His_6-TEV vector, was kindly provided by Prof. Margaret Phillips from the Department of Pharmacology, University of Texas Southwestern Medical Center, US.

Z-competent *E. coli* XL1-blue and One Shot chemically competent *E. coli* BL21star(DE3) were obtained from respectively Zymo Research and Invitrogen.

All buffers and solutions used for procedures involving TbODC were prepared with MilliQ water and filter-sterilized (0.20 µm) before use. Instant low salt LB agar (Duchefa Biochemie) and instant high salt LB broth (Sigma Aldrich) were prepared according to protocol, autoclaved and ampicillin stock solution was added up to a concentration of 100 µg mL^{-1}.

Bradford protein assays were performed using Bio-Rad Protein Assay Kit II with BSA standard. L-ornithine·HCl, used as the substrate, and other chemicals used in this

study were of analytical grade or better and used as received.

Sepabeads EC-EP (epoxide) and EC-HFA (epoxide on an amino spacer) used in this study are epoxy-activated methacrylic beads with pore sizes in the range of 30-40 nm and diameters in the range of 150-300 µm.[59] Both were kindly donated by Dr. Paolo Caimi from Resindion S.R.L. (Mitsubishi Chemical), Milan, Italy. Immobead-150 (epoxide) is an epoxy-activated methacrylic polymer bead with a diameter of 150-300 µm and was kindly donated by Dr. Rob Schoevaart from ChiralVision B.V., Leiden, the Netherlands.[60]

5.4.1 Plasmid amplification

pODC29 plasmids were extracted from the provided filter paper by submersion in 100 µL TE-buffer (15 min, RT). Z-competent *E. coli* XL1-blue cells were transformed with 2 µL of *pODC29* extract according to protocol. After selection on low salt LB agar (16 h, 37°C), the transformants were grown in high salt LB medium (16 h, 37°C, 250 rpm). The plasmids were isolated from the cultures using a GeneJET™ Plasmid Miniprep Kit (Fermentas) according to protocol and stored at 20°C.

5.4.2 L-ornithine decarboxylase overexpression

Chemically competent *E. coli* BL21star(DE3) was transformed with *pODC29*, according to protocol. After selection on low salt LB agar, transformed cells were grown in high salt LB medium (37°C, 200 rpm). After 4 hours incubation (OD_{600} = 0.64), the cultures were used to prepare a 87% glycerol stock, which was stored at -80°C.

The glycerol stock was used to inoculate 25 mL high salt LB medium. After 16 hours incubation (25°C, 250 rpm), the culture (OD_{600} = 1.2) was used to inoculate 0.5 L high salt LB medium. After 4 hours incubation (37°C, 250 rpm), the culture (OD_{600} = 1.2) was induced with IPTG (0.5 mM) and incubated for another 16 hours (25°C, 250 rpm). The cells were harvested in 3 subsequent steps by centrifugation (3750 rpm, 4°C, 25 min) and stored at 80°C.

5.4.3 L-ornithine decarboxylase isolation

The pellet with IPTG induced cells was thawed on ice and suspended in 20 mL lysis buffer (10 mM imidazole, 300 mM NaCl, 0.02% Brij P35, 2 mM PMSF and 2 mM PLP in 50 mM sodium phosphate buffer, pH 7.5). Twenty mg egg white lysozyme was added and the suspension was kept on ice for 30 minutes, while carefully vortexing from time to time. After sonication on ice, 6 times 10 seconds with 10 seconds interval, the suspension was centrifuged (14,000 rpm, 4°C, 20 min). The supernatant was passed through a syringe filter (0.45 µm) and stored on ice.

10 mL cell extract was combined with 2.5 mL Ni-NTA agarose resin (Qiagen) and incubated on a rotator (Cole-Palmer RotoTorque Heavy Duty Rotator) at 4°C for 1 hour. After incubation, the mixture was transferred to a disposable PD-10 column (GE Healthcare) and the flow-through was collected. Subsequently, the resin with trapped TbODC was washed with 3 x 4 mL wash buffer (20 mM imidazole and 300 mM NaCl in 50 mM sodium phosphate buffer, pH 7.5). Finally, the trapped TbODC was eluted with elution buffer (250 mM imidazole and 300 mM NaCl in 50 mM sodium phosphate buffer, pH 7.5) and stored at 4°C. The second half of cell extract was processed in the same way. The purity and protein content of the collected fractions were determined with respectively SDS-PAGE analyses and Bradford protein assays.

Using a stirred cell (Model 8010, Millipore) in combination with a polyethersulfone membrane (PLGC Biomax PES, 10kD cutoff), the fractions were concentrated and their salt content was reduced by 72-fold using a solution of 0.05 mM PLP in 20 mM sodium phosphate buffer pH 7.5. The content of the stirred cell was divided over 1.5 mL-Eppendorf tubes, submerged in liquid nitrogen and the TbODC stocks (1.6 mg mL^{-1}) were stored at -80°C. For TbODC, freshly thawed from -80°C stock, a specific activity of 2 µmol min^{-1} mg^{-1} was determined using the standard activity assay (section 5.4.4).

5.4.4 Activity assay and HPLC analysis

To 180.5 µL reaction buffer (5 mM DTT and 0.5 mM PLP in 10 mM sodium phosphate buffer, pH 7.5), 7.5 µL TbODC stock solution (12 µg) was added. This solution was incubated in an Eppendorf Thermomixer (30°C, 1200 rpm). After 5 minutes the assay was started by addition of 12 µL 25 mM L-ornithine·HCl solution. 10 µL samples were drawn after 2, 4, 6 and 8 minutes and immediately quenched with 10 µL 1 M acetic acid.

L-ornithine and 1,4-diaminobutane concentrations were determined using the fluorescamine derivatization method described by Udenfriend *et al.*[61] To 5 µL of sample 75 µL 100 mM borate buffer pH 9.0 was added and both substrate and product were derivatized by addition of 20 µL 5 mg mL^{-1} fluorescamine in acetonitrile followed by vigorously vortexing.

The HPLC system used for quantification was based on separation on a NovaPak C18-column (60 Å, 4 µm, 3.9 x 150 mm) at 30°C, and detection with a Jasco 820-FP fluorescence detector (λ_{ex} = 390 nm, λ_{em} = 460 nm). Separation was achieved using a solution A (50% 20 mM sodium acetate pH 4.50, 50% methanol) and a solution B (30% 20 mM sodium acetate buffer pH 4.50, 70% methanol) combined in the following gradient: 0-4.5 minutes: 0% B, 5.0-12.5 minutes: 100% B, 13.0-16.0 minutes: 0% B.

5.4.5 Activity and stability

Effect of additives

Using the standard reaction buffer, solutions with different additives were freshly prepared: 5 mM L-cysteine, 5 mM dithiothreitol (DTT), 5 and 10 mM *β*-mercaptoethanol (BME), 0.25 mM L-ascorbic acid, 0.25 mM sodium dithionite, 0.1% Brij P35, and 0.1% bovine serum albumin. Aliquots of 180.5 µL of these reaction buffers were combined with 7.5 µL TbODC stock (12 µg) and incubated for 0 and 4 hours (30°C, 1200 rpm). Subsequently, 2 µL of 5 mM PLP solution was added and activity assays were performed as described in section 5.4.4.

In case of 5 mM DTT and 10 mM BME, the time scale was extended by incubating

additional aliquots for 2, 8, 16 and 24 hours (30°C). Additionally, the effect of adding fresh DTT and BME was investigated by incubating additional aliquots for 18 and 24 hours which were enriched with 5 mM DDT and 10 mM BME, respectively, after 16 hours of incubation.

Effect of pH

To maintain a constant ionic strength, a three-component buffer composed of acetic acid (10 mM), MES (10 mM) and triethanolamine (20 mM) was used following Ellis *et al.*[62] An activation (0.5 mM PLP and 5 mM DTT) and substrate (25 mM L-ornithine·HCl) buffer were prepared using the three-component buffer. Aliquots of both the activation and the substrate buffer were adjusted to the desired pH values (6, 7, 7.5, 8, 9 and 10) with 2 M HCl or NaOH.

To determine the pH dependent activity, 7.5 µL TbODC stock solution (12 µg TbODC) and 2 µL 5 mM PLP solution were added to 180.5 µL activation buffer and this mixture was incubated for 5 minutes (30°C, 1200 rpm). Activity assays were started by adding 12 µL of substrate buffer with a pH similar to that of the activation buffer. To determine the pH dependent stability, the activity assays were preceded by 24 hours incubation (30°C, 1200 rpm). Sampling and analyses were performed as described in section 5.4.4.

Effect of temperature

Aliquots of 7.5 µL TbODC stock solution (12 µg) in 180.5 µL reaction buffer were incubated for 0, 4, and 24 hours in waterbaths at 30, 40, 50, 60 and 70°C. After incubation, 2 µL 5 mM PLP solution was added and the aliquots were transferred to an Eppendorf Thermomixer (1200 rpm) and incubated for 5 minutes at the same temperature as the water bath. The activity assays were performed as described in section 5.4.4.

Effect of ionic strength

Using a 1 M NaCl in 10 mM sodium phosphate buffer pH 7.5 solution, buffers were prepared containing 0.5 mM PLP and 0, 10, 20, 100 mM NaCl. Aliquots were prepared by combining 7.5 µL TbODC stock with 180.5 µL buffer and after 2 hours

of incubation (30°C, 1200 rpm), activity assays were performed and analyzed as described in section 5.4.4.

5.4.6 Covalent immobilization

Using freshly thawed TbODC stock solutions and 0.5 mM PLP and 5 mM DTT in 10 mM sodium phosphate buffer pH 7.5, 12 mL 0.125 mg mL^{-1} TbODC solution was prepared. Of each epoxy support (Sepabeads EC-EP and EC-HFA and Immobead-150) 0.250 gram dry weight was transferred to a 10 mL screw-cap tube (in duplicate). After addition of 2 mL TbODC solution to each tube, all tubes were mounted on a rotator (Cole-Palmer RotoTorque Heavy Duty Rotator) and incubated for 16 hours at 4°C. In time 100 µL samples were taken from the supernatant and stored at 4°C.

After incubation, the supports were filtered over a sintered glass filter with reduced pressure, rinsed with 3 mL buffer, resuspended in another 3 mL buffer and incubated for another hour at room temperature. After washing, the supports were rinsed twice with 3 mL buffer and finally filter-dried. The filter-dry supports were weighed and stored at 4°C.

Additionally, the immobilization procedure as described above was repeated for Sepabeads EC-EP and Immobead-150 using a 250 mM sodium phosphate buffer in the preparation of the TbODC solution.

The protein content in the supernatant during immobilization was determined with Bradford protein assays. Residual activities of the enzyme preparations were determined with 30 mg immobilized arginase in the standard activity assay.

The thermostability of the TbODC preparations was determined at 50°C as previously described in section 5.4.5 except for the replacement of soluble with immobilized TbODC.

