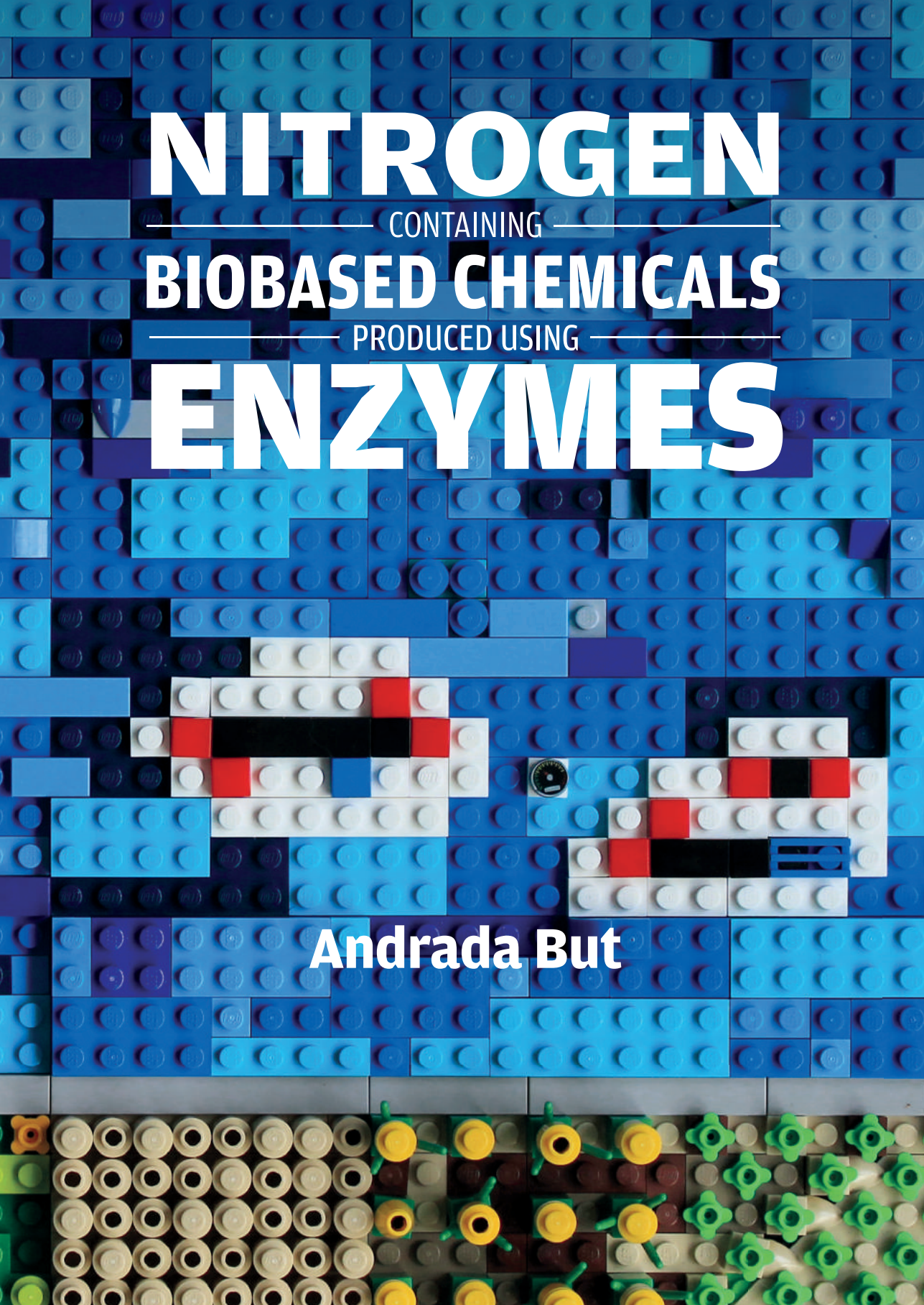
# NITROGEN CONTAINING BIOBASED CHEMICALS PRODUCED USING ENZYMES



Andrada But

**Propositions**

1. The expected similar reactivity of acidic amino acids based on identical functionalities is not in agreement with oxidative decarboxylation.

   (this thesis)

2. Iterative combination of economic and sustainability analysis with experimental data in an early stage, directs the focus for future research towards up- and down-stream the oxidative decarboxylation reaction.

   (this thesis)

3. Sustainability practices for all human activities make ethical concerns redundant.

4. Research that shows how climate change affects personal life, is essential to persuade the general public of the unforeseen effects of using fossil resources.

5. Since impalement is outdated, Dutch bureaucracy can complement education in the fight against corruption.

6. Gardening is an underestimated therapy for chronic diseases.

Proposition belonging to the thesis, entitled:

Nitrogen containing Biobased Chemicals produced using Enzymes

Andrada But

Wageningen, 10 December 2018

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

# NITROGEN CONTAINING BIOBASED CHEMICALS PRODUCED USING ENZYMES

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 10 December 2018
at 11 a.m. in the Aula.

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Nitrogen Containing Biobased Chemicals produced using Enzymes,
152 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2018)
With references, with summary in English

ISBN: 978-94-6343-490-4
DOI: http://doi.org/10.18174/457207

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# Abbreviation list

| | |
|---|---|
| AA | Amino acid |
| Aaa | α-aminoadipic acid |
| AaaCN | 4-cyanobutanoic acid |
| Aba | α-aminobutanoic acid |
| AbaCN | propionitrile |
| ABTS | 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) |
| AOX | Alcohol oxidase |
| Asp | Aspartic acid |
| AspCN | 2-cyanopropanoic acid |
| Br | Bromine |
| C | Carbon |
| ca. | circa |
| Cl | Chlorine |
| CN | Nitrile, nitrile functionality |
| $CO_2$ | Carbon dioxide |
| -COOH | Carboxyl, acid functionality |
| -COOMe | Methyl ester functionality |
| CPO | Chloroperoxidase (Fe-Heme cofactor) |
| DDGS | distiller's dried grains with solubles |
| FAD | Flavin adenine dinucleotide |
| FDCA | 2,5-furandicarboxylic acid |
| GHG | Greenhouse gasses |
| Glu | Glutamic acid |
| GluCN | 3-cyanopropanoic acid |
| GOX | Glucose oxidase |
| H | Hydrogen |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulphuric acid |
| HCN | Hydrogen cyanide |
| HOBr | Hypobromous acid |
| HOCl | Hypochlorous acid |
| HOX | Hypohalous acid |
| HPLC | High performance liquid chromatography |
| HPO | Haloperoxidase |
| HRP | Horseradish peroxidase |
| hSer | homoSerine |
| hSerCN | Hydroxypropionitrile |
| $K_m$ | Michaelis constant |
| Maa | β-methyl aspartic acid |
| MCD | Monochlorodimedone |
| Me, $-CH_3$ | Methyl group |
| MeAsp | Aspartic acid β-methyl ester |
| MeAspCN | Methyl 2-cyanoacetate |
| MeGlu | Glutamic acid γ-methyl ester |
| MeGluCN | Methyl 3-cyanopropanoate |
| N | Nitrogen |
| Na | Sodium |
| NaBr | Sodium bromide |
| NaCl | Sodium chloride |
| NaOCl | Sodium hypochlorite, bleach |
| nLeu | norLeucine |
| nLeuCN | Valeronitrile |
| nVal | norValine |
| nValCN | Butyronitrile |
| O | Oxygen |
| -OH | Hydroxyl functionality |
| Phe | Phenylalanine |
| Ru | Ruthenium |
| Ser | Serine |
| SerCN | Glycolonitrile, Hydroxyacetonitrile |
| Tris | Tris(hydoxymethyl)aminomethane |
| V | Vanadium |
| Val | Valine |
| ValCN | Isobutyronitrile |
| VCPO | Vanadium chloroperoxidase |
| VHPOs | Vanadium haloperoxidases |
| W | Tungsten |
| wt-% | Weight percentage |
| X | Halogens (Cl, Br) |
| $X^+$ | Halogenating species |

# Chapter 1

# Introduction

Modern society is highly dependent on commodity products such as plastics, fuels and medicines. Plastics, for example, are waterproof, lightweight, durable and hygienic, and successfully replace materials such as metal and wood. The plastic bag, the drinks bottle, food packaging, the computer and plastic toys (Figure 1.1) are a few examples of everyday plastic containing items, widely used in the modern world.

Figure 1.1. Examples of plastic items.

Despite their benefits, the excessive use of plastics creates serious issues on our ecosystem.[1] For example, the lightweight plastics, such as the single-use bags, are creating severe waste management issues and environmental pollution. Today, phasing-out measures of the lightweight plastics are being taken around the world.[2] Without doubt, on short term these measures are reducing the amount of single-use plastics to a certain extent. On the long term, it creates awareness among the users,[3] and it allows the development and marketing of alternatives such as compostable plastics.[4] However, behind the scenes issues related to plastics, and to chemicals in general, are not solved by the phasing-out measures. These behind the scenes issues relate to the way chemicals are produced. While the use of chemicals facilitates and simplifies our lives, their production from fossil resources and their use have unforeseen consequences such as pollution, geo-political tensions, and climate change.[5]

## 1.1 From fossil resources to climate change

Nowadays, the production of chemicals (such as plastics) relies mainly on fossil feedstocks and its use changed the modern world as we know it. The exploitation and use of fossil feedstocks as direct products (e.g. kerosene) or conversion into other chemicals (e.g. plastics) generates high amounts of greenhouse gasses (GHG) such as $CO_2$, $CH_4$ and $NO_X$. The global concentration of $CO_2$ rose from 340 ppm to 405 ppm over the period 1980 to 2017 which translates to a rise of the average global temperature of about 0.7°C.[6] This effect is called global warming and the main cause of it is attributed to anthropogenic processing of fossil resources.[7] The rise of global temperature causes climate change with extreme weather conditions (floods and drought), sea level rise and change in pH of oceans.[8] Intensified extreme weather conditions have a high impact on food security but also on oil refineries and as a result of the shutdown of these plants the oil prices are fluctuating. This was shown recently in august 2017 when the oil price in Europe and the rest of the world increased due to the closing of the oil refineries in Texas caused by hurricane Harvey.[9]

The discovery of fossil resources as well as the technological developments of the last century initiated a series of interlinked events which also contribute to the increase of the $CO_2$ level. For example, the Haber-Bosch process which generates ammonia from its elements enabled the use of nitrogen-based fertilisers. The use of these fertilisers triggered social prosperity and a population explosion,[10] which led to an increase in the world's food demand. In some areas of the globe the increase in welfare calls for specialised food such as meat.[11] Consequently, new and/or larger areas for crops and pasture are required and these are made available by deforestation. Forest loss is contributing to global warming effect by releasing the $CO_2$ stored in trees and it is considered the second anthropogenic $CO_2$ source after fossil feedstocks.[12]

To reduce the risks of climate change and to avoid the issues associated with the use of fossil resources, adaptation and mitigation strategies are formulated by governments around the globe.[8] For example, the result of the 21st Conference of the parties of the UN in 2015, was to aim for this century at a rise of the global temperature below 2°C (even 1.5°C) above the pre-industrial value. Unfortunately, world leaders like the USA are withdrawing[13] from the climate change mitigation under the pretext of economic growth. As a result, this is forcing the other signing countries to compensate for the $CO_2$ generated by the USA which is ~15% of the global $CO_2$ emission.[14]

Countries that have signed climate agreements are creating new legislation and economic strategies to accommodate the development of sustainable or green processes in the sectors energy, industry, transportation and agriculture.[15] An overview from 2014 of GHG generated by the major economic sectors (Figure 1.2) shows that the energy sector represents a quarter of the total GHG (25%), followed closely by agriculture and land use with 24% and by industry with 21%.

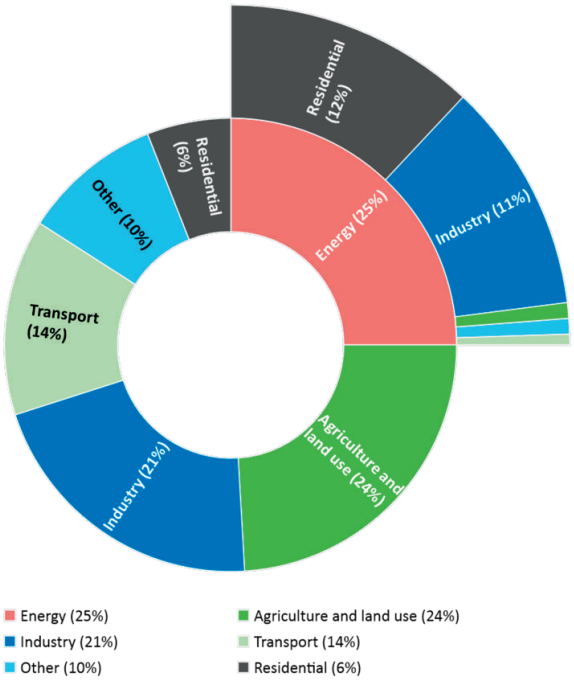



Figure 1.2. Greenhouse gas (GHG) emissions by economic sectors in 2010, adapted from IPCC 2014.[16]

However, about half of the GHG emissions of the energy sector is actually generated due to the energy consumption in the industrial sector. This makes the industrial sector the major producer of GHG with 32% (21% + 11%). Therefore, it is sensible that mitigation strategies in the industrial sector will be the most beneficial against global warming. These mitigation strategies require the reduction of GHG and the enhancement of sinks for GHG by the development of sustainable processes.

## 1.2 The road to sustainability

The word 'sustainability' has become ubiquitous from supermarkets (sustainable farming products) to state governments (sustainable development).[17] The term 'sustainability' is perhaps overused and therefore, is necessary to define it whenever it is used. The explanation given by Kent E. Portney covers what sustainability is understood in this thesis: 'At its heart, sustainability focuses on the use and depletion of natural resources. It is not the same as environmental protection or natural resource conservation; it is more about finding some sort of steady state so that the Earth can support both human population and economic growth'.[18]

An important step to achieve this steady state of sustainability is the replacement of the finite and polluting fossil resources by renewable alternatives such as biomass, wind, solar, hydro and geo-thermal. Next to this, to produce the same goods in a sustainable way benign chemicals and integrated processes which consider the people, the planet and the profit alike are required.

While for the production of energy several renewable alternatives are available, for the production of chemicals only biomass and $CO_2$ are the main alternative sources. Next to the biomass refinery, the use of $CO_2$ as alternative resource for chemicals is also emerging and efforts are made to capture it and to convert it to chemicals.[7] For example, in the Rheticus project, Siemens and Evonik collaborate to produce speciality chemicals from $CO_2$ and bio-electricity in a pilot plant.[19]

### 1.2.1 Biorefinery: from biomass to chemicals

The use of biomass as renewable resource for chemicals is based on the biorefinery approach. Biorefining is the sustainable processing of biomass into a spectrum of biobased products: food, feed, chemicals, materials and bioenergy (biofuels, power and/or heat).[20]

Today, biomass is used as food and feed, construction materials, textiles and energy and these applications have different values relative to their production volume (Figure 1.3).

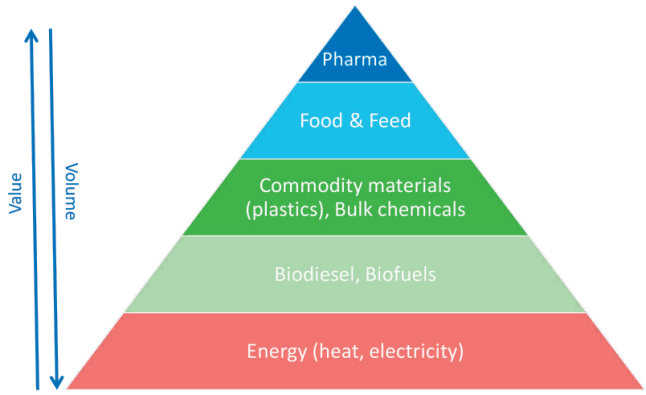



Figure 1.3. The value hierarchy of biomass conversion (adjusted from Peter Westermann).

For example, burning raw biomass for energy has the lowest value and therefore is inefficient. Consequently, biomass should be used for producing more valuable products such as bulk and fine chemicals and only the rests should be used for energy production. [21]

The production of bulk and fine chemicals relies on the availability and accessibility of biomass and on the existing technologies. The availability of biomass is dependent on many factors such as climate, season and/or location, therefore the biorefinery technologies have to be resilient to the variations in biomass composition.

The main components of biomass are carbohydrates, lipids, proteins, lignin, water and some minor components such as vitamins, pigments and minerals (Figure 1.4). The ratio of biomass components varies as a function of biomass type (species) or biomass part (e.g. leaves, grains).

Biomass such as wheat, corn and grasses are refined to produce the same chemicals as the ones obtained from fossil oil (drop-in chemicals) but also new chemicals (dedicated biobased chemicals) that may offer new properties to the same type of materials we are using now, e.g. 2,5-furandicarboxylic acid (Figure 1.4). [22]

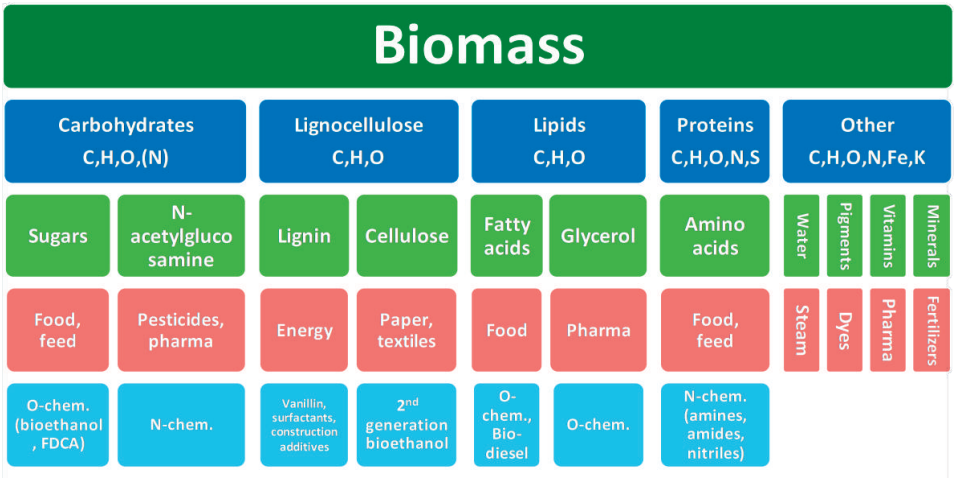



Figure 1.4. Biomass components and their use in a biorefinery approach (O-/N-chem. = oxygen/nitrogen containing chemicals).

A classic example for a drop-in chemical is the production of ethylene[22] from biomass derived ethanol. The production of bioethanol used as fuel initiated the food vs fuel debate[23] which concerns the diversion of farmland for biofuels production to the detriment of the food supply. However, in the context of a sustainable economy, each component of biomass would be used at its highest value satisfying the need for both food and chemicals.

### 1.2.2 Biobased chemicals

All organisms are genetically programmed to grow and develop to pass along their genes. For this, the organism must synthesise highly specialised components starting from simple

molecules available in soil and air and free energy from the sun. The complexity of biomass components is exactly what poses a challenge in the conversion of biomass to chemicals. In the elemental composition of biomass and of fossil resources compared to that required by chemicals (Figure 1.5), a large difference is observed.

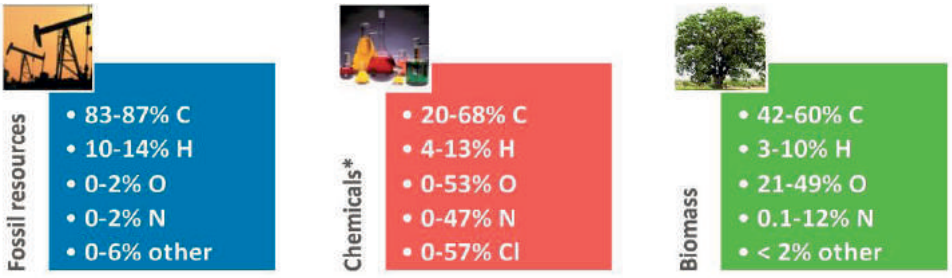



Figure 1.5. Elemental composition of fossil resources,[24] biomass[25] and chemicals of the dry mass. *The elemental composition by weight was calculated for top produced carbon based chemicals. Functionalised platform chemicals were selected by production volume.[26]

On one hand, to produce chemicals from fossil fuels, functionalities (O, N, Cl) must be introduced and this requires high amounts of energy. On the other hand, to produce chemicals from biomass, functionality (in particular O) must be removed or converted. For this, it is recommended to perform redox-neutral conversions of biomass.[21] For example, ethanol ($C_2H_6O$) can be generated from fossil resources by introducing the oxygen functionality into ethylene by acid-hydration under high pressure and temperature. Starting from biomass ethanol can be generated by fermentation of sugars under mild conditions. Here, part of the oxygen functionality of glucose ($C_6H_{12}O_6$) is removed as $CO_2$.

Biomass derived ethanol is used directly as fuel or could be used as a platform chemical and converted to e.g. ethylene glycol and incorporated in copolymers such as polyethylene terephthalate (PET). PET is used for the production of drinks bottles and since 2009 up to 30% biobased PET is used in products such as the 'plant-bottle'.[27] While ethylene glycol and ethylene, two drop-in chemicals, can be made available from biomass, the production of terephthalic acid from biomass is a challenge due to limited aromatic compounds easily available in biomass.

An alternative to terephthalic acid is 2,5-furandicarboxylic acid (FDCA) which is a dedicated biobased chemical. FDCA, the oxidation product of hydroxymethylfurfural (HMF) derived from sugars, can be used in the production of polyesters.[28] The polyethylene furanoate (PEF) polymers have reduced carbon footprint by 50-60%, offers a six times better oxygen barrier and two times better $CO_2$ and water barrier than conventional plastics made from PET.[28] FDCA is an example of a chemical made from biomass which offers overall better properties than the conventional ones derived from petrol. As a result, a joint collaboration 'PEFerence', between 11 companies was announced to develop the PEF value chain and to set up a 50000 t pilot facility for FDCA production.[29]

Many other O-containing chemicals are already commercially produced from biomass: lactic acid, a building block of polylactic acid,[30] is produced by fermentation of sugars or by lactic acid bacteria;[1] pyrolysis oil from biomass;[31] succinic acid from sugar fermentation for the production of polyamides;[1] however, only a few nitrogen containing biobased chemicals are available. Nitrogen containing chemicals such as amines,[32] amides and nitriles are building blocks found in polyamides (PA) or Nylons which offer special properties such as high durability and strength to materials. Only a few PA such as PA-11 and PA-4,10 are produced from biomass.[33] PA-11 is derived from castor oil *via* the pyrolysis of ricinoleic acid followed by a 3 steps synthesis involving HBr, $NH_3$.

Another example of nitrogen containing material is acrylonitrile-butadiene-styrene (ABS), a polymer used for the production of automobile, electronics or toys. ABS is a mouldable polymer that due to the presence of the polar nitrile groups from acrylonitrile becomes a stronger material than pure polystyrene alone. Currently, the three chemical building blocks of ABS are derived from fossil resources, however, biobased alternatives are emerging. Several routes for biobased acrylonitrile are obtained by reacting biobased chemicals such as glycerol[34] or 3-hydroxypropionic acid[35] with ammonia ($NH_3$) to introduce the nitrogen functionality. However, ammonia is produced from $N_2$ in an energy-intensive process, as it will be shown in section 1.2.3.

### 1.2.3 Nitrogen in nature, agriculture and industry

Nitrogen is an essential element for living organisms as it is part of proteins, DNA, RNA, coenzymes (e.g. nicotine amide dinucleotide), etc. but also for the production of chemicals with special properties, e.g. ABS. Remarkably, ~78% of our atmosphere is composed of $N_2$, however, this is an inert gas and only some organisms (bacteria and archaea) can make direct use of it by nitrogen fixation. [36]

Nitrogen fixation in nature is done with the help of nitrogenases.[36] Nitrogenases are metalloenzymes (Fe-Mo or Fe-V) able to bind $N_2$ and convert it to ammonium salts which are taken up by plants and incorporate it in their molecules. The process of converting $N_2$ to ammonia by the Fe-Mo nitrogenases requires 16 molecules of adenosine triphosphate (ATP) showing that high amount of energy is required to achieve the reduction of nitrogen.

$$N_2 + 8\,H^+ + 8\,e^- \xrightarrow[16\,ATP \quad 16\,ADP + P_i]{\text{nitrogenase}} 2\,NH_3 + H_2$$

The nitrogenases are present in species such as *Azotobacter* and *Rhyzobium* which live either free or in symbiosis within the roots of some plants such as the legumes (e.g. beans) and cereal grasses (e.g. rice).[37,38] Other crops that are not capable to access nitrogen directly from air are limited by the availability of nitrogen in soil. In agriculture, nitrogen is supplemented by manure addition and by using nitrogen-based fertilisers such as urea.

Nitrogen-based fertilisers rely on the production of ammonia by the Haber-Bosch process where nitrogen from air is combined with hydrogen from natural gas over iron catalyst.

$$N_2 + 3\,H_2 \xrightarrow[150\text{-}250\ atm]{\text{Fe cat., } 400\text{-}500^\circ C,} 2\,NH_3$$

The reaction takes place at high temperature (400-500°C) and pressure (150-250 atm) in specialised equipment which requires high capital costs but also high operation costs.[39] More than 80% of the total ammonia produced industrially is used for fertilisers and the rest is used for other applications including chemical production.[39]

It appears that ammonia is an indispensable compound in both agriculture and chemical industry, however the production process is dependent on fossil resources and it consumes tremendous amounts of energy. It is estimated that about 2% of the world energy consumption is used for the production of ammonia.[40] To meet the targets of the Paris Climate Agreement regarding the $CO_2$ emission reduction, alternatives to the Haber-Bosch process are emerging e.g. electrochemical production of $NH_3$ which uses sustainable electricity.[41] Alternatives for the production of nitrogen containing chemicals that bypass the direct use of ammonia are developed as well, e.g. the conversion of the nitrogen functionality already present in biomass *via* the biorefinery techniques. Ammonia based fertilisers are used to grow crops which incorporate ammonia into nitrogen functionality mostly as amino acids and proteins. The amino acids can be further converted into nitrogen containing chemicals such as acrylonitrile,[42] *N*-methylpyrrolidone[43] or succinonitrile.[44]

### 1.2.4 Biomass for nitrogen containing chemicals

At the moment not all biomass components are used at their highest value. For example, protein rest streams such as distiller's dried grains with solubles (DDGS) resulted after the production of bio-ethanol, are sold as animal feed. However, from nutritional perspective animals need only the essential amino acids for a healthy diet. Moreover, when the quality is not sufficient for food and feed applications, the proteic fraction is valorised as fertilisers or burnt for energy, two applications which are on the low side of the value chain (Figure 1.3).[45]

Even though the feed application is rather high in the value chain (Figure 1.3) it was shown that when proteins are extracted by certain harsh methods the nutritional value/quality is affected considerably.[46] To increase the value of the proteic fraction it is proposed to break down the proteins into its components (amino acids), separate the amino acids and use them for higher value applications. The essential amino acids (lysine, methionine, tryptophan, etc.) can be used in food and/or feed applications, while the non-essential amino acids (glutamic acid, aspartic acid, etc.) can be converted to a range of nitrogen containing chemicals and further used in other applications. This approach keeps the nutritional benefit and achieves a higher value from the starting material.

Alternative production methods of amino acids such as fermentation are available. Specific amino acids, such as methionine – an essential amino acid, can be obtained by fermentation at high food-grade quality.[47] However, the production costs of these methods are not compatible for chemical applications. Therefore, other sources of amino acids such as rest streams rich in proteins are more suitable.

### 1.2.5 Sources of amino acids

A classic example of a rest stream containing amino acids is DDGS with 30-40% proteins. But other sources rich in proteins are available: vinasse – a side stream from sugar production (20-40% protein), microalgae (50-60%), feathers (>80%), seed cakes after oil press (20-60%)[45] and tea leafs after extraction of tea (20-30%).[48]

On average the most abundant amino acid is glutamic acid (Glu) (~30%) followed by aspartic acid (Asp).[45] But some rest streams have a high concentration of a specific amino acid other than Glu. For example, Protamylasse™ from potato processing is rich in asparagine,[49] or cyanophycin in arginine and Asp but also Glu depending on the composition of the fermentation media.[50]

To access the amino acids, first proteins contained in biomass need to be separated from the other components (Figure 1.6). The separation of proteins from the biomass rest streams was shown to be possible for several biomass rest streams such as tea leaves,[48] grass,[51] rubber plantation residues,[52] and algae.[53,54] Further, the hydrolysis of proteins into its components can be performed by acid, base, enzymatic catalysis or a combination of these.[55] Mixtures of amino acids can be separated using different methods e.g. by electrodialysis into positive, negative and neutral amino acids[56] or by fractional crystallisation using anti-solvents into polar and less polar amino acids.[57]

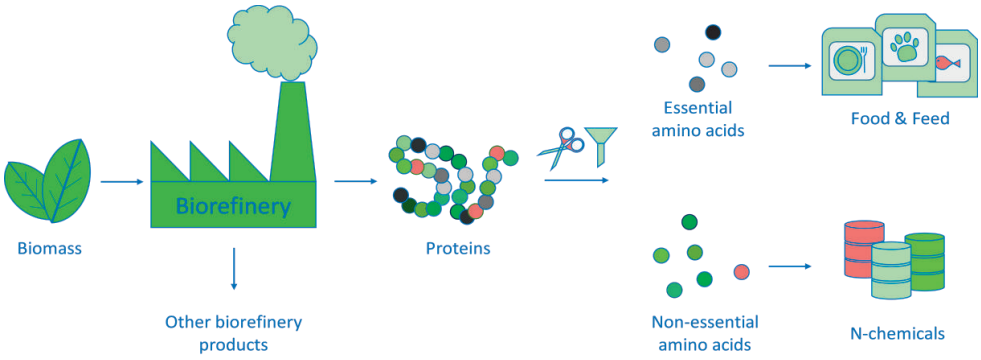



Figure 1.6. From biomass to nitrogen containing chemicals.

Due to the variable concentrations and ratios of amino acids in biomass and the similarity of physical and chemical properties the main bottleneck in the production of biobased chemicals from amino acids is the selective separation of amino acids.[57] A way to overcome this bottleneck is by converting amino acids into other class of compounds with different

properties such as amines[58] or nitriles[59] that allows an effective downstream separation. For example, the conversion of amino acids into nitriles would allow the separation of aliphatic nitriles by distillation or solvent extraction from the water-soluble nitriles which could be further separated by e.g. electrodialysis into neutral and charged nitriles.

## 1.3 Nitriles

Nitriles are organic compounds that contain the -C≡N functionality in their molecule. The carbon triple bond nitrogen is highly polar which leads to intramolecular association. Consequently, the boiling points of nitriles are higher than expected from their molecular mass. Aliphatic nitriles with a short carbon chain (e.g. acetonitrile) are miscible with water but as the carbon chain is increasing, the solubility in water is decreasing. Nitriles may contain other functionalities such as -OH or -COOH which increase their solubility in water. Due to their structure, aliphatic nitriles are good solvents for both polar and nonpolar solutes. Typical reactions of nitriles occur at the electrophilic nitrile group which can react with nucleophiles or at the alpha carbon which is activated by the nitrile group to react to base-catalysed substitution reactions.

### 1.3.1 Nitriles originated from amino acids

Functionalised molecules like nitriles are indispensable compounds in the chemical industry. The different classes of nitriles that can be made available from amino acids are presented in Figure 1.7.