5.4.7 Operational stability

A reaction solution of 10 mM L-ornithine·HCl in 20 mM sodium phosphate buffer (adjusted to pH 7.5) with 0.5 mM PLP and 5 mM DTT, unless stated otherwise, was

prepared. After 5 minutes of incubation of 2.50 mL reaction solution in a stirred (250 rpm) waterbath at the desired temperature, the reaction was started by adding approximately 5 µL TbODC stock solution (8 µg). Sampling and analyses were performed as previously described. The total turnover number (TTN) was calculated by dividing the moles of 1,4-diaminobutane produced by the moles of TbODC spent, based on the homodimeric enzyme configuration with a total molecular weight of 90 kD.

Acknowledgements

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Chapter 6

General discussion

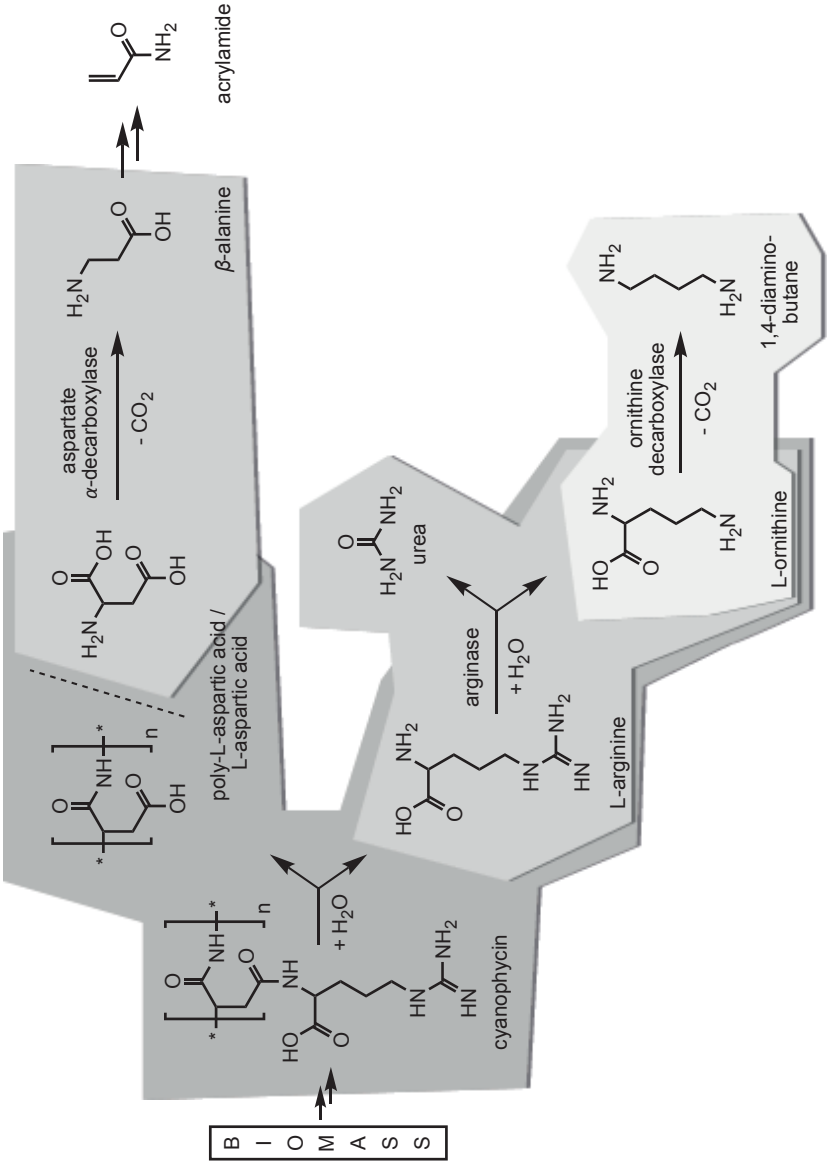


Figure 6.1. Overview of the different (bio)conversions that were investigated in view of the envisioned conversion of cyanophycin into nitrogen containing chemicals. The different shades of grey represent the different conversions addressed in this thesis.

6.1 Introduction

The aim of the research presented in this thesis was to explore and optimize the different (bio)conversion steps involved in the envisioned route from cyanophycin towards nitrogen containing chemicals (Figure 6.1). More specifically, for each conversion that we investigated we aimed to determine the crucial process parameters enabling us to improve productivity or at least identify the bottlenecks of the envisioned route.

In this chapter the results presented in previous chapters will be discussed with respect to their impact on the envisioned route as a whole. Furthermore, recommendations for follow up studies will be given as well as recommendations for the application of biorefineries in general. This chapter ends with general conclusions.

6.2 General discussion and recommendations

6.2.1 Cyanophycin hydrolysis

In Chapter 2, a study on the acid and base catalyzed hydrolysis of cyanophycin (CGP) is presented. The aim was to find the optimal conditions for either complete or selective hydrolysis of the CGP polymer.

Acid and base catalyzed hydrolysis of cyanophycin

Although complete hydrolysis of polypeptides is readily achieved upon reaction with high concentrations of strong acid at elevated temperatures,[1-3] selective hydrolysis of CGP's L-arginine branches has been less well studied. Selective hydrolysis would yield a residue with polyaspartic acid (PASP) properties. PASP is applied as biodegradable replacement for polycarboxylate components, which can serve as scale inhibitor or dispersing agent.[4,5]

Our results prove that acid catalyzed hydrolysis is not selective towards the different peptide bonds and is therefore only suitable for complete hydrolysis, yielding L-aspartic acid and L-arginine. Preliminary experiments (results not

presented) have shown that L-aspartic acid can be separated from L-arginine based on the difference in isoelectric point.

Base catalyzed hydrolysis of CGP resulted in the selective liberation of L-arginine, yielding a residue with reduced L-arginine content. However, in course of time also the L-aspartic acid backbone itself is hydrolyzed. For this reason it was concluded that a choice needs to be made between the degree of PASP functionality and polymer yield. Additionally, under influence of the alkaline environment, liberated L-arginine was instantaneously hydrolyzed to mainly yield ornithine. As described in Chapter 2, PASP can subsequently be precipitated upon addition of methanol, while ornithine remains in solution (Figure 6.5).

Ornithine racemization

The combined liberation of L-arginine and its hydrolysis to L-ornithine would be advantageous in view of the envisioned route, since the enzymatic hydrolysis of L-arginine could be omitted (Figure 6.1). Nonetheless, it appeared that L-ornithine, produced by base catalyzed hydrolysis of CGP, is prone to racemization due to the alkaline environment and elevated temperatures. Since the nitrogen functionalized end-products, in this case 1,4-diaminobutane, are achiral, racemization may not seem a problem. However, chirality is an issue in the subsequent conversion of ornithine to 1,4-diaminobutane. Within the scope of this project the reaction was performed with the enzyme ornithine decarboxylase (Chapter 5), with unparalleled yield and selectivity, but the enzyme is only active on L-amino acids. As a consequence of racemization half of the amino acid feedstock would not be converted, resulting in a maximum yield of 50% of the desired product, if basic hydrolysis of CGP would be employed.

This problem might be solved by dynamic kinetic resolution, in which case the substrate is constantly racemized, while one of the substrate enantiomers is converted (Figure 6.5).[6] Eventually this will result in complete conversion. Racemization could be achieved under similar conditions as applied in the selective hydrolysis of CGP; high pH and temperature. Nonetheless, these conditions do not allow the simultaneous enzymatic decarboxylation of L-ornithine. In this case

an enzymatic racemization which can be performed under mild conditions would be more suitable. Both amino acid racemase (EC 5.1.1.10) and arginine racemase (EC 5.1.1.9) would be suitable candidates for dynamic kinetic resolution of ornithine.[6,7]

Urea

Although it could not be detected with the analytical methods applied, it can be assumed that urea, the other hydrolysis product of L-arginine, was completely hydrolyzed to ammonia and CO_2 under the applied conditions.[8] Urea ($1 \cdot 10^7$ tons $year^{-1}$ (Europe and US combined), 210-230 € ton^{-1}) is used as fertilizer and chemical intermediate.[9] Since it is based on the reaction between ammonia and carbon dioxide at elevated temperatures (170-200°C) and high pressure (1330 MPa), the current production of urea requires high energy inputs.[10,11] Considering its commercial value and the potential energy savings when produced from biomass, the loss of urea is a serious drawback of the base catalyzed hydrolysis of CGP.

Enzymatic cyanophycin hydrolysis

Taking the limited selectivity, the formation of D-ornithine and the loss of urea in consideration, a method for selective hydrolysis which involves milder reaction conditions and has higher product selectivity would be advantageous. In this respect an enzyme would be a suitable catalyst.

Since CGP serves as a storage polymer, enzymes that can either selectively or completely hydrolyze CGP are expected to be present in nature. This means that the organism which creates CGP must also be able to decompose it in times of need.

It is generally assumed that two enzymatic activities are involved in degradation of CGP: (i) an exopeptidase (cyanophycinase) that hydrolyzes the CGP backbone to aspartic acid-arginine dipeptides; and (ii) a peptidase that hydrolyses this dipeptide.[12,13]

Recently, aspartic acid-arginine dipeptides derived from cyanophycin have been recognized for their commercial value as potential natural food additives and

therapeutics.[14] For this reason a production process has been established in which the production of the CGP derived peptides is optimized.[15] By extending this process with peptidase driven dipeptide hydrolysis, this process could serve as a basis for the complete hydrolysis of CGP.

Publications that report selective hydrolysis of CGP, yielding a CGP residue with polyaspartic acid functionality and L-arginine are scarce. In 1984, Allen *et al.*[16] reported that incubation of CGP in extracts of cyanobacterium *Aphanocapsa* 6308 cells starved from nitrogenous compounds yielded mainly L-arginine among other hydrolysis products: 50% L-arginine, 28 to 45% aspartate-arginine dipeptide and less than 5% L-aspartic acid. The aspartic acid not accounted for was most likely present in the form of PASP (≥ 45%). This is certainly an interesting result and it might be suggested to repeat these experiments using modern analyzing techniques and if validated, one could consider the identification of the responsible enzyme(s) for further optimization.

6.2.2 *α*-Decarboxylation of L-aspartic acid

The applicability of aspartate *α*-decarboxylase (ADC) in the conversion of L-aspartic acid into *β*-alanine was studied in Chapter 3.

Stability of aspartate α-decarboxylase

ADC is a remarkably thermostable enzyme and is not affected by product inhibition. Furthermore, it has a pH optimum at pH 7.5 and is instable at pH values below 6. Assuming the use of acid catalyzed hydrolysis of CGP, the resulting solution has to be neutralized by base before enzymatic decarboxylation can take place. Since the carboxylate group of aspartic acid is replaced by a proton, continuous titration with acid is needed during the reaction (Figure 6.5).