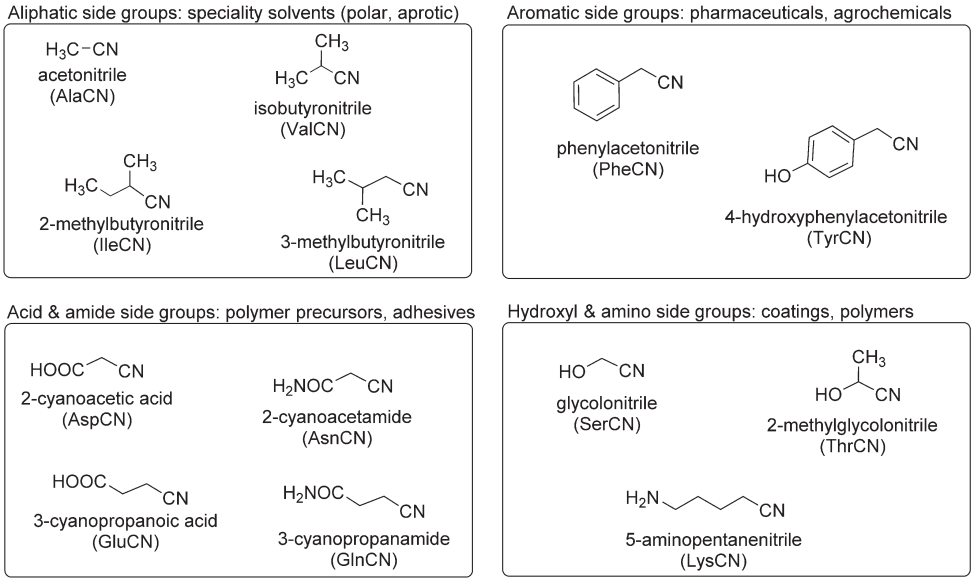



Figure 1.7. Structure of nitriles derived from amino acids. The abbreviation of the nitriles is based on the name of the amino acid from which it is derived. Ala = alanine, Val = valine, Ile = isoleucine, Leu = leucine, Phe = phenylalanine, Tyr = tyrosine, Asp = aspartic acid, Asn = asparagine, Glu = glutamic acid, Gln = glutamine, Ser = serine, Thr = threonine, Lys = lysine.

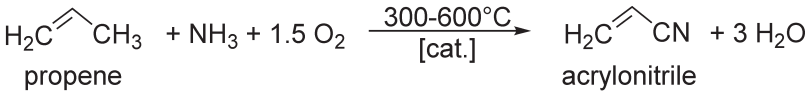
Glutamic acid is the most abundant amino acid present in biomass rest streams in a range between 10% and 63%.[45] Its high availability makes Glu potentially suitable as starting material to produce bulk nitriles such as acrylonitrile. Glu can be converted to 3-cyanopropanoic acid by oxidative decarboxylation and further to acrylonitrile.[42] Acrylonitrile is used in many applications: acrylic fibres, ABS, acrylonitrile-styrene, adiponitrile, acrylamide, nitrile rubber, carbon fibre, etc.[60]

Other amino acids present in biomass in lower percentage than the acidic amino acids can also be converted into nitriles with important applications. For example, phenylacetonitrile (PheCN) is used as intermediate in the production of synthetic penicillins or barbiturates, optically bleaches for fibers, insecticides, perfumes and flavours. Glycolonitrile (SerCN) can be used in coatings and as a bifunctionalised building block in synthesis, and isobutyronitrile (ValCN) or acetonitrile (AlaCN) are mainly used as speciality solvents.[61]

Acetonitrile is one of the main solvents used for extraction of butadiene from crude C4 streams. It is also an important solvent in pharmaceutical manufacture (antibiotics) and highly purified acetonitrile is used in high performance liquid chromatography (HPLC).[61] In synthesis, it is used to produce malononitrile. Currently, acetonitrile is a by-product of ammoxidation of propylene to acrylonitrile, making its production dependent on acrylonitrile.[60]

### 1.3.2 Industrial production of nitriles

Commodity/bulk chemicals such as acrylonitrile are produced on a large scale. Today, more than 95% of acrylonitrile, is produced *via* the ammoxidation reaction in the Sohio process (Scheme 1.1). In this process ammonia together with oxygen are reacted with propene, originated from fossil oil, at high temperatures (300-600°C) in the presence of heterogeneous catalysts containing e.g. bismuth molybdate oxides ($Bi_2O_3$-$MoO_3$).[60]




Scheme 1.1. Ammoxidation of propene in the Sohio process (ΔH=-502 kJ/mol).[26]

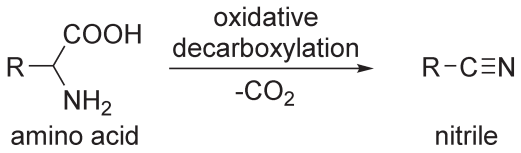
Excess of ammonia and air are used to achieve in one pass a conversion of 98%. Next to acrylonitrile, toxic compounds (HCN) and other side products (acetonitrile and acrolein) are formed. In practice, due to partial combustion of propene, ~760 kJ/mol is released. Unreacted ammonia is neutralised with sulphuric acid to ammonium sulphate and used as a fertiliser.[60]

Fine chemicals, such as malononitrile, cyanoacetic acid, phenylacetaldehyde, are produced on a small scale by cyanidation. These processes involve the use halo-activated substrates, heavy metals and stoichiometric amounts of cyanides (HCN, ClCN, alkali-CN) at temperatures up to 700°C.[61]

These processes generate side products, use highly toxic compounds, are dependent on fossil resources and are usually energy-intensive, therefore, there is a need to produce nitriles in a more sustainable way. This could be achieved by converting biomass derived amino acids into nitriles.

### 1.3.3 Biobased production of nitriles by oxidative decarboxylation

Amino acids can be converted to the corresponding nitriles by oxidative decarboxylation (Scheme 1.2). The oxidative decarboxylation of amino acids was intensely studied in the past. The large majority of methods rely on chemical approaches that involve the use of an activated halogenating species ($X^+$).




Scheme 1.2. Oxidative decarboxylation of amino acids to nitriles.

These chemical approaches use stoichiometric halogenating reagents such as *N*-bromosuccinimide[62] or trichloroisocyanuric acid.[63] These reagents are toxic and/or corrosive, therefore, methods that avoid or replace these reagents are preferred. To produce biobased nitriles in a more sustainable way catalytic methods are considered appropriate.

The proof of concept to produce biobased nitriles was the use of a stoichiometric amount of NaOCl and catalytic amounts of NaBr for the conversion of glutamic acid to 3-cyanopropanoic acid.[42] A techno-economic analysis allowed to conclude whether this approach is feasible.[64] It was found that this approach is unfavourable compared to the current way of producing acrylonitrile. The two main bottlenecks identified were: the necessity of cooling the reaction which requires high amounts of energy and the production of stoichiometric amounts of salt (NaCl) which results in environmental issues.

An alternative method for the oxidative decarboxylation of amino acids is the use of haloperoxidases.[59,65] This is possible for the conversion of several amino acids (valine, phenylalanine, glutamic acid) by using a haloperoxidase which can oxidise a halide with hydrogen peroxide and form the corresponding hypohalous acid. The hypohalous acid reacts with the amino acids in a chemical manner to form the nitrile. This reaction is operated at room temperature without the need for cooling and due to the use of $H_2O_2$ no salt is generated. This chemo-enzymatic reaction that avoids the drawbacks of the hypochlorite method was selected as a suitable approach to investigate the production of biobased nitriles.

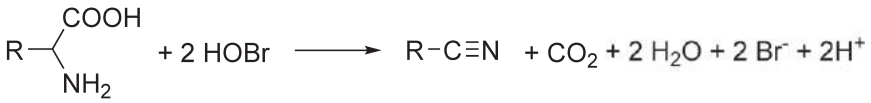
## 1.4 Biobased nitriles by chemo-enzymatic oxidative decarboxylation

Oxidative decarboxylation of amino acids to produce nitriles can be achieved by chemo-enzymatic catalysis.[59,65] This reaction happens in two steps at room temperature (Scheme 1.3). The first step is the oxidation of $Br^-$ with $H_2O_2$ to HOBr which is catalysed by a haloperoxidase which in this research was vanadium chloroperoxidase (VCPO). HOBr is produced in the active site of the enzyme and it is thought to be released into the reaction mixture.[66,67] In the second, chemical step two equivalents of HOBr react with the amino acid to form the nitrile.

**Step 1.** *In situ* production of HOBr

$$Br^- + H_2O_2 + H^+ \xrightarrow[\text{pH 5.6, r.t.}]{\text{VCPO}} HOBr + H_2O$$

**Step 2.** Oxidative decarboxylation of amino acids into nitriles



Scheme 1.3. The oxidative decarboxylation of amino acids into nitriles by chemo-enzymatic catalysis, VCPO = vanadium chloroperoxidase, r.t. = room temperature.

### 1.4.1 Haloperoxidases

VCPO is part of the haloperoxidases (HPO) which is a class of enzymes capable of two-electron oxidation of halides to the corresponding hypohalous acid (HOX) using a peroxide such as hydrogen peroxide (Scheme 1.4, equation 1). The hypohalous acid is the chemical equivalent of the electrophilic $X^+$ species[68] which can further react with organic substrates to give halogenated compounds (Scheme 1.4, equation 2) or can react in an oxidative reaction such as the case of amino acids (Scheme 1.4, equation 3). When these substrates are not present HOX will react with hydrogen peroxide to form singlet oxygen (Scheme 1.4, equation 4).

(1) $X^- + H_2O_2 + H^+ \xrightarrow{HPO} HOX + H_2O$

*spontaneous chemical reactions with HOX:*

(2) $HOX + S_1 \longrightarrow S_1\text{-}X + H_2O$

(3) $HOX + S_2 \longrightarrow [S_2\text{-}X] \longrightarrow S_2(ox) + X^-$

(4) $HOX + H_2O_2 \longrightarrow {}^1O_2 + X^- + H_2O + H^+$

**$X^-$:** $Cl^-$, $Br^-$, $I^-$, $SCN^-$; **HOX:** $X_2$, $X^+$-like species, etc. **$S_1$:** monochlorodimedone, barbituric acid, alkenes, etc. **$S_2$:** amino acids, thiols, thioesters, etc.

Scheme 1.4. Specific reactions of haloperoxidases.

The activity of HPO is based on the monochlorodimedone (MCD) assay (Scheme 1.4, equation 1 and 2, $S_1$ = MCD).[69] The enzyme activity can be observed also qualitatively using the phenol red assay[69] where a change in colour from yellow to blue-purple under slightly acidic pH indicates a positive result. This assay is also used for screening for haloperoxidases. Both phenol red and MCD are good scavengers for the HOX.

Depending on the most electronegative halide that can be oxidised these enzymes are divided in chloroperoxidases (CPO) able to oxidise $Cl^-$, $Br^-$, $I^-$; bromoperoxidases (BPO) able to oxidise $Br^-$, $I^-$ and iodoperoxidases (IPO) which oxidise only $I^-$. $F^-$ cannot be oxidised by peroxides due to the lower oxidation potential therefore, it is inert for the enzyme. A pseudo halide, the thiocyanate ion ($SCN^-$) can also be oxidised by some haloperoxidases.

Haloperoxidases are found in nature in various origins: mammalians (milk, saliva, tears, neutrophils, eosinophils, thyroid), marine algae (*Ascophyllum nodosum*) and other marine species (*Murex* snails), marine fungus (*Caldariomyces fumago*) and terrestrial fungi (*Curvularia inaequalis*).[70–72] The function of these enzymes in nature is mostly involved in protection mechanisms against different pathogens e.g. against biofouling, either by direct action of the HOX or by halogenation of organic substrates to generate bactericidal agents including bromoform or dibromomethane.[73] In terrestrial fungi the role of CPO is aiding pathogenic mechanisms. For example, HOCl produced by CPO can oxidise lignin and degrade it. It is believed that in unfavourable conditions the fungi will secrete more CPO to degrade the lignocellulose in the cell walls of plants. This will facilitate the infiltration of the fungus in the host.[74]

Haloperoxidases have in their active site as prosthetic group either a Fe-Heme molecule or a vanadate ($VO_4^{3-}$) molecule.[75] The Fe-Heme moiety is covalently bound to the protein usually by two ester linkages to aspartate and glutamate. The vanadium atom from the vanadate is coordinated to the nitrogen from a histidine residue and stabilised by other residues of the protein. The metal ion in the vanadium haloperoxidases (VHPOs) does not change oxidation state during the catalytic cycle unlike the Fe-Heme dependent haloperoxidases, which suffer from oxidative inactivation. Beside this, VHPOs present tolerance for organic solvents[69] and are able to perform regio- and stereospecific halogenation.[76,77] As a result of these characteristics VHPOs received increasing interest in the pharmaceutical[68] and the chemical[59] sector.

### 1.4.2 Vanadium chloroperoxidase (VCPO)

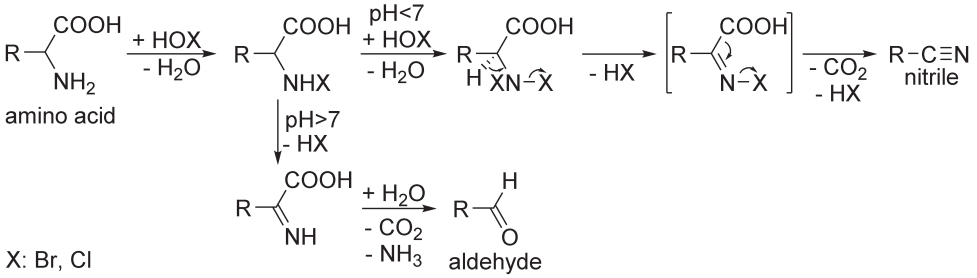
In this research the enzyme vanadium chloroperoxidase (VCPO) was used for the conversion of amino acids to nitriles. VCPO was originally found in the fungus *Curvularia inaequalis* and it was expressed in *Saccharomyces cerevisiae*[78] and *Escherichia coli*.[79] VCPO is a monomeric enzyme with one molecule of vanadate per unit and has a molecular mass of 67 kDa. The enzyme catalyses the oxidation of halides in a substrate inhibited bi bi ping-pong mechanism.[68,74] The inhibition constant ($K_i$) for chloride was 5 mM and for bromide

was 0.5 mM based on MCD assay.[80] To maintain high enzymatic activity for reactions similar to MCD the concentration of the halide should be kept below the $K_i$ value. The vanadate can be replaced by phosphate with a 50% loss of its original activity, therefore the use of phosphate buffers is not indicated. In the correct formulation VCPO[72] is maintaining its activity for months and after many freezing/thawing cycles. It was shown that VCPO has tolerance towards oxidants and organic solvents and elevated temperature.[72]

VCPO is a relatively newly discovered enzyme that most likely can be used in industrial processes due to its characteristics: tolerance towards oxidants, organic solvents and elevated temperature and high storage stability.

### 1.4.3 Oxidative decarboxylation reaction mechanism

Different reaction mechanisms have been proposed in literature for the oxidative decarboxylation of amino acids by activated halogenating species ($X^+$) and the most accepted mechanism is presented in Scheme 1.5.




Scheme 1.5. Reaction mechanism of the oxidative decarboxylation of amino acids as presented before by Claes *et al.*[81]

To convert one equivalent of amino acid into nitrile, two equivalents of activated halogenated species are required.[82] The activated halogenated species can be: HOX, NaOX, $XO^-$, $H_2O^+X$, $X_2$, Enzyme-X, etc. Most literature agrees that the halogenation happens at the amino functionality of the amino acids in two steps. In the first halogenation step a *N*-monohalogenated amino acid is formed. In slightly acidic conditions, this compound will further react with the second equivalent of halogenating species to form the *N,N*-dihalogenated amino acid which will spontaneously decarboxylate to an unstable iminium intermediate and further oxidised to the nitrile and regenerate the halide.

The selectivity towards the nitrile or the aldehyde is influenced by the pH of the reaction.[81] If the reaction conditions are alkaline, the second step of halogenation does not occur, the halide is regenerated earlier, and the corresponding aldehyde is formed as product.

The type of halide used for the oxidative decarboxylation of amino acids was shown to be another parameter that influences the selectivity.[59] VCPO can oxidise both $Cl^-$ and $Br^-$ to the corresponding hypohalite which can further react with organic substrates including amino acids. As HOCl is a weaker oxidant than HOBr the reaction rates with organic substrates will

be slower giving the *N*-monohalogenated intermediate the chance to form the iminium intermediate rather than to react with a second HOCl molecule. This was shown for phenylalanine which is directed towards the formation of phenylacetaldehyde with HOCl and towards phenylacetonitrile with HOBr.[59]

In Scheme 1.4, equation 4 is shown that singlet oxygen can be formed, particularly if an excess of $H_2O_2$ is present in the reaction mixture. Singlet oxygen is a high energy form of oxygen which is reactive towards organic compounds leading to side reactions. To limit the possibilities of side reactions with singlet oxygen the addition of hydrogen peroxide needs to be controlled e.g. by gradual addition or *in situ* generation.

## 1.5 Hydrogen peroxide production

The production of biobased nitriles by the chemo-enzymatic oxidative decarboxylation requires the use of hydrogen peroxide ($H_2O_2$). $H_2O_2$ is considered a clean or 'environmentally benign' oxidising agent mainly because it is non-toxic and it decomposes to water and oxygen. In the last decades $H_2O_2$ is replacing bleach or other oxidants such as *tert*-butylhydroperoxide, permanganate and nitric acid, which prevents generation of halogenated compounds and disposal of halogen-rich, $NO_X$ or other waste effluents.[83] Worldwide more than 95% of $H_2O_2$ is produced on a large scale by the oxidation of alkyl anthraquinone.[26]

### 1.5.1 The anthraquinone oxidation process

In the industrial process, in the first step the anthraquinone is hydrogenated on Ni/Pd catalysts at 40-50°C to generate anthrahydroquinone.[26] In the next step anthrahydroquinone reacts with oxygen which is reduced to $H_2O_2$ and the anthraquinone is regenerated. $H_2O_2$ is extracted from the organic solution in demineralised water at a concentration of ca. 30% (wt-%). The main advantage of this process is the high yield of $H_2O_2$ per cycle. However, a number of side reactions lead to a net consumption of anthraquinone which requires to regenerate the solution. The presence of organic impurities creates challenges on purification and next to this the catalyst regeneration is required.

The world production of hydrogen peroxide in 2005 was 3.6 million t.[26] Due to the large scale of the process only a few production sites are operated worldwide. For more efficient transportation, $H_2O_2$ solution is distilled to 70% (wt-%) $H_2O_2$.[83] As a result, reactions that require dilute streams of $H_2O_2$, such as organic synthesis or oxidation reactions involving HPO, would result in an inefficient use of energy due to sequential concentration and dilution steps. Inherent safety issues are also associated to concentrated $H_2O_2$ which require special storage conditions. To circumvent some of these issues, improvements to the steps of anthraquinone process and alternative methods are being developed.

### 1.5.2 Alternatives to anthraquinone process

Due to the low concentrations of $H_2O_2$ required in the conversion of amino acids to nitriles, alternative methods of producing $H_2O_2$ *in situ* will be further discussed. The *in situ* production of $H_2O_2$ can be achieved by the use of cascade reactions. This will ensure low and homogeneous concentration of $H_2O_2$, and it can prevent the deactivation of the downstream catalyst such as HPO[84] and the generation of side reactions. Alternatives to the anthraquinone process that make use of molecular oxygen for the *in situ* production of $H_2O_2$ have been developed and these involve catalytic, direct synthesis, electrochemical, photochemical and enzymatic methods.[83,85]

To match the reaction conditions of the $H_2O_2$ production and the VCPO reaction the enzymatic method was selected for this research. The combination of two enzymes

prevents the possibility of inactivation or inhibition of VCPO by the reagents used upstream and allows the possibility of a one-pot cascade reaction. The enzymes able to produce $H_2O_2$ are called oxidases.

### 1.5.3 Oxidases-Haloperoxidases cascade

To produce $H_2O_2$, the oxidases are making direct use of oxygen and it takes the necessary electrons from a substrate (Scheme 1.6).

$$\text{Substrate} + O_2 \xrightarrow{\text{oxidase}} \text{Substrate}_{ox} + H_2O_2$$

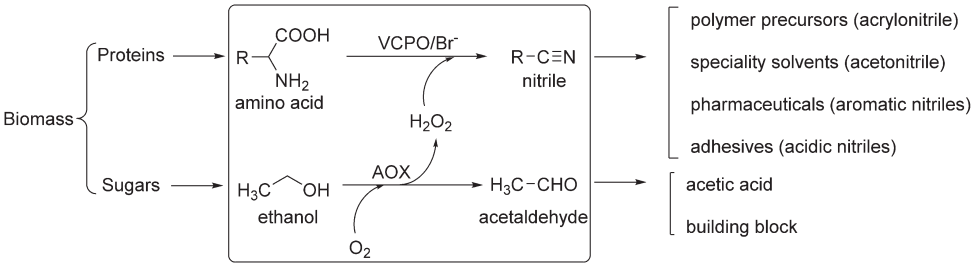
Scheme 1.6. General reaction scheme of oxidases.

Glucose oxidase (GOX) is the enzyme most used for the production of $H_2O_2$. GOX is used in cascade reactions with peroxidases for numerous applications: quantification of glucose for medical analysis and in bioreactors, but also in oxidation of thioanisole, barbituric acid and other chemicals. The great advantage of using GOX is the self-regeneration of the cofactor flavin adenine dinucleotide (FAD). GOX is using as substrate glucose which is converted to gluconic acid. The production of gluconic acid results in the acidification of the reaction mixture which requires pH control and this results in salt by-products.

Other FAD-containing oxidases could lead to the same performance as GOX for the *in situ* production of $H_2O_2$ but without the drawbacks related to gluconic acid. For example, alcohol oxidases (AOX)[86] convert short chain primary alcohols into (highly) volatile aldehydes. The substrate affinity of AOXs decreases with the length of the carbon chain of the alcohol, therefore, AOXs have the highest activity with methanol and ethanol as substrates.[87] Formaldehyde is the oxidation product of methanol, while ethanol is converted to acetaldehyde; as formaldehyde is more toxic compared to acetaldehyde,[88] ethanol is the preferred substrate. It was shown that acetaldehyde can be easily separated by pervaporation[89] or air stripping.[90] Using AOX instead of GOX and ethanol instead of glucose for the *in situ* generation of $H_2O_2$ would avoid complex downstream processing. Furthermore, the cascade reaction of AOX with an HPO which will use $H_2O_2$ for the production of $X^+$ species would give access to more sustainable reactions involving halogenation reagents.

## 1.6 Aim of this thesis

The aim of this thesis is to explore the biocatalytic conversion of amino acids to nitriles as an alternative to the petrochemical route by understanding the reaction conditions and the reaction mechanism and by further improving the sustainability of the method. An overview of the aim can be seen in Scheme 1.7.




Scheme 1.7. Conversion of amino acids originated from biomass to nitriles by *in situ* production of $H_2O_2$ and the applications of nitriles.

The enzyme vanadium chloroperoxidase (VCPO) together with $H_2O_2$ and a bromide source were used for this conversion. The acidic amino acids, in particular Glu was chosen as the first substrate due to the high availability in proteic rest streams.[45] Other amino acids with other functionalities on the side chain were tested to understand the influence of the side chain functionality on the conversion. To further improve the sustainability of the process, $H_2O_2$ was produced *in situ* by an alcohol oxidase from ethanol and oxygen. In the end, an evaluation of the chemo-enzymatic oxidative decarboxylation of amino acids to nitriles was conducted from technical and economic perspective.

### 1.6.1 Research questions

**1.** Is it possible to produce biobased nitriles using chemo-enzymatic catalysis in a sustainable way?

**2.** Why do chemically similar amino acids, aspartic acid and glutamic acid, react differently under the same reaction conditions?

**3.** How does the functionality of the side chain influence the reactivity of the α-functionalities?

**4.** Can $H_2O_2$ be produced *in situ* by an AOX in a cascade reaction with VCPO for nitrile production?

**5.** Is it technically and economically viable to implement the chemo-enzymatic oxidative decarboxylation of amino acids as a biobased alternative to the petrochemical route?

### 1.6.2 Thesis outline

**Chapter 2. Acidic amino acids.** The conversion of chemically similar amino acids, aspartic acid (Asp) and glutamic acid (Glu), is described. Different reaction parameters are investigated to understand the differences in reactivity observed between Asp, an amino acid with carboxylic side chain functionality but one carbon atom less than Glu. This will answer research question 2.

**Chapter 3. The influence of the side chain functionality.** Here, the conversion of amino acids with different side chain functionality into nitriles is investigated. This is aimed to understand the influence of the side chain functionality over the conversion and selectivity. This will answer research question 3.

**Chapter 4. *In situ* $H_2O_2$ production.** In this chapter the setup and reaction conditions required by a cascade of alcohol oxidase and vanadium chloroperoxidase (VCPO) for the *in situ* production of $H_2O_2$ to convert Glu into its corresponding nitrile are presented. This will answer research question 4.

**Chapter 5. Techno-economic assessment of the process.** This chapter is a techno-economic evaluation for the production of 3-cyanopropanoic acid from Glu using VCPO. Biobased methods are compared between each other in different scenarios. The aim of this chapter is to identify the bottlenecks of these methods and make recommendations for future research strategies. This will answer research question 5.

**Chapter 6. General discussion and recommendations.** The impacts of the results obtained in chapter 2-5 on the conversion of amino acids to nitriles using biocatalysis are evaluated. The chapter is ending with recommendations for further research. This will answer research question 1.

# Chapter 2

# Unusual differences in the reactivity of glutamic and aspartic acid in oxidative decarboxylation reactions



This chapter was published in adapted form as: Unusual differences in the reactivity of glutamic and aspartic acid in oxidative decarboxylation reactions, A. But, E. van der Wijst, J. Le Nôtre, R. Wever, J. P. M. Sanders, J. H. Bitter, E. L. Scott, *Green Chemistry*, 19, **2017**, 5178-5186

## Abstract

Amino acids are potential substrates to replace fossil feedstocks for the synthesis of nitriles *via* oxidative decarboxylation using vanadium chloroperoxidase (VCPO), $H_2O_2$ and bromide. Here the conversion of glutamic acid (Glu) and aspartic acid (Asp) was investigated. It was observed that these two chemically similar amino acids have strikingly different reactivity. In the presence of catalytic amounts of NaBr (0.1 equiv.), Glu was converted with high selectivity to 3-cyanopropanoic acid. In contrast, under the same reaction conditions Asp showed low conversion and selectivity towards the nitrile, 2-cyanoacetic acid (AspCN).

It was shown that only by increasing the amount of NaBr present in the reaction mixture (from 0.1 to 2 equiv.), could the conversion of Asp be increased from 15% to 100% and its selectivity towards AspCN from 45% to 80%. This contradicts the theoretical hypothesis that bromide is recycled during the reaction.

NaBr concentration was found to have a major influence on reactivity, independent of ionic strength of the solution. NaBr is involved not only in the formation of the reactive $Br^+$ species by VCPO, but also results in the formation of potential intermediates which influences reactivity. It was concluded that the difference in reactivity between Asp and Glu must be due to subtle differences in inter- and intramolecular interactions between the functionalities of the amino acids.

## 2.1 Introduction

Environmental and geopolitical issues associated with the use of fossil resources are driving the transition from fossil to biobased processes for the production of transportation fuels and chemicals.[5] Conventional petrochemical synthesis of nitriles involves the introduction of the nitrogen functionality using ammonia. This requires high amounts of energy and often generates toxic by-products.[26] Therefore sustainable alternatives are required.

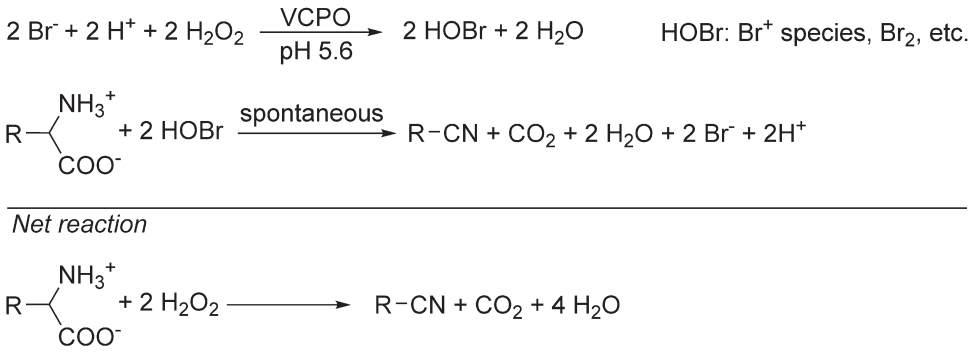
Biobased alternatives, circumventing the use of fossil resources, could make use of biomass derived amino acids for the production of nitrogen containing chemicals.[45] The use of amino acids has the advantage that nitrogen is already present in the molecule. It has been reported that glutamic acid (Glu) can be successfully converted to the corresponding nitrile, 3-cyanopropanoic acid (GluCN) chemically by oxidative decarboxylation.[42] GluCN has been shown to be an intermediate for the production of succinonitrile[44] and acrylonitrile.[42] The oxidative decarboxylation of Asp gives 2-cyanoacetic acid (AspCN) which is a starting material for cyanoacetates.[91] Functionalised molecules, such as GluCN and AspCN, are suitable in many applications including the production of polymers, adhesives and pharmaceuticals.[61,91] Therefore the use of amino acids could be useful for the production of biobased chemicals.

The oxidative decarboxylation of amino acids relies on the *in situ* generation of oxidising reagents, such as the halogenating species: '$X^+$' ($Br^+$, $Cl^+$). For the production of biobased acrylonitrile from Glu *via* GluCN, $Br^+$ was generated using hypochlorite and catalytic amounts of NaBr.[42] However, this approach resulted in stoichiometric amounts of salts and requires cooling. This was detrimental for the overall economic feasibility and sustainability of the process.[64,92] Alternative halide promoted reactions avoiding the use of hazardous and stoichiometric amounts of reagents have been proposed. These involve a heterogeneous tungsten catalyst,[81] electrochemical bromide oxidation[93] and enzymatic catalysis using haloperoxidases.[59] The use of molecular oxygen in the presence of a Ru catalyst without the use of halides, has recently been reported for the reaction with a number of amino acids.[94]

Theoretically, each equivalent of amino acid requires two equivalents of $X^+$ species to be converted to the nitrile (Scheme 2.1). Interestingly the reactivity of Glu conforms to this stoichiometry, while it was observed that Asp required a two-fold increase in the number of equivalents of $X^+$ to reach full conversion.[82] Other catalytic reactions also reported lower reactivity of Asp compared to Glu.[93,94] The difference in reactivity of these similar acidic amino acids appears remarkable but it was not further investigated. It has already been shown that vanadium chloroperoxidase (VCPO) can fully convert Glu into GluCN with high selectivity.[59] To investigate the difference in reactivity of Glu and Asp, an enzymatic reaction using VCPO was selected.

VCPO from the fungus *Curvularia inaequalis* is a robust haloperoxidase.[66,95] This enzyme oxidises halides to the corresponding hypohalous acid (HOX) using $H_2O_2$. HOX, or the active species $X^+$, is produced at the active site and thought to be released into the reaction mixture.[66,67] The $X^+$ species is responsible for the oxidative decarboxylation of amino acids. Currently no evidence suggests that VCPO is directly involved in this reaction. It can be assumed that oxidative decarboxylation is a chemical reaction and the role of the enzyme is to produce the oxidant ($X^+$). Thus this chemo-enzymatic approach may allow conversion of both Glu and Asp.

In the first step, VCPO oxidises $Br^-$ to $Br^+$ using $H_2O_2$ at pH 5.6 (Scheme 2.1, reaction 1). In the second step, two equivalents of $Br^+$ are required for the oxidative decarboxylation of one equivalent of amino acid (Scheme 2.1, reaction 2). The resultant $Br^-$ is then regenerated to $Br^+$ by VCPO. This allows the conversion of amino acids in presence of catalytic amounts of $Br^-$.




Scheme 2.1. Oxidative decarboxylation of amino acids into nitriles assisted by vanadium chloroperoxidase (VCPO).