Although ADC is remarkably thermostable, it suffers from a rather low turnover number. It has been suggested that deactivation of ADC in presence of substrate occurs via decarboxylation-dependent transamination of the catalytically essential pyruvoyl group, resulting in a catalytically inactive alanine residue at the *N*-terminus (Figure 6.2).[17,18]

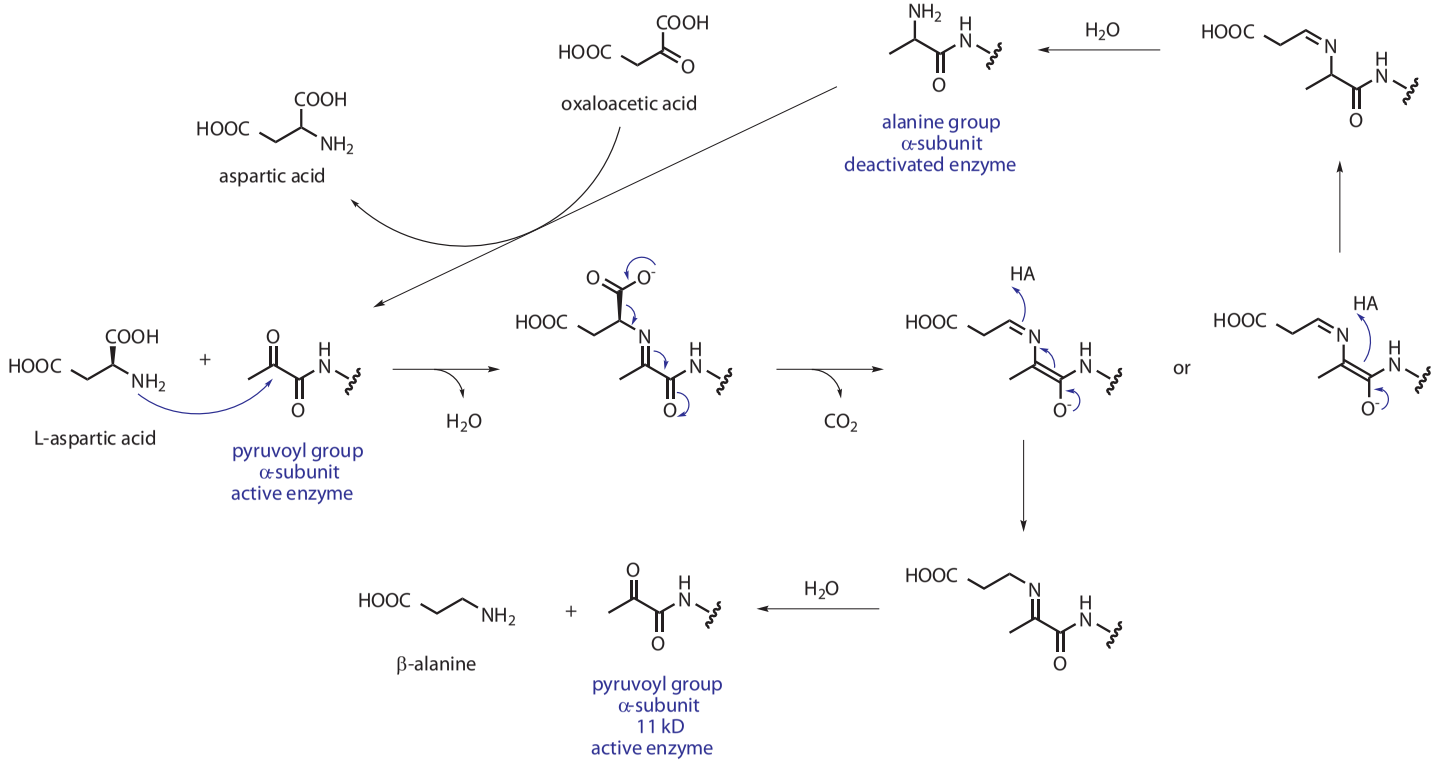


Figure 6.2. Proposed reaction mechanism of aspartate α-decarboxylase (ADC), based on the work of Albert *et al.*[25], and the proposed transamination reaction leading to its deactivation. ADC could not be reactivated by means of a reversed transamination with oxaloacetic acid.

The assumed transamination reaction is reported for several other decarboxylases[19-22] and could in some cases be reactived upon reaction with an α-keto acid.[23,24] Nonetheless, tests with oxaloacetic acid, the α-keto acid equivalent of aspartic acid, revealed that ADC could not be reactivated by means of a reversed transamination (Figure 6.2) (results not published).

Suggestions for total turnover number improvement

If the low total turnover number of ADC is not enhanced in one way or another, the decarboxylation using this particular enzyme does not seem to be an economically feasible method for the conversion of L-aspartic acid to β-alanine. Since examples of non-enzymatic methods for the α-decarboxylation of L-aspartic acid and analogues such as L-glutamic acid are rather limited,[26,27] further investigation of the enzymatic conversion is still of interest.

It has been suggested in Chapter 3 that protein engineering could be a solution to this problem. A study in which protein engineering is applied to overcome substrate deactivation in pyruvoyl-dependent enzymes is to date not known to the author. Nonetheless, McElroy and Robertus[28] showed that at least the opposite is possible; with help of site-directed mutagenesis they were able to induce deactivation due to transamination in pyruvoyl-dependent *Lactobacillus* 30a histidine decarboxylase, where it was not present before.

Another option would be to apply protein engineering on an enzyme that has decarboxylase activity for compounds similar to L-aspartic acid and is dependent on non-covalently bound and thus replaceable pyridoxal-5`-phosphate (PLP) as cofactor. A potential candidate might be L-glutamate α-decarboxylase (GAD). Lammens *et al.*[23] showed that transamination induced deactivation of GAD could be reversed by the addition of small amounts of α-ketoglutarate or addition of fresh PLP.

6.2.3 Hydrolysis of L-arginine

For the purpose of hydrolyzing L-arginine to yield urea and L-ornithine, the application of the manganese dependent *Bacillus subtilis* arginase (EC 3.5.3.1) was

investigated using a continuously stirred membrane reactor setup. The results of this study are reported in Chapter 4.

Although *B. subtilis* arginase already has a remarkably high operational stability in the presence of 0.5 mM manganese chloride (TTN = $1.13 \cdot 10^8$), addition of dithionite significantly improved its stability (TTN > $1 \cdot 10^9$). Since addition of dithionite does not prevent Mn^{2+} oxidation, it might be speculated that not Mn^{2+} availability, but the reduced redox potential that results from dithionite addition has its effect on the operational stability of *B. subtilis* arginase.

Covalent immobilization on epoxy activated Sepabeads EC-EP resulted in enhanced thermostability of the enzyme. Since the operational stability of soluble *B. subtilis* arginase is already very good, the choice whether to use entrapment in a membrane reactor or covalent immobilization will mainly depend on the expertise and the equipment available at the production facility and should be subject of studies on the pilot scale.

B. subtilis arginase works optimally in alkaline environments and this is advantageous since both the substrate and product are basic compounds. This means that the pH of the L-arginine feedstock, obtained after acid precipitation of L-aspartic acid, needs to be adjusted with base, but subsequently remains in the alkaline region (Figure 6.5).

Before the reaction products, L-ornithine and urea, are isolated, it will probably be necessary to remove the manganese ions and any manganese dioxide present in the reaction solution. Manganese dioxide is a brown solid that is formed upon oxidation of Mn^{2+} and might hamper downstream processing. Mn^{2+}, which is prone to oxidation, can be deliberately oxidized to solid manganese oxide upon extensive contact with air, after which all manganese oxide and thus Mn^{2+} can be removed from the product solution by means of filtration (Figure 6.5).

The separation of L-ornithine and urea can be performed using ion-exchange chromatography, as described by Bommarius *et al.*[29] In this process L-ornithine is retained on a cation exchange column while urea passes, after which L-ornithine is eluted with an ammonia solution. The application of chromatographic techniques

might, however, be too costly in the production of nitrogen containing bulk chemicals. Therefore, the search for alternative separation techniques such as reactive extraction[30] or precipitation[31] should be one of the priorities in future research.

6.2.4 Decarboxylation of L-ornithine

Since enzymatic decarboxylation of L-ornithine to yield 1,4-diaminobutane occurs at mild reaction conditions and is highly selective, the application of *Trypanosoma brucei* ornithine decarboxylase (TbODC) (EC 4.1.1.17) was investigated as described in Chapter 5.

Unfortunately, in general ODCs are known to be unstable enzymes.[32-34] Testing different additives, it appeared that TbODC's stability was substantially improved upon addition of dithiothreitol (DTT), which not only has a stabilizing, but also an activating effect. Although DTT is already effective at a relatively low concentration of 5 mM, reuse of this costly additive might be desirable. In this case the use of immobilized DTT, *e.g.* on a poly-acrylate resin,[35] could be investigated.

For optimal TbODC performance, the pH of the reaction environment should be controlled at pH 8. Since both potential substrate inputs, the one from the selective CGP hydrolysis and the one from the L-arginine hydrolysis, will be highly alkaline, an initial pH adjustment with acid will be necessary. Additionally, since the pH will increase upon decarboxylation of L-ornithine, acid needs to be added during conversion to keep the pH of the reaction solution constant (Figure 6.5).

Immobilization of TbODC on Sepabeads EC-HFA led to an almost threefold improvement in operational stability (TTN = $1.95 \cdot 10^5$) as compared to the soluble enzyme (TTN = $6.90 \cdot 10^4$). Although this is a notable improvement, it is questionable if this threefold improvement in operational stability will make up for the costs that need to be invested for the immobilization itself. The TTN of soluble TbODC ($6.90 \cdot 10^4$), although vastly improved in the study described in Chapter 5, might not be sufficient for economically feasible application in the production of bulk chemicals.

The direct cause of the limited TTN of TbODC is not known. Deactivation observed during conversion could for example not be reversed upon addition of fresh pyridoxal-5'-phosphate and therefore does not seem to be related to a transamination side reaction as assumed for ADC. Possibly, the limited stability of TbODC is related to the strength of the intersubunit binding. Kitani *et al.*[36] reported that the ODC subunits are readily dissociated upon increased ionic strength. Therefore, the limited operational stability of TbODC might be subject of a mechanistic follow up study with special attention for intersubunit binding.

Alternatively, the application of resting cells with elevated ODC levels could be studied.[37] The cellular environment could be beneficial in terms of stability and whole cell biocatalysts could be economically more attractive in this case.

6.2.5 Enzyme immobilization on epoxy activated supports

On an industrial scale immobilization of enzymes might be beneficial, because this enables reuse and facilitates product recovery. Additionally, immobilization might enhance enzyme stability towards conditions encountered in industrial processes.[38]

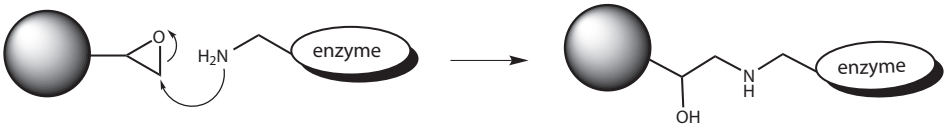
Epoxy activated supports

Among the different immobilization techniques, there are not so many that allow immobilization of large amounts of enzymes on solid supports, using easy-to-perform methodologies under mild conditions.[39] In this respect, epoxy supports are ideal immobilization supports. Upon simple incubation in presence of an epoxy support, enzymes are covalently bound to the supports upon reaction of their nucleophilic groups, *e.g.* amino, hydroxy, or thiol moieties, with the epoxy groups attached to the supports.[40]

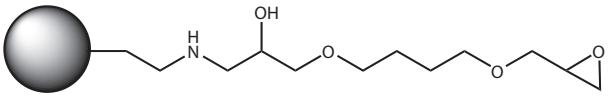
It is proposed that protein immobilization on epoxy supports follows a two-step mechanism: (i) first the enzyme is adsorbed to the surface of the support and (ii) subsequently the protein's nucleophilic groups (amino, thiol, or hydroxyl groups) react with the epoxy groups on the support to form covalent bonds (Figure 6.3). Based on this two-step mechanism, the hydrophobicity of the surfaces should play a role in the adsorption and subsequent binding of the enzymes on the supports

and thus in the performance of the immobilized enzyme.[39]

In this thesis, the immobilization of three enzymes, namely aspartate α-decarboxylase (ADC) (Chapter 3), arginase (Chapter 4) and ornithine decarboxylase (TbODC) (Chapter 5), on different commercially available and industrially relevant epoxy-activated supports is described: Eupergit (C 250L), Immobead (IB-150) and Sepabeads (EC-EP and EC-HFA).



EC-EP, IB-150 and C 250L



EC-HFA

Figure 6.3. Top. Mechanism of covalent enzyme immobilization on epoxy activated supports as demonstrated for supports with short spacer arms, such as EC-EP, C 250L and IB-150. **Below.** Schematic representation of EC-HFA with its relatively long, polar spacer arm.

All supports reported in this thesis are based on relatively hydrophobic cross-linked copolymer beads of methacrylamide (methacrylate esters in case of IB-150), and monomers with epoxy functionality.[42-44] The overall hydrophobicity of the supports is therefore determined by the spacer, which connects the epoxy functionality with the support. The spacer in EC-EP, C 250L and IB-150 is only two bond lengths long and therefore does not contribute much to the hydrophobicity of the support as a whole. EC-HFA has a spacer of fifteen bonds long with polar groups, which makes this support relatively hydrophilic (Figure 6.3).[45,46] Additional to the hydrophobicity of the support, the support-enzyme spacer also determines the degree of conformational freedom once the enzyme is bound to the support.[47,48]

Immobilization results

The enzymes were immobilized by incubating buffered enzyme solutions in

presence of the epoxy activated support (see Chapter 3, 4 and 5 for details). After immobilization, the immobilization yield and recovered activity were determined. Subsequently, the thermal and operational stability of immobilized enzyme was compared with soluble enzyme (Table 6.1).

Table 6.1. Overview of the immobilization results reported in this thesis.

Enzyme	Support	Type	Immobilization yield (%)[b]	Recovered activity (%)[c]	Thermostability enhancement[d]	TTN improvement upon immobilization (%)[e]
ADC	Sepabeads	EC-HFA	88	89	-	60
	”	EC-EP	95	80	0	60
Arginase	”	EC-HFA	98	84	0	n.d.[a]
	”	EC-EP	100	75	+	0
	Eupergit	C 250L	99	44	+	n.d.
ODC	Sepabeads	EC-HFA	100	17	0	180
	”	EC-EP	100	35	-	n.d.
	Immobead	IB-150	100	20	-	n.d.

[a] Not determined; [b] Immobilization yields give the amount of enzyme remaining on the support after washing; [c] Recovered activity is the activity relative to the expected activity, taking into account the amount of enzyme bound to the support; [d] Thermostability enhancement is relative to soluble enzyme: +: improved thermostability; -: lowered thermostability; 0: no change in thermostability; [e] Total turnover number (TTN) improvement is relative to the TTN of soluble enzyme.

Immobilization yields. Following the standard protocol, buffers with low ionic strength (5 mM) were used for immobilization on EC-HFA whereas buffers with high ionic strength (0.5 M) were used for the other relatively hydrophobic supports. In the latter case, increased ionic strengths would be necessary to force hydrophobic adsorption, facilitating subsequent covalent binding.[39,48] Since all immobilizations were performed with high immobilization yields, the ionic strengths suggested in the standard protocols seemed to work well (Table 6.1).

Recovered activities. When comparing the recovered activities for each enzyme individually, it is notable that the activities obtained upon immobilization on C 250L or IB-150 are significantly lower than those obtained with EC-EP. Considering their physical properties, these supports appear to be quite similar. Nonetheless, EC-EP differs from C 250L and IB-150 in epoxy group density; the densities are

respectively 106, 300 and 485 mmol g^{-1} wet support.[49,50] As a result of their relatively high epoxy group density, immobilization on C 250L and IB-150 may lead to an increased amount of covalent multipoint attachment of the enzyme to the support, thereby increasing the risk of altering the enzyme conformation and distorting its active site as well as hampering the conformational changes needed for catalysis.[50] For ADC and arginase, the support with the long spacer (EC-HFA) gives slightly better recovered activities than EC-EP; for ODC the situation is just opposite.

Thermostability. The effects of the different supports on thermostability are very diverse for the different enzymes (Table 6.1). While immobilization of ADC on EC-HFA resulted in a decrease in thermostability and immobilization on EC-EP left the thermostability unaffected, immobilization of TbODC on the same supports led to the opposite results. In case of arginase, the thermostability was enhanced upon immobilization on EC-EP, while EC-HFA did not affect the thermostability of the enzyme.

Taking into account the length and the hydrophobicity of the support's spacer arms (Figure 6.4), the differences in thermostability might be explained by the following: i. the orientation of enzyme binding. While binding on EC-EP, C 250L and IB-150 would occur *via* the most hydrophobic areas, binding to EC-HFA would occur through its more hydrophilic areas;[45] ii. the different possibilities of multipoint covalent attachment as result of the spacer arm length. Short spacer arms might provide rigidity, while longer spacer arms allow enzyme molecules to distribute and accommodate themselves sufficiently distant from the support surface;[51] iii. the interaction of the enzyme with the support surface upon heat-induced conformational changes. Internal hydrophobic pockets might interact with the surface of hydrophobic supports, distorting the active center.[48]

Operational stability. Covalent immobilization of ADC and ODC led to enhancements in their total turnover number (TTN) by 60 and 180%, respectively (Table 6.1). This enhancement in TTN is probably the result of the protection that the supports offer against shear stress due to prolonged stirring. Although

immobilization of arginase lead to an increased thermal stability, its already impressive TTN was not further improved upon immobilization.

Feasibility of enzyme immobilization

Besides a possible enhancement in both thermal and operational stability, covalent immobilization of enzymes on spherical beads allows the application of a packed-bed reactor in which higher degrees of conversion or higher space-time yields can be achieved than in a CSTR.[52]

Nonetheless, if the costs for the immobilization materials are too high with respect to the price of the product, immobilization would not be useful. Using the TTNs determined for the different enzymes, the enzyme and, if immobilized, immobilization support costs were calculated for each bioconversion that was investigated in this thesis (Figure 6.4). When possible, the costs were calculated for three situations: soluble enzyme before optimization, soluble enzyme under optimized conditions and immobilized enzyme under optimized conditions.

Looking at the results for ADC, it becomes immediately clear that the enzyme cost price (627 € $kmol^{-1}$) is about three times higher than the selling price of the envisioned end-product, acrylamide (213 € $kmol^{-1}$).

Since the improvement in TTN, which is already low, is limited upon covalent immobilization of ADC, the combined costs upon immobilization are considerably high with 2,192 € $kmol^{-1}$ product. To bring down both enzyme and immobilization material costs in the enzymatic α-decarboxylation of L-aspartate, the TTN of ADC needs to be drastically improved.

Looking at the enzyme costs related to the hydrolysis of L-arginine, the costs for the non-optimized reaction are already low with 0.027 € $kmol^{-1}$. Upon optimization the costs were decreased with almost a factor 10. Upon immobilization the total material costs are 0.017 € $kmol^{-1}$ L-arginine hydrolyzed. Since arginase has a high TTN it would be economically attractive to recycle this enzyme.

As described in Chapter 4, the application of soluble arginase in a membrane reactor could be one possible technique to enable its reuse. However, the capital costs of a membrane reactor are rather high. Another option would be

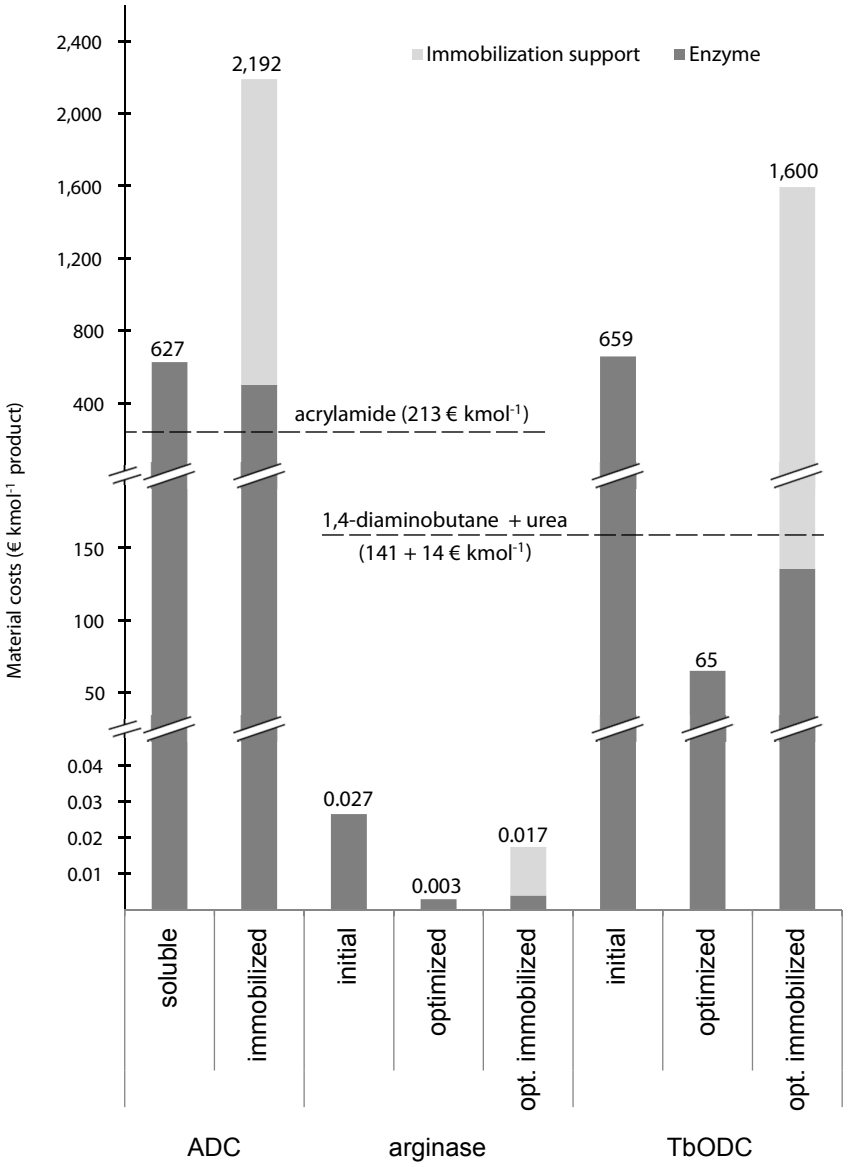


Figure 6.4. Representation of enzyme and immobilization materials costs involved per kmol product formed, in case of immobilized or soluble enzyme. The costs were calculated on basis of an enzyme cost price of 100 € kg^{-1}, support loading of 31 g enzyme kg^{-1} dry support and recycling was assumed until the enzymes were completely deactivated. The cost prices are presented in € $kmol^{-1}$ instead of the more commonly used € ton^{-1}, which enables the direct comparison between the combined enzyme and immobilization support cost price per equivalent substrate converted with the price per equivalent end product throughout the different conversions.