As well as the synthesis of the nitrile, the corresponding aldehyde can be formed.[65,96] Various factors can influence the selectivity of the reaction. One of these factors is the type of halide. For example, it was shown in the reaction of phenylalanine that the use of bromide resulted in selectivity towards the nitrile and chloride to the aldehyde.[59] As well as this, selectivity to the nitrile is enhanced at acidic pH.[81]

Other factors influencing conversion and selectivity towards the nitrile is the chemical (electronic) nature of the amino acid and steric effects. It was speculated that the nature of the functionality at the β-carbon (based on electronic effects) affects the reactivity of the amino acid towards oxidative decarboxylation.[81] Furthermore, it could be possible that due to the steric hindrance created by the functionality of Asp at the β-carbon, an excess of hypobromite (four equivalents),[82] was necessary to achieve full conversion.

Amino acids can undergo oxidative decarboxylation to nitriles using $Br^+$ generated *in situ* using VCPO.[59] However, it remains unclear how the type of halide or chemical structure of the amino acid can influence the reactivity. The focus of this chapter is to investigate

differences in reactivity of acidic amino acids with similar chemical structure during oxidative decarboxylation to gain more molecular insight on the reaction. Here reactions of Glu and Asp and the influence of various halides and their concentration on the selectivity towards nitrile formation were studied. It will also be discussed that subtle differences in inter- and intramolecular interactions between the functionalities of the amino acids are most likely to account for the difference in reactivity between Glu and Asp.



## 2.2 Experimental

### 2.2.1 Materials

L-Aspartic acid (98.5% pure), L-glutamic acid (98.5% pure), 2-cyanoacetic acid (≥99% pure), NaF (99% pure), NaCl (99.5% pure), NaBr (99% pure), $H_2O_2$ (35 wt%), citric acid, $Na_3VO_4$ (99.98% pure), and Trizma base were purchased from Sigma-Aldrich. 3-Cyanopropanoic acid (95.9% pure) was provided by Interchim and diethylaminoethyl (DEAE) Sephacel by GE Healthcare and monochlorodimedone (MCD) by BioResource Products.

### 2.2.2 Vanadium chloroperoxidase (VCPO)

Vanadium chloroperoxidase was expressed in *Escherichia coli* cells containing the VCPO plasmid using a protocol described elsewhere.[79] The VCPO was isolated from the lysed *E. coli* cells by two purification steps. The first purification step was heat treatment at 70°C for ca. 10 min, followed by centrifugation. The second step was a batch purification of the supernatant using a DEAE resin and a stepwise elution with 100 mM Tris/$H_2SO_4$ buffer pH 8.2 and a 100 mM Tris/$H_2SO_4$ buffer pH 8.2 containing 1 M NaCl. The obtained partially purified VCPO was concentrated on 30 kDa membrane and stored in 100 mM Tris/$H_2SO_4$ buffer pH 8.2 containing 100 µM $Na_3VO_4$ at -20°C. The concentrated VCPO has an activity of 92 U/mL based on a modified MCD assay[79] (50 µM MCD, 0.5 mM NaBr, 1 mM $H_2O_2$, 100 µM $Na_3VO_4$ in 50 mM sodium citrate buffer pH 5.5, 25°C) and it was validated on the conversion of Glu to GluCN (Figure A.1).

### 2.2.3 HPLC

Amino acids were analysed by derivatisation as described before.[59,97] The nitriles were analysed without dilution or derivatisation by HPLC using an UltiMate 3000 equipment from Thermo Scientific. Detection was achieved using a RI-101 detector from Shodex set at 35°C. The column used was a Rezex ROA Organic acid H+ (8%) column (7.8 × 300 mm) from Phenomenex, at 35°C with an eluent flow of 0.5 mL/min of 12 mM $H_2SO_4$.

### 2.2.4 Standard procedure for oxidative decarboxylation of amino acids by VCPO

In a glass vial a 2 mL solution containing 5 mM of amino acid, 0.5 mM of NaBr, 0.36 U/mL of VCPO (based on MCD assay) and 20 mM citrate buffer pH 5.6 was stirred at 400 rpm, at room temperature (20-22°C). To this reaction mixture 18 mM $H_2O_2$/h (66 µL of 0.56 M $H_2O_2$/h) were added using a NE-1600 syringe pump from ProSense. The reaction was quenched after 1 hour by adding $Na_2S_2O_3$. For the time course reactions individual reactions

were performed for each time point. Samples were taken for HPLC analysis. Deviations from this standard procedure are presented in the results and discussion section. The reaction rates of Glu and Asp were calculated as the slopes of the linear section of the time course reactions. Conversion and selectivity were calculated using the formulas:

$$\text{Conversion} = 100 \times ([\text{Substrate}_{initial}] - [\text{Substrate}_{final}]) / [\text{Substrate}_{initial}]$$

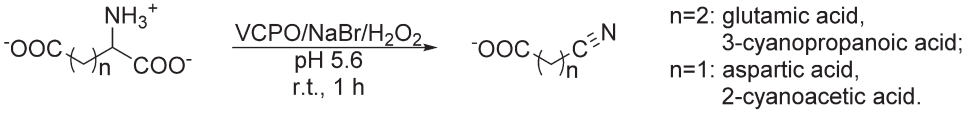
$$\text{Selectivity} = 100 \times [\text{Product}_{final}] / ([\text{Substrate}_{initial}] - [\text{Substrate}_{final}])$$

## 2.3 Results & discussion

The oxidative decarboxylation reactions of Glu and Asp were studied at low concentration of NaBr. Then the optimal reaction conditions for Asp were found. To elucidate the difference in reactivity between Glu and Asp, the influence of the ionic strength and the type of halide on the reactivity of amino acids was studied.

### 2.3.1 Reactivity of Glu *vs.* Asp

The conversion of Glu to GluCN (Scheme 2.2, n=2) was initially performed using VCPO expressed in *E. coli* (Figure A.1), catalytic amounts of NaBr and continuous addition of $H_2O_2$.




Scheme 2.2 Glutamic acid (Glu) and aspartic acid (Asp) conversion into 3-cyanopropanoic acid (GluCN) and 2-cyanoacetic acid (AspCN) by VCPO.

The conversion of Glu to the corresponding nitrile was followed in time using 0.5 mM NaBr and the results are displayed in Figure 2.1.a. As it can be observed, the concentration of Glu is decreasing in time as the concentration of GluCN is increasing. The oxidative decarboxylation of Glu is complete after about 40 min and the only product of the reaction is GluCN. These results are consistent with previous findings where VCPO was expressed in *Saccharomices cerevisiae*.[59]

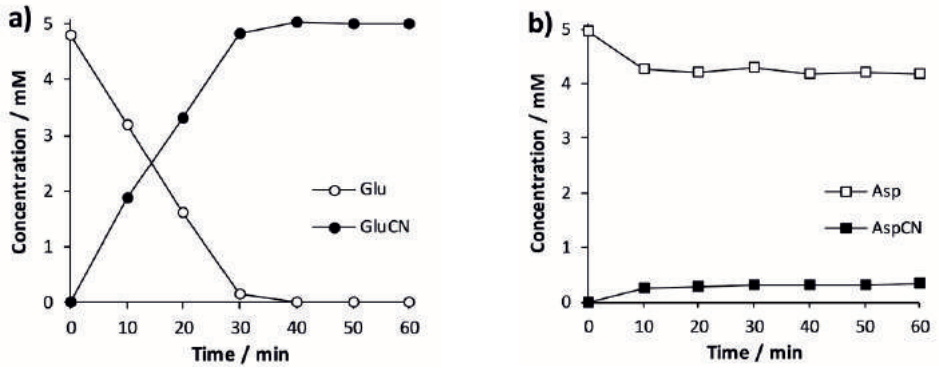



Figure 2.1. Conversion of a) glutamic acid (Glu) into 3-cyanopropanoic acid (GluCN) and b) aspartic acid (Asp) into 2-cyanoacetic acid (AspCN) by vanadium chloroperoxidase at 0.5 mM NaBr. Reaction rate of Glu is 0.15 mM/min.

The same reaction conditions used for Glu, were applied for the conversion of Asp to AspCN, (Scheme 2.2, n=1). In Figure 2.1.b the concentrations of Asp and AspCN can be observed as a function of time. It can be seen that in the first 10 min of the reaction, the concentration of Asp is decreasing from 4.96 to 4.27 mM which is equivalent to a conversion of ca. 14% and further no conversion is observed. Under the same reaction conditions the conversion of Glu was ca. 33% after 10 min and 97% after 30 min. The amount of AspCN produced after 1 h (Figure 2.1.b) corresponds to a selectivity of only 45% which indicates that by-products are formed.

Limited data is available regarding the oxidative decarboxylation of Asp and usually not in comparison with Glu. Only two examples are known to investigate both acidic amino acids under the same reaction conditions.[93,94] Both papers reported lower conversions of Asp compared to Glu which is comparable with the above results. The difference in reactivity of acidic amino acids is surprising given that Glu and Asp differ in structure only by one methylene group. Despite their structural similarity and close $pK_a$ values of the ω-COOH (3.86 for Asp and 4.07 for Glu), the two acidic amino acids display significantly different reactivity in the oxidative decarboxylation reaction. Up to now no clear explanation is provided in literature for the observed differences between Asp and Glu.[82]

It is generally accepted that to convert one equivalent of amino acid into nitrile, two equivalents of $Br^+$ and therefore two equivalents of $H_2O_2$ are required (Scheme 2.1). However, it was shown that Asp requires about four equivalents of $Br^+$.[82] In the present experimental setup, although an excess of $H_2O_2$ was used (amino acid:$H_2O_2$=1:3.6), still low conversions of Asp (<20%) were observed (Figure 2.1.b). Therefore, the next step to investigate the difference in reactivity between Asp and Glu was to explore whether the reaction conditions chosen were suitable for the conversion of Asp. The reaction time and the amounts of $H_2O_2$, VCPO and NaBr were varied as shown in Table 2.1. The conversion of Asp and the selectivity for AspCN after 1 h under standard conditions (Table 2.1, entry 2) can be compared with those after modification of reaction conditions (Table 2.1, entry 3-9). First, the overall $Br^+$ production was increased, either by extending the time of the reaction or by adding higher amounts of $H_2O_2$ to the reaction mixture (Table 2.1, entry 3-7). The use of larger amounts of $H_2O_2$ may give rise to other effects. It is known that excess of $H_2O_2$ can react with HOBr to generate singlet oxygen which can give undesired side reactions.[98] Moreover, haloperoxidases can be inhibited by excess of $H_2O_2$[84] and this was also observed in this research during the conversion of Glu using different amounts of $H_2O_2$ in aliquots (Figure A.2). To avoid these possible issues, both the amounts of VCPO and $H_2O_2$ were increased. This ensures an increase in the overall amount of $Br^+$ generated per unit of time. However, only a small increase in the conversion of Asp was observed but resulted in a small decrease in nitrile selectivity (Table 2.1, entry 4 and 7).

Table 2.1. Reaction condition screening (reaction time, concentration of H2O2, VCPO and NaBr) for oxidative decarboxylation of aspartic acid (Asp) and comparison with glutamic acid (Glu). The data represent the results of single experiment.

| Entry | Amino acid | Reaction time (h) | $H_2O_2$ (equivalents*/h) | VCPO (U/mL) | [NaBr] (mM) | Conversion (%) | Selectivity (%) |
|---|---|---|---|---|---|---|---|
| 1 | Glu | 1 | 3.6 | 0.36 | 0.5 | 100 | >98 |
| 2 | Asp | 1 | 3.6 | 0.36 | 0.5 | 15 | 45 |
| 3 | Asp | 2 | 3.6 | 0.36 | 0.5 | 14 | 53 |
| 4 | Asp | 4 | 3.6 | 0.36 | 0.5 | 22 | 35 |
| 5 | Asp | 1 | 7.2 | 0.71 | 0.5 | 14 | 51 |
| 6 | Asp | 2 | 7.2 | 0.71 | 0.5 | 18 | 40 |
| 7 | Asp | 4 | 7.2 | 0.71 | 0.5 | 24 | 31 |
| 8 | Asp | 1 | 3.6 | 0.36 | ($NH_4Br$) 0.5 | 17 | 43 |
| 9 | Asp | 1 | 3.6 | 0.36 | 10 | 100 | 80 |

*based on starting concentration of amino acid (5 mM)

NaBr was used as a bromide source in the reaction. However, others found the optimal reaction conditions using $NH_4Br$ as bromide source.[81,93] After changing NaBr to $NH_4Br$ (Table 2.1, entry 8) and comparing the conversion of Asp and selectivity to AspCN no significant change was observed. This shows that at low concentration of bromide source the nature of the cation does not significantly affect the reaction. Only on increasing the amount of NaBr from 0.5 mM to 10 mM (Table 2.1, entry 9) could 100% conversion and 80% selectivity towards AspCN be achieved after 1 h. From this it is concluded that the bromide concentration plays a significant role in the reactivity of Asp.

This effect of the bromide concentration was not previously observed probably due to much higher concentrations of bromide used.[81,82,93] To confirm this observation, experiments with the $Br^+$ species generated using NaOCl and different amounts of NaBr were also performed (Figure A.3). These experiments confirmed that the higher the concentration of NaBr used, the higher the conversion and the selectivity towards the nitrile. Thus the concentration of NaBr in the reaction plays an important role in the conversion of amino acids towards nitriles and not necessarily the $Br^+$ equivalents present as was reported previously.[82]

By increasing the concentration of NaBr also the ionic strength of the solution is increased which could influence the reactivity of the amino acid. The influence of the ionic strength of the solution was investigated further.

### 2.3.2 The influence of ionic strength on the reactivity of Glu & Asp

In this section, the hypothesis that an increase in concentration of NaBr would increase the ionic strength of the solution and influence the reactivity of amino acids[99] was investigated. This is especially true for the amino acids with charged functionalities on the side chain.[100]

The effect of the ionic strength of the solution on the reactivity of Asp was studied by doping the reaction mixture with anions other than bromide, namely fluoride and chloride.

Here reactions at fixed concentration of bromide (0.5 mM) in the presence of fluoride and chloride were performed for both Asp and Glu. This ensures that a catalytic amount of bromide is present and that the ionic strength of the solution is increased by the addition of the other anions. For comparison, control reactions with only one halide present were performed.

Fluoride cannot be oxidised by $H_2O_2$ to the $F^+$ species due to a higher redox potential. Therefore, fluoride increases the ionic strength of the solution while not generating a reactive species. Although chloride is a substrate for VCPO and produces a reactive $Cl^+$ species that can react with the amino acid to form nitriles,[96] the reactivity of $Br^+$ species is superior compared to that of $Cl^+$.[101] Hence, when chloride is added with the bromide to the reaction mixture, mainly an ionic effect is expected. The minor effect due to the reactivity of chloride can be subtracted using the control reactions containing only chloride. The conversion and selectivity of Asp as a function of halide type and concentration are presented in Figure 2.2.

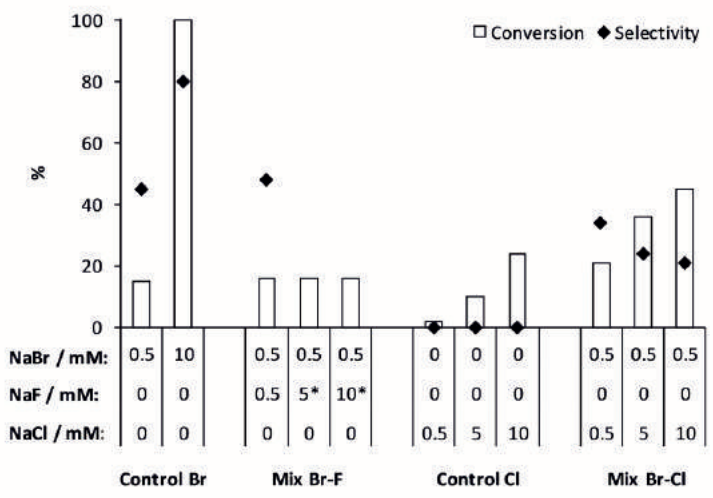



Figure 2.2. Conversion of aspartic acid (Asp) and selectivity towards 2-cyanoacetic acid (AspCN) as function of halides concentration. *detection of AspCN not available due to NaF interference with the analysis method. The data represent results from single experiment.

In the presence of NaF the conversion of Asp has the same value as at low concentration of NaBr (ca.15%). This remains unchanged with an increasing concentration of NaF. Thus, addition of NaF increases ionic strength but does not influence the reactivity of Asp. It was observed that in the control reactions when only NaCl is present, Asp is converted but with no selectivity towards AspCN. This may be explained by the lower reaction rates in chlorination reactions[101] which could promote other oxidation reactions of amino acids.