immobilization on epoxy supports, which in terms of material costs would be relatively inexpensive. Additionally, arginase immobilized in spherical beads allows the application of a packed-bed reactor in which higher degrees of conversion or higher space-time yields can be achieved than in a CSTR.[52]

In case of TbODC, the enzyme costs under non-optimized conditions are with 659 € $kmol^{-1}$ of decarboxylated L-ornithine too costly for the production of one kmol 1,4-diaminobutane and urea for the combined price of 155 € $kmol^{-1}$. Addition of dithiothreitol lowered the enzyme costs to 65 € $kmol^{-1}$ converted. This is below the price of one kmol of 1,4-diaminobutane and urea, but one must consider that these are only the enzyme costs (*e.g.* the costs of DTT are excluded). A larger margin will be necessary to cover the costs upstream and especially downstream of the L-ornithine conversion. Immobilization of ODC at a combined cost price of 1600 € $kmol^{-1}$ is of course out of the question. As with ADC, the TTN of ODC needs to be further improved, possibly as described in sections 6.2.2 and 6.2.4, to make the enzymatic decarboxylation of ODC economically feasible.

6.2.6 From *β*-alanine towards acrylamide

As mentioned throughout this thesis, *β*-alanine is envisioned as an intermediate in the L-aspartic acid based, and thus biobased, production of acrylamide. The envisioned route from *β*-alanine towards acrylamide was not studied extensively. However, some promising preliminary results were obtained and it was decided to continue this research as part of another research project. Due to issues of confidentiality the preliminary results obtained within the N-ergy project cannot be disclosed.

Information about the feasibility of this conversion would be of key importance in the decision whether to put more emphasis on the production of PASP or products derived from L-aspartic acid.

6.2.7 Process impression

Since the research presented in this thesis was mainly focused on individual reactions, the process design presented in Figure 6.5 is limited to an impression of the combined conversions including the crucial parameters and the most obvious unit operations. The route based on acid catalyzed hydrolysis of CGP is depicted with dotted lines, while the route based on base catalyzed hydrolysis is depicted with solid lines.

In a follow up study some crucial points in the process design should be investigated. First of all, the process route from CGP to nitrogen containing chemicals is built up from reactions that require environments with different pH values. As a consequence this requires pH adjustments with most reaction steps, which would involve addition of large quantities of mineral acid and base and would subsequently result in large amounts of salts. It would therefore be useful to determine the pH values at which the reactions can run at sufficient rate and selectivity, leading to an optimum with respect to the degree of pH adjustment and productivity.

Secondly, the isolation and reuse of the different additives, required in the different reaction steps, should be investigated. Reuse can be attractive from both an economical and environmental standpoint. Furthermore, the isolation of additives might be required to overcome interference downstream of the reaction. As mentioned in section 6.2.4 the DTT required for the stability of TbODC could be used in an immobilized form such as on a polyacrylate resin.[35] Furthermore, one should for example think about recycling the methanol required in PASP precipitation, the regeneration of oxidized Mn^{2+} and DTN for enzymatic L-arginine hydrolysis and the PLP required for L-ornithine decarboxylation.

Thirdly, one should consider recycling of the process heat necessary for the different reaction steps. Especially the hydrolysis of CGP requires a lot of heat that could subsequently be used for reactions that require lower temperatures.

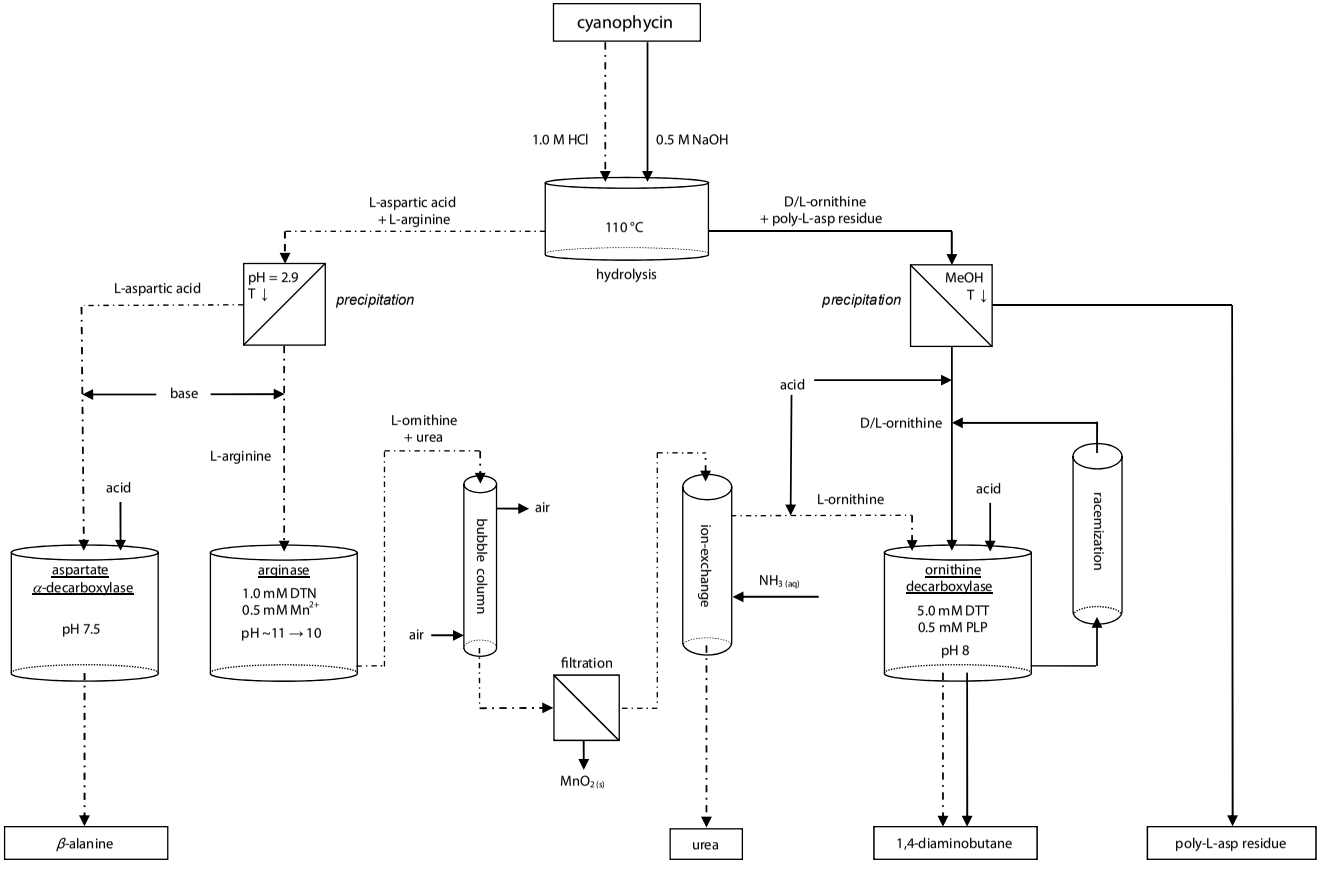


Figure 6.5. Impression of the process routes that could lead from CGP towards nitrogen containing chemicals. The dotted lines represents the route based on acid catalyzed hydrolysis of CGP, while the solid lines represent the route based on base catalyzed hydrolysis of CGP.

6.3 General recommendations in view of the biorefinery approach

In the years that we were working on this subject some general recommendations for further research were identified. These recommendations involve methods for the selective isolation of amino acids from complex residual biomass streams and a method for the isolation of nitrogen containing chemicals from relatively simple product mixtures in aqueous environments.

6.3.1 Methods for the isolation of amino acids from complex biomass streams

As explained in the General introduction (Chapter 1), methods are required which enable isolation of the desired amino acids from complex biomass streams for their application in the production of nitrogen functionalized chemicals. However, since protein hydrolysates are complex, dilute mixtures of many amino acids, this is not a straightforward task.

The work described in this thesis is based on the elegant isolation of L-aspartic acid and L-arginine from complex biomass streams via the formation of CGP. Nonetheless, to be able to make use of all functionalized compounds present in residual biomass, additional isolation methods need to be developed. In this paragraph the use of CGP with alternative amino acid compositions and siderophores are addressed.

Alternative cyanophycin compositions

In general, CGP is known to consist of equimolar amounts of L-aspartic acid and L-arginine. However, recently alternative CGP compositions have been reported: 4%-18% lysine, 20% citrulline and 8% ornithine instead of arginine (Figure 6.6).[53-55] The difference in amino acid content is related to the use of different host organisms for the CGP synthetase enzyme and the omission and/or addition of amino acids in the growth medium.[54]

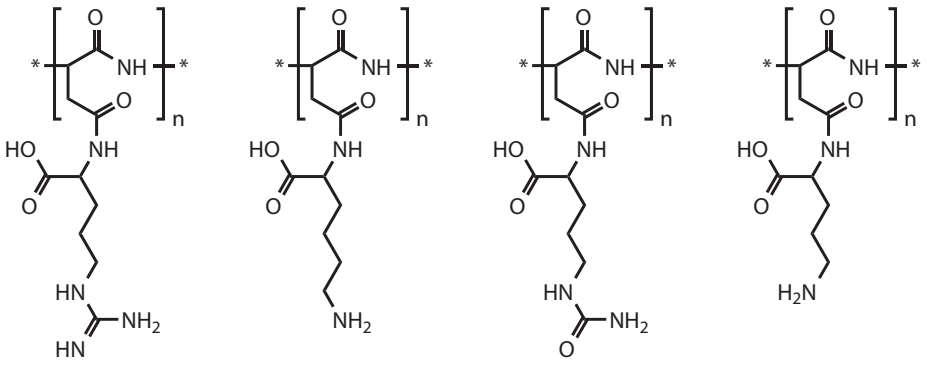


Figure 6.6. Cyanophycin molecules built up from different amino acids.