The conversion of Asp is dependent on the concentration of NaCl and follows the same trend as Glu in presence of NaCl (Figure A.4). By subtracting the control reactions with chloride from the conversion of Asp in mixtures of NaCl and NaBr, it can be observed that

the conversion of Asp is not influenced by the presence of NaCl. The same value is observed as at low concentration of NaBr (ca. 15%). In contrast, the selectivity towards AspCN using both bromide and chloride is decreasing with the increase in the concentration of NaCl. It can be concluded that the oxidative decarboxylation reaction is dependent on the nature of the halide and that by using chloride the selectivity favours other oxidation products. This was observed before for phenylalanine which in presence of NaCl was converted to the corresponding aldehyde.[59] Glu, however, remains a special case where high selectivity towards nitriles can be maintained even in presence of chloride (Figure A.4).

The hypothesis that a higher ionic strength could influence the reactivity of Asp to the corresponding nitrile was investigated in this section. Anions other than bromide, fluoride or chloride, were added to increase the ionic strength. However, in the presence of these anions the reactivity of Asp was not increased. It can be concluded that not the ionic strength is influencing the reaction, but the type of halide and its affinity for the amino acid. This suggests that NaBr is involved not only in the formation of the reactive $Br^+$ species by VCPO, but has close interaction with the amino acid which influences the conversion of amino acids into nitriles. It is speculated that non-covalent interactions between NaBr and the amino acid could influence the reactivity of amino acids.

### 2.3.3 Oxidative decarboxylation of a mixture of Glu and Asp

Based on the results obtained for individual amino acids it was hypothesised that varying the concentration of NaBr a selective oxidative decarboxylation for mixtures of Glu and Asp could be achieved. By increasing the concentration of NaBr during the reaction time, it is proposed that Glu should initially be converted followed by the conversion of Asp. This would allow a selective reactive conversion and simplify the downstream processing within mixtures. To investigate this, the oxidative decarboxylation reaction was performed with an equimolar mixture of Glu and Asp with a low initial concentration of NaBr (0.5 mM). After 30 min the concentration of NaBr was increased up to 10 mM.

The change in concentration of the substrates and of the products as a function of time is presented in Figure 2.3. Surprisingly, after 10 min of reaction, the conversion of both Asp and Glu does not proceed further. This was observed before in case of individual reaction of Asp when low concentration of NaBr was used which indicates that there is a shortage of NaBr in solution. Indeed, a further addition of NaBr up to 10 mM at 30 min resulted in further conversion of both amino acids. Complete conversion was achieved for both acidic amino acids after a total reaction time of 60 min. The ratio between the two nitriles at full conversion for a equimolar mixture of Glu and Asp is similar to that reported by the electrochemically generated $Br^+$.[93]

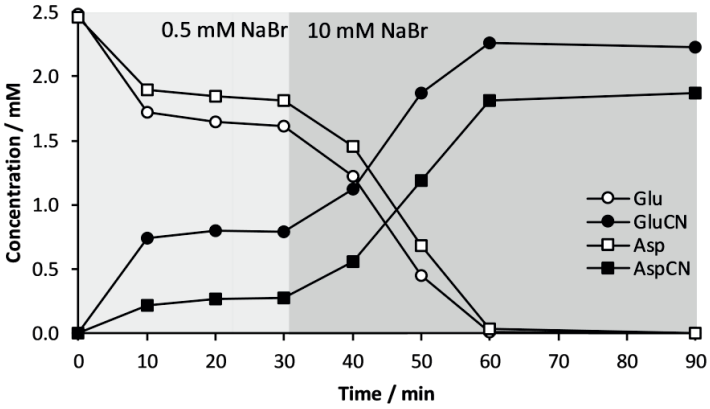



Figure 2.3. Oxidative decarboxylation of mixture of glutamic acid (Glu) and aspartic acid (Asp) (Glu:Asp = 1:1, molar ratio; 5 mM amino acid in total). The reaction was started with 0.5 mM NaBr followed by addition of extra 9.5 mM NaBr after 30 min.

It can be observed that both acidic amino acids follow the same trend during the reaction which is contrary to the selective reactive conversion that was expected. This shows that the hypothesis formulated above is not valid and that competition between the two acidic amino acids exists. It can be seen in Figure 2.3, the selectivity of Glu at both low and high concentration of NaBr are higher than those of Asp, which corresponds to the reactions of individual amino acids. This illustrates that Glu is more reactive and more selective for nitrile formation. When only Glu is present in the reaction NaBr is reused (catalytic) by VCPO as shown in Scheme 2.1. Remarkably, when Asp is present in the reaction NaBr is not reused by VCPO. It appears that NaBr is not available in the reaction mixture and that could be consumed by side reactions such as bromination or scavenged/rendered unreactive by other means. If NaBr is consumed in other bromination reactions, VCPO cannot further produce the reactive species, $Br^+$, and no more nitriles are formed until extra NaBr is supplied. The possibility of bromide depletion by side reactions *via* bromination was investigated further.

### 2.3.4 Bromide consumption by side reactions

The possibility of bromide consumption by side reactions *via* bromination was studied. For this the conversion of Asp was performed at high concentration of NaBr in order to achieve higher conversions which would allow the identification of possible side products. Furthermore, stability tests of the identified products were performed under the oxidative decarboxylation reactions.

Initially the conversion of Asp was followed in time at high concentrations of NaBr (10 mM) as presented in Figure 2.4. It can be observed that, the concentration of Asp is decreasing and the concentration of AspCN is increasing. Asp is fully converted in about 50 min and the amount of nitrile formed corresponds to a selectivity of 80%. When sufficient bromide (10

mM NaBr) is present in the reaction, Asp can reach full conversion. The reaction rate of Asp with 10 mM NaBr is 0.12 mM/min and this is somewhat lower than that of Glu at 0.5 mM NaBr (0.15 mM/min). It is known that VCPO is inhibited by bromide[80] and this could be the cause of the lower reaction rate observed for Asp, however, this was not investigated further.

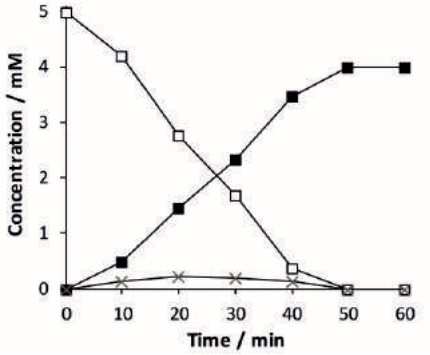
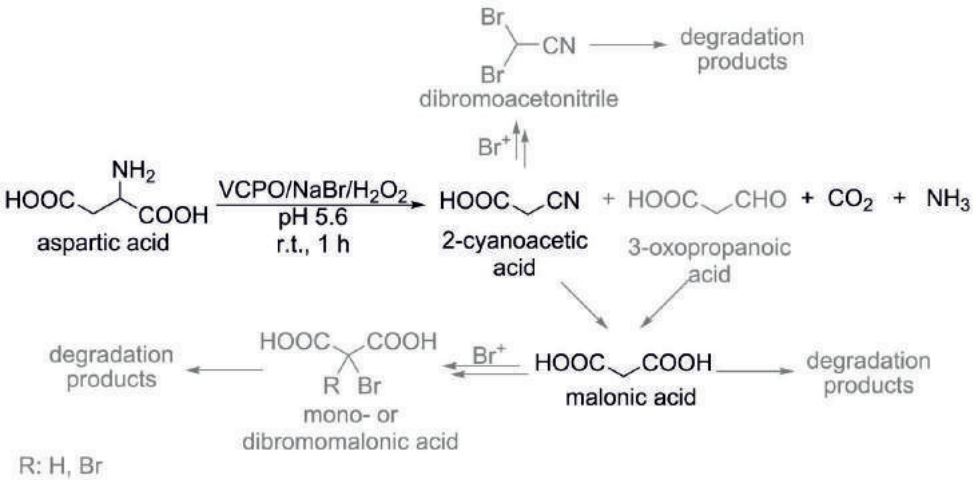



Figure 2.4. Aspartic acid (Asp) conversion into 2-cyanopropanoic acid (AspCN) by vanadium chloroperoxidase (VCPO) at 10 mM NaBr. Reaction rate of Asp is 0.12 mM/min. □ aspartic acid, ■ 2-cyanoacetic acid, × malonic acid.

Malonic acid was identified as a by-product of the oxidative decarboxylation of Asp (Figure 2.4). Its formation as a by-product by photolysis of Asp has also been reported.[102] However, bromide depletion cannot be explained by formation of malonic acid.

Figure 2.4 also shows that in the first minutes of the reaction the concentration of malonic acid is slowly increasing followed by a decrease until malonic acid is no longer detectable. This trend suggests that malonic acid is formed at the beginning of the reaction and that it is consumed in a secondary reaction. During the oxidative decarboxylation of Asp, another compound was detected by HPLC next to AspCN and malonic acid but this could not be identified either by HPLC or LC-MS. The formation of singlet oxygen could result in such reactions however this was not investigated. An overview of the (possible) side products of the oxidative decarboxylation of Asp is presented in Scheme 2.3.




Scheme 2.3. Oxidative decarboxylation of aspartic acid and the possible side products (with black – identified compounds, with grey – unidentified compounds or unproved pathways).

It is known that the aldehyde is the most likely by-product formed during the oxidative decarboxylation of amino acids.[65,96] As mentioned earlier, the corresponding aldehyde of Asp would be 3-oxopropanoic acid. However in presence of oxidising reagents such as $Br^+$ it could be converted to the corresponding dicarboxylic acid and it is expected that 3-oxopropanoic acid is an unstable and reactive compound. It was hypothesised that malonic acid originates from the oxidation of the aldehyde rather than the hydrolysis of the nitrile. For this the stability of AspCN was investigated under the oxidative decarboxylation reaction conditions, in presence of $Br^+$ species and of $H_2O_2$ (Figure A.5.a). After one hour the concentration of AspCN decreased by a maximum of 5%. However, the formation of malonic acid was not detected. Moreover, the hydrolysis of the AspCN would require more acidic conditions[103] than that used in this reaction (pH 5.6).

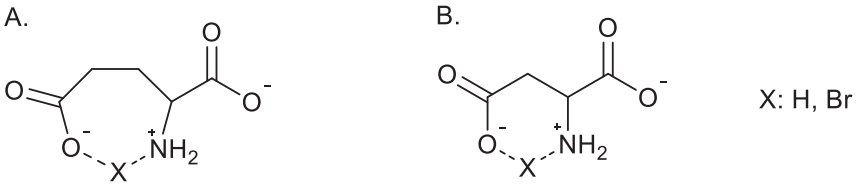
Malonic acid, AspCN and 3-oxopropanoic acid, all have two acidic hydrogen atoms at α-carbon which opens the possibility for side reactions to occur in the presence of halogenating reagents. It is possible that some of the bromide might be consumed by the bromination of these compounds to form mono- or dibrominated by-products.[104,105] Additionally to the stability of AspCN, the stability of malonic acid was investigated under the oxidative decarboxylation reaction conditions in presence of different amounts of bromide (Figure A.5.b). This showed that during one hour in presence of $Br^+$ species, malonic acid is a stable compound. It can be concluded that the bromide is not consumed by the bromination of malonic acid but it could be consumed by the bromination of AspCN or 3-oxopropanoic acid. To identify any brominated compounds in the reaction solution after the oxidative decarboxylation of Asp, LC-MS analyses were performed. However, analysis did not reveal any brominated compounds. It has been reported that AspCN can form dibromoacetonitrile in presence of *N*-bromosuccinimide.[105] Nonetheless, it was shown that dibromoacetonitrile is not a stable compound in presence of reducing reagents such

as sodium thiosulfate[106] which was used to quench the oxidative decarboxylation reaction (see Experimental). Therefore, to confirm the possibility of depletion of bromide by bromination reactions further investigation is necessary. For example, larger scale reactions would increase the amount of by-products and allow easier identification, as well as using a different method than sodium thiosulfate to terminate the reaction. Although bromination of products is possible, currently there is no evidence to support this hypothesis.

Recent publications speculate that the presence of functional groups at the β-carbon of the amino acid diminishes the reactivity of that amino acid, due to electronic effects closer to the reaction site[81] or a chelating effect towards the metal catalyst.[94] This suggests that the structure of the amino acid and possibly the conformation adopted in solution is responsible for the low reactivity of Asp.

Such differences in reactivity of two structurally similar amino acids may be explained by different molecular interactions of NaBr with Asp compared to Glu. This could be due to different conformations of the two acidic amino acids in solution. Hence, intra- and/or intermolecular interactions may become important.

Stabilisation of molecules is possible due to electrostatic or dipolar interactions such as carbonyl-carbonyl,[107,108] n->π* interactions and H-bonding between the two carboxylic groups or between the amine and carboxylic side chain[109–112] which could lead to ring formation (Scheme 2.4). Stabilisation by ring formation has been suggested previously for *N*-brominated amino acids such as *N*-bromo-β-alanine and *N*-bromo-γ-aminobutyric acid.[113] This behaviour could be possible for both Glu and Asp and is illustrated in Scheme 2.4. A six membered ring formed by Asp would be more stable compared to a seven membered ring formed by Glu. Additionally, the possibility of forming a ring is higher for Asp for which the amino and the γ-carboxylic group are closer in the open structure and oriented on the same side of the molecule.




Scheme 2.4. Proposed ring structure of A. glutamic acid or of *N*-brominated glutamic acid and B. aspartic acid or *N*-brominated aspartic acid.

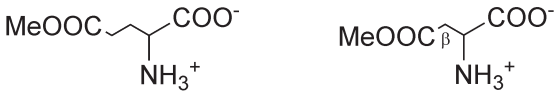
From spectroscopic analysis in the gas phase of conformers of amino acids, such as γ-aminobutyric acid, it was concluded that the stabilisation by H-bonding decreases with increase in the carbon chain length between -$NH_2$ and –COOH.[114] Thus Glu, which has an extra methylene group in its side chain, is not or is less stabilised than Asp. Therefore, full conversion of Glu can be achieved by using only catalytic amounts of NaBr. In contrast,

higher concentrations of NaBr were required for complete conversion of Asp. It can be hypothesised that either the increase in bromide ions or the increase of the ionic strength of the solution could break or weaken the interactions which stabilise Asp. The influence of ionic strength on the reactivity of Asp was presented earlier (Figure 2.2). This showed that the ionic strength of the solution does not influence the reactivity of Asp and Glu.

Intramolecular interactions can be investigated by Raman spectroscopy.[115] The Raman measurements of aqueous solutions of Glu and Asp in presence and absence of NaBr resulted in very weak Raman signals (results not presented). This is due to the low solubility of Glu and Asp in aqueous solutions.[115] Therefore other methods should be used to investigate such interactions. An indirect way to investigate the role of bromide in the conversion of Asp is by the use of computational methods. This would allow the simulation of possible conformers which could reveal more insights on the low reactivity of Asp and the role of bromine in the oxidative decarboxylation of amino acids. As most of such studies are performed just in vacuum it is advised to conduct this investigation also in aqueous media.

It may be concluded that the difference in reactivity between Asp and Glu, two similar amino acids, are likely to be due to inter- and/or intramolecular interactions between the functionalities of the amino acids.

The possibility of intramolecular interaction as presented in Scheme 2.4 was further explored experimentally. For this, glutamic acid γ-methyl ester (MeGlu) and aspartic acid β-methyl ester (MeAsp) (Scheme 2.5) were used. By derivatisation of the ω-carboxylate, it is proposed that inter- and/or intramolecular interactions may be influenced.




Scheme 2.5. Glutamic acid γ-methyl ester, MeGlu (left) and aspartic acid β-methyl ester, MeAsp (right).

Individual oxidative decarboxylation reactions of MeGlu and MeAsp were followed in time and compared with results of individual reaction of Glu and Asp under the same reaction conditions (Figure 2.5).