Although citrulline and ornithine are not abundantly present in proteins, these results show that there is potential for the development of CGP polymers with alternative amino acid contents. This means that CGP could potentially be applied for the specific isolation of other amino acids than L-aspartic acid and L-arginine which broadens its application in the biorefinery approach.

Further research on this topic should be focused on identification of the factors involved in determination of CGP's composition and possibilities to incorporate a broader spectrum of amino acids.

Siderophores

An alternative approach for the isolation of amino acids from residual biomass, would be through their incorporation in siderophores. Siderophores (from the Greek: "iron carriers") are defined as relatively low molecular weight, ferric ion specific chelating agents, which are naturally synthesized by bacteria and fungi growing under low iron stress. The role of siderophores in such situation is to scavenge iron from the environment and to make the mineral available to the microbial cell.[56]

The structures of siderophores are distinguished by their iron binding functionality. The most common functionalities are catecholates, hydroxymates and

hydroxycarboxylates (Figure 6.7, respectively).[57] Depending on the overall structure of the siderophore a hexadentate, tetradentate or bidentate binding with iron is possible.

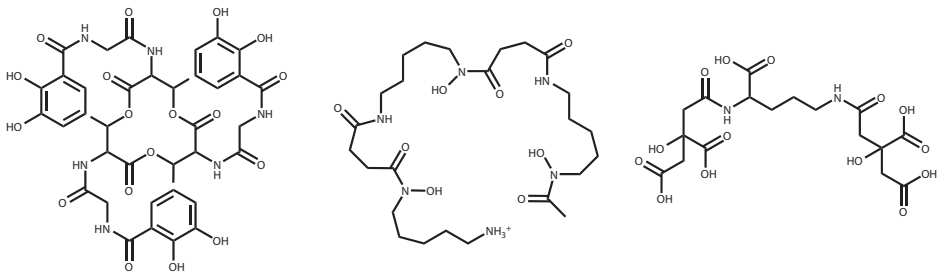


Figure 6.7. Examples of siderophore structures containing, respectively, catecholate, hydroxymate and hydroxycarboxylate functionalities.[57] All three siderophores depicted in this figure can bind iron in a hexadentate fashion (Figure 6.8).

It has been recognized that amino acids are particularly good sources of nitrogen in the production of siderophores.[58] Díaz de Villegas *et al.*[59] reported that addition of glutamic acid induced siderophores productivity. Upon screening different amino acids, Albesa *et al.*[60] found that all, except for methionine, histidine, serine and phenylalanine, led to higher siderophore yields when compared to inorganic nitrogen sources. The highest yields were obtained using proline and arginine, being, respectively, 0.41 and 0.35 g g^{-1} dry cell weight (*Pseudomonas fluorescens* ATCC 13525, pH 7).

A challenge would be to find a feasible method for the isolation of the generally water soluble siderophores from the reaction mixture. There are several protocols that describe their extraction using organic solvent subsequent to acidification.[60-62] An alternative method for the isolation of siderophores would involve affinity chromatography. Inspired by the application of immobilized metal affinity chromatography (IMAC) for the isolation of recombinant histidine-tagged (His-tag) proteins, Braich *et al.*[63] developed an affinity purification technique for

siderophores based on their affinity with metal ions. It is, however, debatable if such technique would be feasible in the production of bulk chemicals.

Once produced and isolated, hydrolysis of the siderophore structures will be necessary to allow subsequent conversion to nitrogen functionalized chemicals. A possibility would be the use of esterases. Abergel *et al.*[64] reported the application of *E. coli* ferric enterobactin esterase (Fes) in the specific hydrolysis of the trilactone backbone of enterobactin, leading to the formation of 2,3-dihydroxybenzoylserine monomers (Figure 6.8).

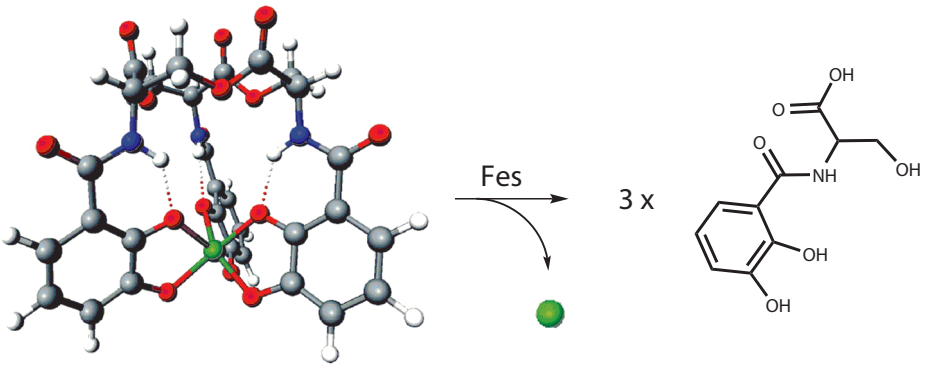


Figure 6.8. Schematic representation of the enzymatic hydrolysis of enterobactin by *E. coli* ferric enterobactin esterase (Fes). Adapted from Raymond *et al.*[64]

Other enzymes known to be capable of selective hydrolysis of siderophores are limited; two other examples of enzymes capable of hydrolyzing siderophores are encoded by the *iroD* and *iroE* genes.[65] Alternatively, siderophores might be selectively hydrolyzed using acid or base under controlled conditions.

6.3.2 Isolation of amino compounds from aqueous environments

The amino acids obtained from biomass residues and subsequently converted in biorefineries will initially be surrounded by aqueous environments. Due to the polarity of most of the amino acids and compounds originating therefrom in combination with the possible preference for biocatalytic conversions, the

presence of water does not necessarily pose problems during the conversion steps. Nonetheless, if a conversion requires a non-aqueous environment or when required for formulation of the final product, isolation of the product from residual substrate or co-products, preceding dewatering, will be essential.

As stated in the General introduction (Chapter 1), the use of ion-exchange chromatography (IEC) techniques would be too costly in the production of bulk chemicals and therefore alternatives need to be developed. Reactive extraction would be a suitable method for the problem described above.

Reactive extraction

As shortly discussed in Chapter 1, reactive extraction is a promising method for the selective extraction of amino compounds from aqueous environments. Cascaval *et al.*[30] investigated the application of this technique in the separation of amino acids.

Via the pH of the solution and thus the protonation state, it can be determined which amino acid will react with the extractant, *e.g.* di-(2-ethylhexyl)phosphoric acid, by means of an interfacial chemical reaction of the ionexchange type and thereby allowing its extraction from the aqueous phase (Figure 6.9).

It would be worthwhile to investigate the application of this technique with amino compounds other than amino acids. Ideally, the method would be designed in such way that both the organic solvent and extractant could be recycled.

6.4 N-ergy project

As mentioned in the introduction, the research described in this thesis is part of the N-ergy project. The aim of the N-ergy project was to develop a process for the integrated production of ethanol and nitrogen containing chemicals from amino acid rich residues. N-ergy involved three PhD research projects hosted by Wageningen University and the Westfälische Wilhelms-Universität Münster and the N-ergy project as a whole was subjected to a life cycle assessment study (LCA) conducted by the Energy research Centre of the Netherlands (ECN).

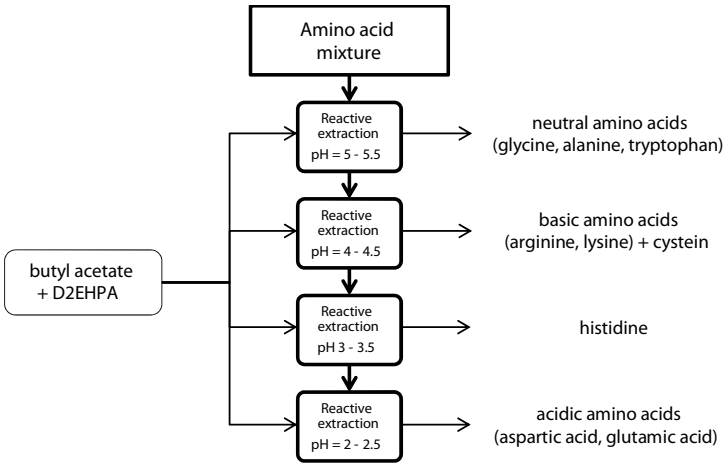


Figure 6.9. Schematic overview of the application of reactive extraction in the separation of amino acids. Adapted from Cascaval *et al.*[30] D2EHPA = di-(2-ethylhexyl)phosphoric acid.

Within the project strains of *Saccharomyces cerevisiae* and *Rhizopus oryzae* were developed that were able to incorporate nitrogen in CGP. The highest productivity achieved resulted in a cellular CGP content of 21% (w/w) in *Saccharomyces cerevisiae*.[66] The CGP content and the stability of the developed strains need to be further improved. Furthermore, a simple and cost effective method was developed for the isolation of CGP from the production organisms.[66] Additionally, as reported in this thesis, methods were developed for the hydrolysis of CGP and their subsequent conversion to nitrogen containing chemicals such as 1,4-diaminobutane.

A techno-economical assessment conducted by ECN revealed that coproduction of ethanol, nitrogen containing chemicals and electricity has economical potential when CGP could be produced at levels of 50% (w/w) and a conversion efficiency of 90% in further optimized chemo-enzymatic conversions would be achieved. Additionally, it was concluded that this process will be more likely to be successful when applied to an existing ethanol producing site. From the LCA it was concluded that the envisioned process has an environmental performance which is superior when compared to the petrochemical production of nitrogen containing chemicals and an individual ethanol plant.

6.5 General conclusions

Aim of the research presented in this thesis was to explore and optimize the different (bio)conversion steps involved in the envisioned route from cyanophycin (CGP) towards nitrogen containing chemicals (Figure 6.1).

Acid catalyzed hydrolysis of CGP yields both L-aspartic acid and L-arginine at comparable rates and is therefore suitable for complete hydrolysis. Upon base catalyzed hydrolysis, the rate of L-arginine liberation is overall significantly higher than that of L-aspartic acid, which enables the production of a CGP residue with polyaspartic acid functionality.

In the α-decarboxylation of L-aspartic acid, aspartate α-decarboxylase (ADC) has several advantages, such as its perfect selectivity, lack of product inhibition and high thermostability. However, ADC's low operational stability needs to be addressed before large scale applications become feasible.

In view of its application in the hydrolysis of L-arginine, *Bacillus subtilis* arginase was successfully stabilized and immobilized. Addition of sodium dithionite to the substrate solution resulted in very good stabilization, while immobilization of *B. subtilis* arginase on Sepabeads EC-EP resulted in an enhanced thermostability.

In its application in the conversion of L-ornithine in 1,4-diaminobutane, the stability and activity of *Trypanosoma brucei* ornithine decarboxylase (TbODC) was substantially improved upon addition of dithiothreitol. Although TbODC's immobilization on Sepabeads EC-HFA led to an almost threefold improvement in operational stability, additional research to improve the operational stability of TbODC is recommended.