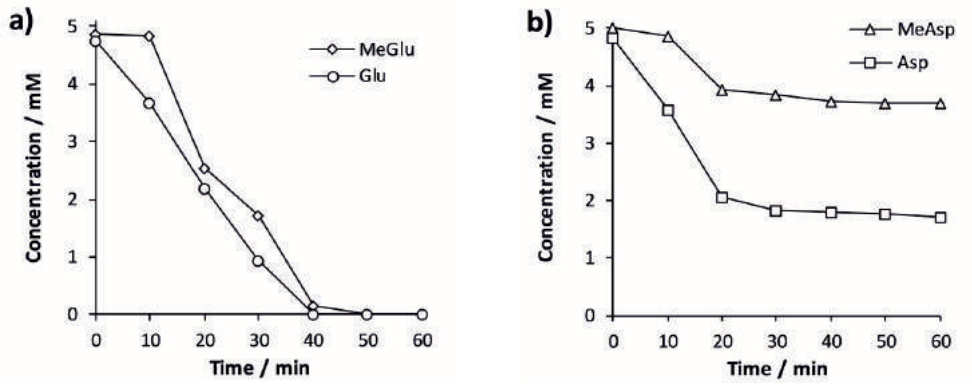



Figure 2.5. The conversion of a) glutamic acid (Glu) and glutamic acid γ-methyl ester (MeGlu) as average data of duplicate experiment and b) aspartic acid (Asp) and aspartic acid β-methyl ester (MeAsp) by oxidative decarboxylation using VCPO at 2 mM NaBr as a function of time.

In Figure 2.5.a the concentration of the MeGlu is constant for the first 10 min of reaction and then starts to decline. MeGlu reached full conversion after 50 min of reaction. However, the concentration of Glu declines linearly, reaching full conversion after 40 min. In Figure 2.5.b, the initial ca. 10 min of reaction of MeAsp displays a similar trend to MeGlu. From 20 to 60 min the concentration of MeAsp remains unchanged. It can be seen that the reaction of MeAsp stops after 20 min as it was observed also for Asp. At 2 mM NaBr, both MeAsp and Asp do not achieve full conversion in the same way as experiments of Asp at 0.5 mM NaBr (Figure 2.1.b).

By derivatising the side chain carboxylic functionality to disrupt conformations as shown in Scheme 2.4, the reactivity of both amino acids shows a delay in reactivity. This is more pronounced for MeAsp, which has a shorter carbon chain. This would suggest that the reactive groups around the α-carbon are now compromised. It can be further speculated that the length or the functionality present on the side chain could influence the reactivity of amino acids, however this is the focus of future research.

## 2.4 Conclusions

The aim of this chapter was to gain insight in the remarkable difference in the reactivity of chemically similar amino acids regarding the enzymatic oxidative decarboxylation towards the nitrile formation.



Here the conversion of glutamic acid (Glu) and aspartic acid (Asp) was investigated as a function of bromide concentration. In presence of catalytic amount of bromide (0.1 equiv.), Glu resulted in full conversion and high selectivity. In contrast, under the same reaction conditions Asp showed low conversion and selectivity towards the nitrile. It was shown that by increasing the amount of bromide present in the reaction mixture to 2 equiv., the conversion of Asp was increased from 15% to 100% and its selectivity towards 2-cyanoacetic acid (AspCN) from 45% to 80%. The data indicate that during the conversion at low bromide concentration, bromide was scavenged from the reaction mixture by unknown mechanisms. It was shown that the ionic strength of the solution was not responsible for the higher conversion of Asp at higher bromide concentrations. However, the type of halide is influencing the reactivity of amino acids towards oxidative decarboxylation. Experiments using amino acids with the derivatised side chain functionality such as glutamic acid γ-methyl ester and aspartic acid β-methyl ester showed delay in reactivity compared to the acidic amino acids, Glu and Asp. It was concluded that most likely the difference in reactivity between Asp and Glu, must be due to inter- and/or intramolecular interactions between the functionalities of the amino acids. Using this knowledge, Asp can be converted with high selectivity into AspCN, a potential intermediate for the production of biobased functionalised chemicals such as cyanoacetates.

# Chapter 3

# The effect of amino acid side chain length and functionality on oxidative decarboxylation



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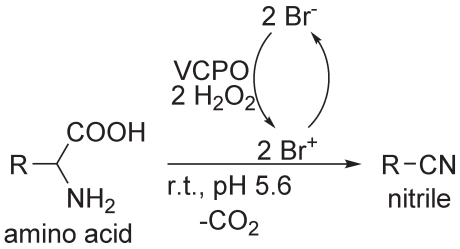
## Abstract

The production of biobased nitriles by oxidative decarboxylation of amino acids using chemo-enzymatic catalysis was investigated. Up to now, the effect of the side chain functionality of amino acids on the conversion towards nitrile by oxidative decarboxylation was not fully understood. Amino acids with different side chain functionalities (aliphatic, hydroxyl, carboxyl and methyl ester) and different carbon length (3C-6C) were investigated. It was shown that while functional groups like aliphatic, hydroxyl or methyl ester show no significant influence on the selectivity of amino acids, the carboxyl functionality has a positive effect during the initial part of the oxidative decarboxylation reaction. Additionally, the position of the functionality on the carbon chain of the amino acids plays an important role in the conversion to nitriles. The initial reaction rate for aspartic acid (-COOH side group) was 0.125 mM/min, while for homoserine (-OH side group) was 0.030 mM/min and for α-aminobutanoic acid (aliphatic side chain) was slightly lower (0.020 mM/min). It is proposed that a self-catalysis mechanism occurs between the side chain carboxyl and the amino functionality during the intermediate bromination steps.

## 3.1 Introduction

To reduce environmental pressure due to processes using fossil feedstocks,[26] amino acids from bioderived rest streams have been proposed as a biobased alternative to produce nitrogen containing chemicals such as nitriles.[42,44] Oxidative decarboxylation of amino acids can give access to biobased nitriles. These include drop-in chemicals (acetonitrile, acrylonitrile) and speciality or dedicated chemicals (isobutyronitrile, 2-cyanoacetatic acid).

A number of catalytic oxidative decarboxylation reactions of amino acids have been described.[59,81,93,94] The majority of catalytic methods rely on the *in situ* generation of $Br^+$ species.[59,81,93] Among these is the enzymatic conversion of amino acids[59] which uses the enzyme vanadium chloroperoxidase (VCPO), $H_2O_2$ and $Br^-$ to generate *in situ* HOBr or $Br^+$ species.[66] Theoretically, two equivalents of the $Br^+$ species react further with the amino acid to yield the corresponding nitrile and to regenerate the $Br^-$ (Scheme 3.1).[116]




Scheme 3.1. Enzymatic oxidative decarboxylation of amino acids to nitriles.

The conversion and selectivity towards the nitriles is influenced by several parameters such as pH, temperature, the nature of the halide and the nature of the amino acid. It is already known that next to the nitrile, the corresponding aldehyde is formed from amino acids by oxidative decarboxylation followed by deamination of a reaction intermediate.[65,96] Mildly acidic conditions[81,82] and lower temperatures[82] are more favourable for the production of nitriles. Using VCPO, full conversion and high selectivity was achieved for glutamic acid (Glu) and phenylalanine at room temperature and slightly acidic conditions (pH of 5.6).[59] It was reported that the choice of halide is important for the oxidative decarboxylation of amino acids and that bromide stimulates the production of nitriles.[59,116]

While the effects of pH, temperature and halide on the reactivity of amino acids have already been reported, the influence of the side chain functionality has been investigated less. In Chapter 2, it was shown that by using VCPO, Glu rapidly reaches full conversion with excellent nitrile selectivity using only catalytic amounts of bromide.[59,116] Under the same reaction conditions aspartic acid (Asp), an amino acid with a similar chemical structure, resulted in low conversion (~15%) and selectivity (45%).[116] Only, by increasing the concentration of bromide up to stoichiometric amounts, high conversion (100%) and selectivity (~80%) were achieved. It was proposed that the difference in reactivity between Asp and Glu is due to intramolecular interactions between the side chain carboxylic group

and the α-amino group which could lead to stabilisation by ring formation.[116] Further, reactions with ω-carboxyl groups of Asp and Glu blocked as methyl ester showed a decrease in conversion, in particular for Asp. It was concluded that the length of the side chain and the apparent hindrance by space (steric effect) of the side functionality must influence the reactivity of amino acids.

For aliphatic amino acids, it was found that by increasing the length of the carbon side chain by one or two carbons, the selectivity towards the nitrile also increases by a few percentages.[82,117] Also others observed deviations in the reactivity of amino acids. For example, using a W-based heterogeneous catalyst, amino acids with hydroxy functionality (serine, threonine) or branched aliphatic amino acids (valine, isoleucine) resulted in lower conversion compared to linear amino acids.[81] In this case the reduced conversion towards oxidative decarboxylation was attributed to the presence of functionality at the β-carbon of the amino acid. Up to now, the effect of the side chain functionality and its position on the carbon chain of the amino acid on the conversion and nitrile selectivity has not been fully understood.

The aim of this research is to understand the influence of the side chain length and functionality on the reactivity of amino acids to nitriles by oxidative decarboxylation. Using VCPO as means to generate the $Br^+$ species *in situ*, amino acids with different side chain length and functionality were converted at different concentrations of bromide into their corresponding nitriles. The differences in selectivity towards nitriles and the conversion of amino acids with carboxylic and hydroxyl functionalities were compared with aliphatic groups. The reactivity of amino acids with methyl ester protected side chain carboxylic functionality was compared to unprotected acidic amino acids.

## 3.2 Experimental

### 3.2.1 Materials

L-Glutamic acid (>98.5%), L-aspartic acid (≥98%), L-serine (≥99%), L-valine (98.5%), L-norvaline (≥99%), L-norleucine (≥98%), glutamic acid γ-methyl ester (98.5%), malonic acid (99%), 2-cyanoacetic acid (≥99%), 4-cyanobutanoic acid (98%), hydroxyacetonitrile (70%), isobutyronitrile, isobutyraldehyde, NaBr (>99%), $H_2O_2$ (35 wt-%), citric acid, $Na_3VO_4$, and Trizma base were purchased from Sigma-Aldrich. *n*-Butyronitrile (99%), propionitrile (99%), 3-hydroxypropionitrile (99%) were purchased from Acros Organics and L-homoserine, L-2-aminobutyric acid, L-2-aminoadipic acid and valeronitrile (99%) from Alpha Aesar. 3-Cyanopropanoic acid (95.9%) was provided by Interchim and monochlorodimedone by BioResourse Products. Aspartic acid β-methyl ester hydrochloride was purchased from Sigma-Aldrich and before use HCl was removed using AG 4-X4 Resin from Bio-Rad.

### 3.2.2 Enzyme

The enzyme vanadium chloroperoxidase (VCPO) was expressed and purified using a protocol described elsewhere.[116] The VCPO stock had an activity of 92 U/mL (based on MCD assay).

### 3.2.3 MCD assay

In a UV disposable cuvette were added 1 mL of MCD mix (50 µM MCD, 1 mM $H_2O_2$, 0.5 mM NaBr, 100 µM $Na_3VO_4$, 50 mM citrate buffer pH 5.5) and 10 µL of VCPO with appropriate dilution. The bromination of monochlorodimedone (MCD) was followed in time at 290 nm.

### 3.2.4 Standard procedure for oxidative decarboxylation of amino acids by VCPO

A typical procedure is as follows: in a glass vial 5 mM of amino acid, 0.5 mM of NaBr, 0.36 U/mL (90 nM) of VCPO and 20 mM citrate buffer pH 5.6 contained in a total volume of 2 mL were stirred at 400 rpm, at room temperature (21°C). To this reaction mixture 16 mM $H_2O_2$/h (66 µL of 0.5 M $H_2O_2$/h) were added using a NE-1600 syringe pump from ProSense. The reaction was stopped after 1 hour by adding $Na_2S_2O_3$. Samples were taken from the reaction mixture for HPLC analysis. For the time course reactions, individual vials were prepared for each time point. Deviations from this procedure are mentioned in the caption of figures.

### 3.2.5 HPLC

Amino acids were analysed by derivatisation as previously described.[59] Nitriles were analysed without dilution or derivatisation by using an UltiMate 3000 from Thermo Scientific. Detection was achieved using a RI-101 detector from Shodex set at 35°C. The columns used were a Rezex ROA Organic acid H+ (8%) column (7.8 × 300 mm) from Phenomenex, at 35°C with a flow of 0.5 mL/min for 3-cyanopropanoic acid, 2-cyanoacetic acid, 4-cyanobutanoic acid, hydroxyacetonitrile and 3-hydroxypropionitrile; and an Acquity UPLC® BEH C18 column (2.1 × 150 mm, 1.7 µm particle size) at 80°C with a flow of 0.35 mL/min for isobutyronitrile, isobutyraldehyde, *n*-butyronitrile, propionitrile. The elution was carried out with 12 mM $H_2SO_4$ aqueous solution and the quantification was performed by external standard method.

## 3.3 Results & discussions

To explore the influence of the side chain length and functionality, amino acids with different carbon length (3C-6C) were selected from three different categories: with aliphatic, hydroxyl, acidic and methyl ester functionality (Table 3.1).

Table 3.1. Characteristics of amino acids and the corresponding nitriles.

| Amino acid | Amino acid code | Side chain functionality | Carbon number | Nitrile |
|---|---|---|---|---|
| α-Aminobutanoic acid | Aba | $-CH_2-CH_3$ | 4 | Propionitrile |
| Norvaline | nVal | $-(CH_2)_2-CH_3$ | 5(b*) | Butyronitrile |
| Valine | Val | $-CH-(CH_3)_2$ | 5 | Isobutyronitrile |
| Norleucine | nLeu | $-(CH_2)_3-CH_3$ | 6 | Valeronitrile |
| Serine | Ser | $-CH_2-OH$ | 3 | Hydroxyacetonitrile |
| Homoserine | hSer | $-(CH_2)_2-OH$ | 4 | 2-Hydroxypropionitrile |
| Aspartic acid | Asp | $-CH_2-COOH$ | 4 | 2-Cyanoacetic acid |
| Glutamic acid | Glu | $-(CH_2)_2-COOH$ | 5 | 3-Cyanopropanoic acid |
| α-Aminoadipic acid | Aaa | $-(CH_2)_3-COOH$ | 6 | 4-Cyanobutanoic acid |
| Aspartic acid β-methyl ester | MeAsp | $-CH_2-COOMe^{\bullet}$ | 4+1 | Methyl ester of 2-cyanoacetic acid |
| Glutamic acid γ-methyl ester | MeGlu | $-(CH_2)_2-COOMe^{\bullet}$ | 5+1 | Methyl ester of 3-cyanopropanoic acid |

* b = branched, $^{\bullet}$Me = methyl group

### 3.3.1 The influence of the side chain length and functionality on reactivity of amino acids

Previously it was found that Glu can be fully converted in presence of catalytic amounts of NaBr (0.5 mM). In contrast Asp required higher concentrations of NaBr (10 mM) to be fully converted.[116] To explore the oxidative decarboxylation reaction of the amino acids with aliphatic, hydroxyl and acidic functionality, a range of concentrations of NaBr (0.5-20 mM) was selected. To avoid possible inhibition of VCPO by halide ions[66] the concentration of NaBr was kept below 20 mM. The conversion of each amino acid and the selectivity towards the nitrile were registered after one hour of reaction and plotted at different concentrations of NaBr in Figure 3.1.A-D.

From Figure 3.1.a it can be observed that at 0.5 mM NaBr the amino acids with aliphatic and hydroxy side chain functionality reached on average about 40% conversion and between 50% to 60% selectivity. Among the three acidic amino acids tested only two, Glu and Aaa, were fully converted within 1 h and have excellent nitrile selectivity. Remarkably lower conversion and selectivity was observed for Val and Asp, compared to other amino acids with aliphatic or acidic functionality. Among the amino acids with hydroxy functionality, hSer, which has 4 carbons, achieved lower selectivity (43%) than Ser (56%), which has 3 carbons in the molecule.