Of the three enzymes studied in this thesis only reuse of *B. subtilis* arginase by immobilization on epoxy supports would be feasible in terms of material costs. In view of the overall process design, three subjects for further study were identified: (i) minimization of pH adjustments, (ii) isolation and reuse of additives and (iii) recycling of process heat. In view of the biorefinery approach in general, investigation of additional methods to selectively isolate amino acids from complex

biomass mixtures and methods to isolate amino compounds from aqueous environments is encouraged.

In conclusion, CGP appears to be a valuable molecule in the production of nitrogen containing chemicals from residual biomass streams. This thesis provides routes from CGP towards nitrogen containing chemicals, indicating the strengths of these routes and emphasizing where further optimization is required.

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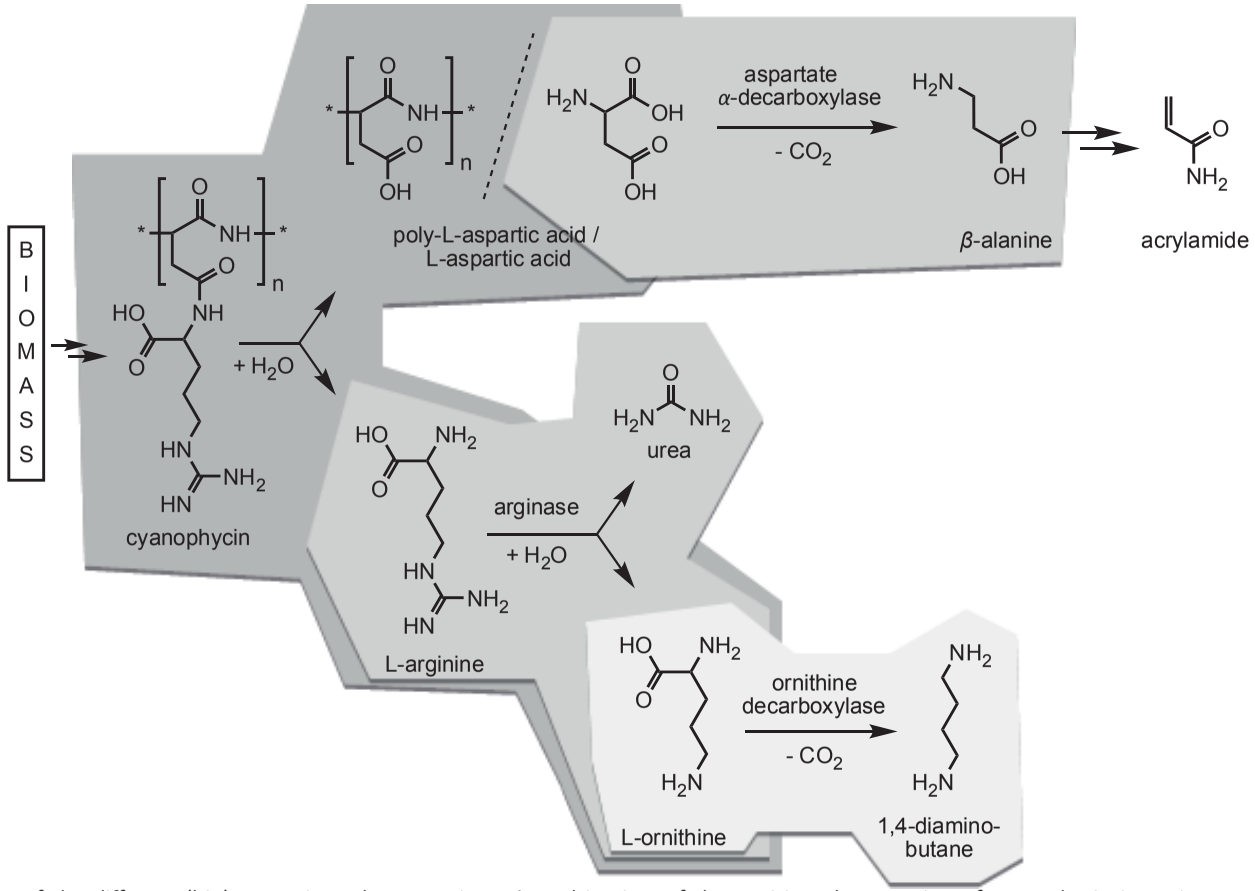
Summary

Currently nitrogen containing bulk chemicals are produced from naphtha. However, as explained in **Chapter 1** it would be more energy efficient, less capital intensive and eventually more economical to start from functionalized compounds that already have nitrogen incorporated, such as amino acids. Recent developments have made it possible to specifically fix and thus isolate L-aspartic acid and L-arginine from agricultural waste streams in the form of the polypeptide cyanophycin (CGP). The aim of the research presented in this thesis was to explore and optimize the different (bio)conversion steps involved in the envisioned route from CGP towards nitrogen containing chemicals.

In **Chapter 2** a study is presented on the acid and base catalyzed hydrolysis of CGP. Acid catalyzed hydrolysis of CGP yields both L-aspartic acid and L-arginine at comparable rates and is therefore suitable for complete hydrolysis. It was observed that upon base catalyzed hydrolysis of CGP the rate of L-arginine liberation is overall significantly higher than that of L-aspartic acid, which enables selective hydrolysis of CGP. Over time L-aspartic acid liberation and thus hydrolysis of the polyaspartic acid backbone of CGP will occur and therefore a choice needs to be made between the degree of polyaspartic acid functionality and the polymer length of the CGP residue.

The results of a study on the applicability of *Escherichia coli* L-aspartate α-decarboxylase (ADC) for the production of β-alanine from L-aspartic acid are presented in **Chapter 3**. The α-decarboxylation of L-aspartic acid using ADC has several advantages, such as its high selectivity, ease of production, lack of product inhibition and high thermostability under storage conditions. In addition, covalent immobilization of ADC on Sepabeads EC-EP epoxy supports is straightforward and makes the enzyme slightly more stable. However, ADC's low operational stability, probably caused by irreversible transamination of its catalytically essential pyruvoyl group, needs to be addressed before large scale applications become feasible.

The results of a study on stabilization and immobilization of *Bacillus subtilis*



Overview of the different (bio)conversions that were investigated in view of the envisioned conversion of cyanophycin into nitrogen containing chemicals. The different shades of grey represent the different conversions addressed in this thesis.

arginase are presented in **Chapter 4**. In view of its application in the hydrolysis of L-arginine to L-ornithine and urea, *B. subtilis* arginase was successfully stabilized and immobilized. Initial pH of the substrate solution, addition of aspartic acid and reducing agents all had an effect on the operational stability of *B. subtilis* arginase. A remarkably good operational stability (total turnover number, TTN = $1.13 \cdot 10^8$) was observed at the pH of arginine free base (pH 11.0), which was further improved with the addition of sodium dithionite (TTN > $1 \cdot 10^9$). Furthermore, *B. subtilis* arginase was successfully immobilized on three commercially available epoxy-activated supports. Immobilization on Sepabeads EC-EP was most successful resulting in a recovered activity of 75% and enhanced thermostability.

In **Chapter 5** a study on *Trypanosoma brucei* ornithine decarboxylase (TbODC) is described. The stabilization and immobilization of TbODC were investigated for its application in the conversion of L-ornithine to 1,4-diaminobutane. The stability of TbODC is substantially improved upon addition of dithiothreitol (DTT), which not only has a stabilizing, but also an activating effect. For optimal performance of TbODC, the pH should be controlled at pH 8 and the ionic strength should be kept to a minimum. Furthermore, TbODC shows an optimum in productivity at 40°C with respect to its temperature dependent activity and stability. Although TbODC's immobilization on Sepabeads EC-HFA leads to an almost threefold improvement in operational stability, additional research to improve the operational stability of TbODC is recommended.

The impact of the results described in the previous chapters on the overall route from CGP to nitrogen containing chemicals is discussed in **Chapter 6**. Of the three enzymes studied in this thesis only reuse of *B. subtilis* arginase by immobilization on epoxy supports would be feasible in terms of material costs. In view of the overall process design, three subjects for further study were identified: (i) minimization of pH adjustments, (ii) isolation and reuse of additives and (iii) recycling of process heat. In view of the biorefinery approach in general, investigation of additional methods to selectively isolate amino acids from complex biomass mixtures and methods to isolate amino compounds from aqueous

environments is encouraged.

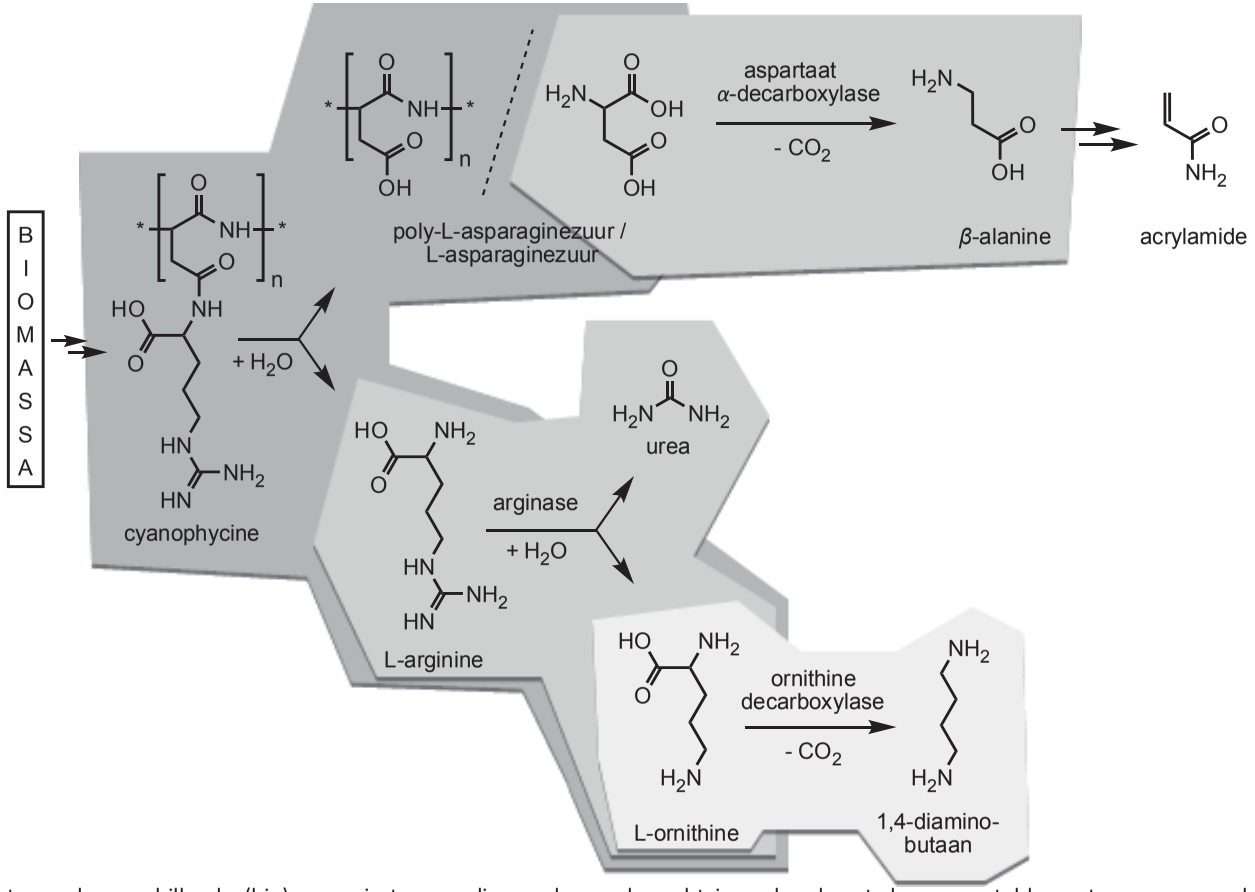
In conclusion, CGP appears to be a valuable molecule in the production of nitrogen containing chemicals from residual biomass streams. This thesis provides routes from CGP towards nitrogen containing chemicals, indicating the strengths of these routes and emphasizing where further optimization is required.

Samenvatting

Vandaag de dag worden stikstofhoudende bulkchemicaliën veelal geproduceerd uit nafta. Het zou echter, zoals omschreven in **Hoofdstuk 1**, energie-efficiënter, minder kapitaalinvestering vergend en uiteindelijk economischer zijn om te beginnen met grondstoffen die al stikstof bevatten, zoals aminozuren. Recente ontwikkelingen maken het mogelijk om specifiek L-asparaginezuur en L-arginine te binden in de vorm van het polypeptide cyanophycine (CGP) en daarmee te isoleren uit agrarische reststromen. Het doel van het onderzoek dat gepresenteerd wordt in dit proefschrift was om de verschillende (bio)conversiestappen van de beoogde route van CGP naar stikstofhoudende chemicaliën te bestuderen en te optimaliseren.

In **Hoofdstuk 2** wordt een studie naar de zuur en base gekatalyseerde hydrolyse van CGP gepresenteerd. Bij zuur gekatalyseerde hydrolyse van CGP komen L-asparaginezuur en L-arginine met vergelijkbare snelheden vrij en daarom is zuur gekatalyseerde hydrolyse geschikt voor complete hydrolyse van CGP. Bij base gekatalyseerde hydrolyse van CGP bleek L-arginine met een significant hogere snelheid te worden vrijgemaakt dan L-asparaginezuur, wat selectieve hydrolyse mogelijk maakt. Na verloop van tijd wordt dus ook de poly-asparaginezuurstructuur van CGP gehydrolyseerd en er zal daarom een keuze gemaakt moeten worden tussen de mate van poly-asparaginezuurfunctionaliteit en de polymeerlengte van het CGP residu.

De resultaten van een studie naar de toepasbaarheid van *Escherichia coli* L-aspartaat α-decarboxylase (ADC) in de productie van β-alanine vanuit L-asparaginezuur worden weergegeven in **Hoofdstuk 3**. Het toepassen van ADC in de α-decarboxylering van L-asparaginezuur heeft verschillende voordelen, zoals een hoge selectiviteit, het gemak van productie, het ontbreken van product inhibitie en een hoge thermostabiliteit in afwezigheid van substraat. Daarbij is covalente immobilisatie van ADC op Sepabeads EC-EP eenvoudig en resulteert het in een hogere stabiliteit. Echter, ADC's lage operationele stabiliteit, waarschijnlijk het gevolg van een onomkeerbare transaminering van de katalytisch essentiële



Overzicht van de verschillende (bio)conversiestappen die werden onderzocht in verband met de voorgestelde routes van cyanophycine naar stikstofhoudende chemicaliën. De grijstinten onderscheiden de verschillende conversiestappen die in dit proefschrift worden omschreven.

pyruvoylgroep, zal moeten worden aangepakt voordat toepassing van ADC op grote schaal haalbaar wordt.

De resultaten van een studie naar de stabilisatie en immobilisatie van *Bacillus subtilis* arginase worden gepresenteerd in **Hoofdstuk 4**. Met het oog op de toepassing in de hydrolyse van L-arginine naar L-ornithine en urea, werd *B. subtilis* arginase succesvol gestabiliseerd en geïmmobiliseerd. De beginwaarde van de pH van de substraatoplossing, toevoeging van L-asparaginezuur en reducerende stoffen hadden allemaal invloed op de operationele stabiliteit van *B. subtilis* arginase. Een opmerkelijk hoge operationele stabiliteit (turnovernummer, TTN = $1.13 \cdot 10^8$) werd geobserveerd bij de pH van de vrije base van arginine (pH 11.0). Deze operationele stabiliteit werd nog verder verbeterd door toevoeging van natrium diothionite (TTN > $1 \cdot 10^9$). Daarnaast werd *B. subtilis* arginase succesvol geïmmobiliseerd op drie commercieel verkrijgbare, epoxy-geactiveerde dragers. Immobilisatie op Sepabeads EC-EP was het meest succesvol, en resulteerde in een resterende activiteit van 75% en een toename van de thermostabiliteit.

In **Hoofdstuk 5** wordt een studie naar *Trypanosoma brucei* ornithine decarboxylase (TbODC) omschreven. De stabilisatie en immobilisatie van TbODC werden onderzocht voor toepassing in de omzetting van L-ornithine naar 1,4-diaminobutaan. De stabiliteit van TbODC werd substantieel verbeterd door toevoeging van dithiothreitol (DTT), wat niet alleen een stabiliserend, maar ook een activerend effect heeft. Voor het optimaal presteren van TbODC moet de pH constant worden gehouden op pH 8 en de ionsterkte tot een minimum worden beperkt. Verder presteert TbODC optimaal bij 40°C met betrekking tot zijn temperatuur afhankelijke activiteit en stabiliteit. Hoewel immobilisatie op Sepabeads EC-HFA leidt tot een bijna drievoudige verbetering van de operationele stabiliteit, wordt aanvullend onderzoek naar de verbetering van TbODC's stabiliteit aangeraden.

De impact van de in de eerdere hoofdstukken omschreven resultaten op de complete route van CGP naar stikstofhoudende chemicaliën wordt bediscussieerd in **Hoofdstuk 6**. Van de drie in dit proefschrift bestudeerde enzymen is alleen

hergebruik van *B. subtilis* arginase door middel van immobilisatie op epoxy-geactiveerde dragers economisch haalbaar met betrekking tot de materiaalkosten. Met oog op het totale procesontwerp, werden drie onderwerpen voor verdere studie geïdentificeerd: (i) minimalisatie van het aantal pH aanpassingen, (ii) isolatie en hergebruik van additieven en (iii) hergebruik van proceswarmte. Met het oog op de bioraffinage aanpak in het algemeen, wordt het onderzoek naar additionele methoden voor de selectieve isolatie van aminozuren uit complexe biomassamengsels en methodes voor de isolatie van aminocomponenten uit waterige milieus aangemoedigd.

Concluderend kan worden gesteld dat CGP een waardevol molecuul is voor de productie van stikstofhoudende chemicaliën uit agrarische reststromen. Dit proefschrift beschrijft de routes van CGP naar stikstofhoudende chemicaliën, waarbij de sterke punten van deze routes worden aangegeven en daarnaast wordt benadrukt waar verdere verbeteringen nodig zijn.

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Curriculum vitae

Paul Mathijs Könst was born on November 5, 1981, in Heemstede, the Netherlands. In 2000 he obtained his gymnasium diploma at the Dr. Mollercollege in Waalwijk, after which he began to study Biotechnology at Wageningen University.

While he specialized in bioprocess engineering, he showed a great interest for the field of biocatalysis. He did his minor thesis at the Bioprocess Engineering Group, Wageningen University, where he investigated α-tocopherol production in the micro-algae *Dunaliella tertiolecta*. For his major thesis he investigated the stereoselective hydrolysis of epimeric 1-oxaspiro[2.5]octanes by yeast epoxide hydrolase at the Laboratory of Organic Chemistry, Wageningen University. After an internship at the Biological Chemistry Group, University of Toronto, Canada, where he worked on cross-linked bis-hemoglobins, he obtained his Master's degree at the end of 2005.

In January 2006, he started his PhD research on the production of nitrogen containing chemicals from cyanophycin at the Valorisation of Plant Production Chains Group and the Laboratory of Organic Chemistry, both at Wageningen University. The results of this work are presented in this thesis.

Since October 2010, he is employed as a postdoctoral researcher at the Biocatalysis and Organic Chemistry Group, Delft University of Technology.

List of publications

P.M. Könst, M.C.R. Franssen, E.L. Scott and J.P.M. Sanders, "Stabilization and immobilization of *Trypanosoma brucei* ornithine decarboxylase for the biobased production of 1,4-diaminobutane", *Green Chemistry*, 2011, **13**, 1167-1174.

P.M. Könst, E.L. Scott, M.C.R. Franssen and J.P.M. Sanders, "Acid and base catalyzed hydrolysis of cyanophycin for the biobased production of nitrogen containing chemicals", *Journal of Biobased Materials and Bioenergy*, 2011, **5**, 102-108.

P.M. Könst, P.M.C.C.D. Turras, M.C.R. Franssen, E.L. Scott and J.P.M. Sanders, "Stabilized and immobilized *Bacillus subtilis* arginase for the biobased production of nitrogen-containing chemicals", *Advanced Synthesis and Catalysis*, 2010, **352**, 1493-1502.

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C.A.G.M. Weijers, **P.M. Könst**, M.C.R. Franssen and E.J.R. Südholter, "Stereoselectivity and substrate specificity in the kinetic resolution of methyl-substituted 1-oxaspiro[2.5]octanes by *Rhodotorula glutinis* epoxide hydrolase", *Organic and Biomolecular Chemistry*, 2007, **5**, 3106-3114.

Overview of completed training activities

Discipline specific activities

Biocat2006, Hamburg, Germany, 2006

6th European Motor Biofuels Forum 2008, Rotterdam, 2008

IXth Netherlands' Catalysis and Chemistry Conference, Noordwijkerhout, 2008

Renewable Resources and Biorefineries 4, Rotterdam, 2008

NWO-OS/SR/BC meeting, Lunteren, 2008, 2009, 2010

Netherlands' Process Technology Symposium 8, Veldhoven, 2008

Advanced Course Downstream Processing, BSDL, Delft, 2008

Biotrans 2009, Bern, Switzerland, 2009

Advanced Course Biocatalysis, BSDL, Delft, 2011

General courses

Afstudeervak organiseren en begeleiden, WGS, Wageningen, 2006

PhD Competence Assessment, WGS, Wageningen, 2007

Project and Time Management, WGS, Wageningen, 2007

Scientific Writing, WGS, Wageningen, 2008

Basic Statistics, SENSE, Wageningen, 2008

Mini symposium 'Bibliometrics at Wageningen UR', Wageningen, 2009

NWO Talent Day, Utrecht, 2009, 2010

Optionals

Preparation of research proposal, 2006

Open day Biotechnology, Wageningen University, Wageningen, 2007, 2008

Organization Biotechnology Excursion France, 2008

Biotechnology Excursion France, 2008

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