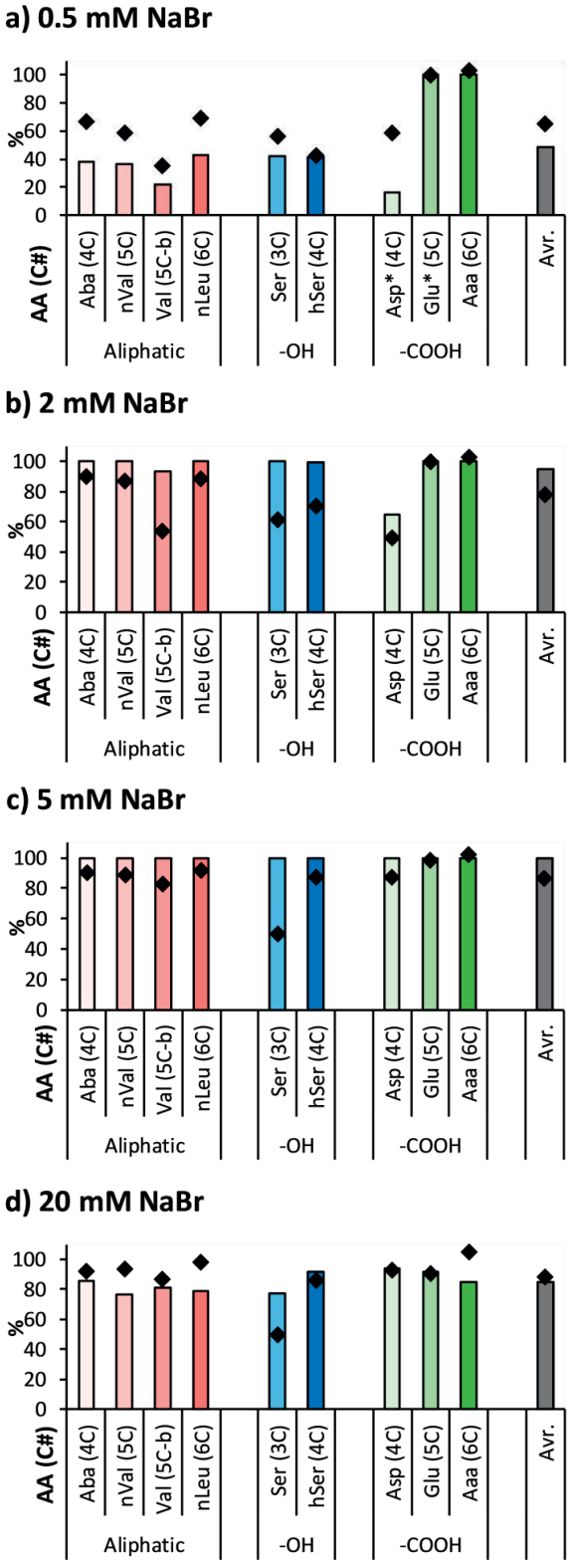



Figure 3.1. Amino acids conversion and selectivity into nitriles as a function of the concentration of NaBr after 1 hour reaction time. The concentration of NaBr was a) 0.5 mM, b) 2 mM, c) 5 mM and d) 20 mM. Bars represent conversion and ◆ selectivity. The data represent the average of at least two individual experiments except for nVal and hSer at 5 and 20 mM NaBr which are single data points. Experiments with 10 and 15 mM NaBr can be found at Appendix B (Figure B.1). *data replicated according to[116] (own work). AA = amino acid, C# = carbon number.

At 2 mM NaBr almost all amino acids, except Val and Asp, reached full conversion (Figure 3.1.b). As the concentration of NaBr was increased by 4-fold (from 0.5 to 2 mM) the conversion of Val and Asp also increased by 4-fold (from 22% to 93% for Val and from 16% to 65% for Asp). From Figure 3.1.b it can be observed that the selectivity of the linear aliphatic amino acids (Aba, nVal and nLeu) is ca. 88% while that of the amino acids with hydroxyl side chain is lower at ca. 65%.

At 5 mM NaBr all amino acids, including Val and Asp, were fully converted (Figure 3.1.c) and Val, Ser and Asp result in the lowest selectivity. The reactivity of amino acids was also performed at 10 and 15 mM NaBr (Figure B.1). Overall, a peak in conversion was observed at 5 and 10 mM NaBr for all amino acids, except Glu and Aaa. At higher concentrations of NaBr (15 and 20 mM) the conversion of all

amino acids drops. The average conversion of all amino acids dropped from 100% at 5 mM NaBr (Figure 3.1.c) to 85% at 20 mM NaBr (Figure 3.1.d). It can be observed that at higher concentrations of NaBr, amino acids with longer carbon chain (nLeu, Aaa) have not reached 100% conversion which indicates a lower enzymatic activity. The drop in conversion at high concentrations of NaBr is attributed to inhibition of VCPO by NaBr,[118] combined with a possible inhibition of VCPO created by amino acids with long carbon chain.

Although at high concentrations of NaBr, the conversion after 1 h of reaction is lower due to lower reaction rates, the selectivity is maintained at high levels (on average 88%). It seems that the high concentration of NaBr has a beneficial effect on the selectivity towards the nitriles, especially on the aliphatic amino acids.

Similar results were obtained by the use of a W-based heterogeneous catalyst[81]: high selectivity towards nitriles (99%), full conversion for Glu and most aliphatic amino acids and, lower conversions for the branched aliphatic amino acids (Val: 92%, Ile: 95%) and the amino acids with hydroxyl functionality (Ser: 95%, hSer: 87%). The use of a Ru catalyst and $O_2$ in the absence of a halide salt resulted as well in large difference in conversions between aliphatic functionalities (ca. 99%) and hydroxyl (ca. 50%) or acidic (Glu: 80%, Asp: 18%) and selectivity around 80%.[94]

Previously it was found that reaching full conversion using VCPO, catalytic amounts of NaBr can be used in case of Glu but equimolar amounts of NaBr are required for Asp.[116] In this chapter it was observed that only the acidic amino acids with long carbon chain, Glu and Aaa, were fully converted in presence of catalytic amounts of NaBr (0.5 mM). To confirm that NaBr is indeed required in catalytic amounts, reactions with 0.2 mM NaBr and in the absence of NaBr were performed for Glu and Aaa. At 0.2 mM NaBr, both Glu and Aaa reached full conversion and high selectivity (Figure 3.2).

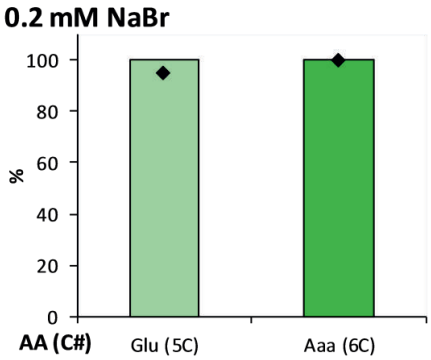



Figure 3.2. The conversion of glutamic acid (Glu) and aminoadipic acid (Aaa) into nitriles at 0.2 mM NaBr after 1 hour reaction time. Bars represent conversion, ◆ selectivity, AA = amino acid, C# = carbon number.

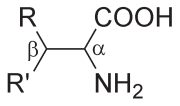
When no NaBr is present in solution the reaction does not occur (results not shown). It can be concluded that NaBr is indeed required for the conversion of Glu and Aaa and that the

conversion towards nitriles is proceeding *via* bromide/bromine species as shown previously.[65]

Despite its apparent chemical similarity with Glu and Aaa, Asp is the only acidic amino acid tested that cannot be fully converted in presence of catalytic amounts of NaBr. Additionally among all amino acids, at the highest NaBr concentration tested (20 mM) Asp has the highest conversion (~94%) and maintains high selectivity. The only significant difference between Asp, Glu and Aaa is the length of the side chain and the position of the carboxylic functionality on the carbon chain.



Previously, it was speculated that the presence of functionality at the β-carbon (Scheme 3.2) is reducing the reactivity of amino acids.[81] This was also observed here for Val, Ser and Asp at different concentrations of NaBr tested (Figure 3.1). It was sugested[81] that an electron donating group at the β-carbon such as methyl (Val) and hydroxyl (Ser) will influence the selectivity in a negative way. However, an electron withdrawing group, such as carboxyl in Asp or methyl ester in aspartic acid β-methyl ester (MeAsp) also reduces the reactivity of amino acids.[116] It can be concluded that the electronic effect of the side chain functionality is not likely to be responsible for the lower reactivity observed for Asp, nor for Ser and Val.




Val: R=$CH_3$, R'=$CH_3$,
Ser: R=H, R'=OH,
Asp: R=H, R'=COOH,
MeAsp: R=H, R'=COOMe.

Scheme 3.2. Structure of amino acids with different functionalities at β position; Me = methyl.

In Chapter 2 it was hypothesised that intra- and intermolecular interactions between the side chain functionality and the α-functionalities are responsible for the low conversion and selectivity of Asp.[116] Next to this, branching points in the carbon chain like in the case of Val can cause steric hindrance. At low concentrations of NaBr, steric hindrance could slow down the reaction rate between the $Br^+$ species and the amino acid and thus, promote by-product (aldehyde) formation. However, at high concentrations of NaBr the steric hindrance seems to be overcome by the positive effect of NaBr concentration on selectivity. It can be concluded that the reactivity at the α-functionalities of the amino acids will be influenced in a negative way through space (steric effect or/and intramolecular interaction) by the functionality present at the β-carbon.

The hypothesis of intramolecular interaction between the side chain carboxylic and the α-functionalities was briefly investigated[116] by derivatisation of the ω-carboxylate of two acidic amino acids, Asp and Glu (Figure 2.5). In Figure 3.3 the conversion of the two acidic amino acids which have the side chain carboxylic functionality blocked as methyl esters was further investigated at different concentrations of NaBr.

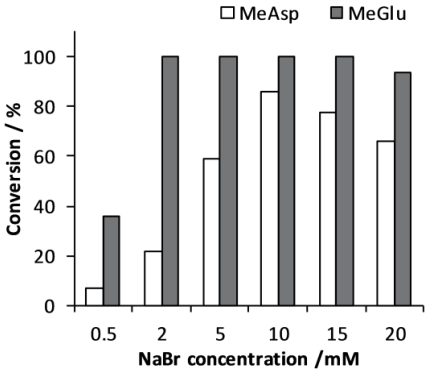



Figure 3.3. The conversion of MeGlu and MeAsp by oxidative decarboxylation as a function of the concentration of NaBr, after 1 h reaction time. *data taken from [12].

In Figure 3.3, the conversion of MeGlu at 0.5 mM NaBr is 36%. As the concentration of NaBr was increased to 2 mM, the conversion of MeGlu increased to 100% and it remains stable up to 15 mM NaBr. At 20 mM NaBr the conversion is declining by a few percentages to 93%. To reach full conversion of MeGlu a minimum concentration of 2 mM NaBr was required, while Glu was fully converted in presence of catalytic amounts of NaBr (0.2 mM, Figure 3.2). The conversion of MeGlu follows a similar trend to that of the amino acids with aliphatic and hydroxyl functionality at the side chain which is in contrast to that of Glu.

The conversion of MeAsp is slowly increasing (Figure 3.3) up to 10 mM NaBr where MeAsp is reaching a peak in conversion (86%). As the concentration of NaBr is increasing up to 20 mM the conversion of MeAsp is declining to 66%. The conversion trend observed for MeAsp is most similar to that of Asp and Val but the conversion of MeAsp is significantly lower. As full conversion was not reached for MeAsp it seems that the loss of the side chain carboxyl functionality is affecting the conversion even more than in case of MeGlu, perhaps due to the presence of the side chain functionality at the β-carbon.

The conversion of MeGlu and MeAsp at 2 mM NaBr (Figure 2.5), revealed a so-called induction period of 10 min before the reaction starts,[116] however, this was not shown before for the conversion of MeGlu by NaOCl and 10 mol% NaBr.[44] The induction period and the reaction rate of amino acids was therefore further investigated for all the other amino acids.

### 3.3.2 The reaction rate as a function of the side chain of amino acids

The induction period and the reaction rate of amino acids was followed in time at 2 mM NaBr. To exemplify, the reactions of two amino acids with five carbon atoms but different functionality, nVal (aliphatic) and Glu (carboxyl), are presented in Figure 3.4.

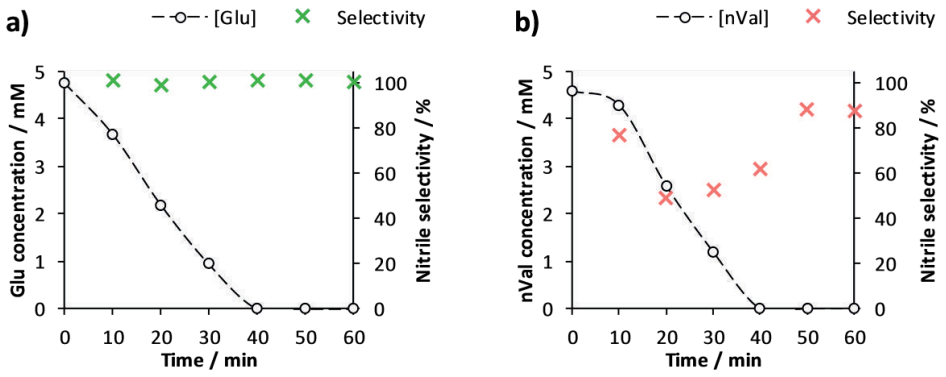



Figure 3.4. The conversion of a) glutamic acid (Glu) and b) norvaline (nVal) at 2 mM NaBr.

In Figure 3.4.a, it can be seen that as a function of time the concentration of Glu is decreasing linearly while the selectivity for nitrile is remaining constant around 100%. In Figure 3.4.b, the concentration of nVal is starting to decrease only after 10 min of reaction. Despite the induction period observed for nVal, after 40 min of reaction full conversion is achieved for both nVal and Glu. As the time is increasing, the selectivity is increasing gradually to its highest value (87%). In case of nVal, low conversion but high selectivity is registered at 10 min. However, the high selectivity is likely due to an error in measurements that originates from the use of two different HPLC methods to quantify the substrate and the product.

The conversion of the other amino acids was also followed in time at a concentration of 2 mM NaBr (Figure B.2.a-g). The conversion of the amino acids with carboxyl functionality at the side chain started directly without an induction period. All the other amino acids except nLeu, present an induction period of 10 min. For most amino acids, full conversion was achieved after 40 min, hSer after 50 min and Val after 90 min. Asp was partially converted and it is known that Asp does not reach full conversion at low concentration of NaBr not even after longer reaction times (2 h).[116]

During the induction period the reaction rate is lower than the maximum reaction rate. The initial reaction rates during the induction period and the maximum reaction rates were calculated and plotted in Figure 3.5 for all amino acids tested at 2 mM NaBr. A summary of these data is also presented in Table B.1.

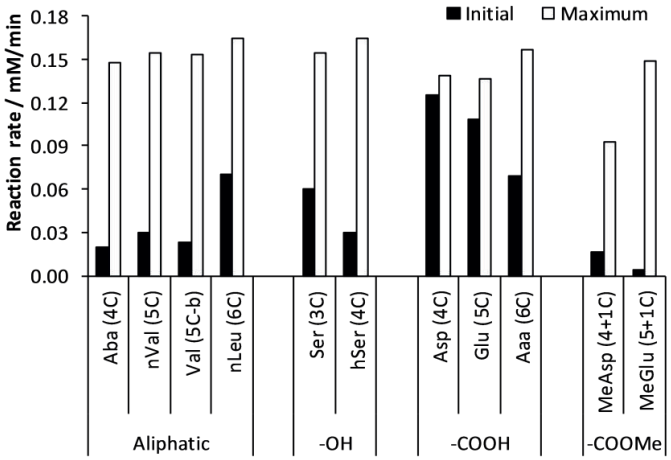



Figure 3.5. Initial and maximum reaction rates of the oxidative decarboxylation of amino acids at 2 mM NaBr. The initial reaction rate is calculated for the first 10 min of reaction. The maximum reaction rate is calculated for the time interval 10-40 min.

On average, the amino acids with aliphatic side chain have an initial reaction rate of 0.036 mM/min, which is 4.3-fold lower compared to the maximum reaction rate achieved (0.156 mM/min). For the amino acids with the hydroxyl group on the side chain a similar difference (3.5-fold) is observed as well. Remarkably, for the acidic amino acids the difference between the initial and the maximum reaction rate is only 1.4-fold. On a closer look, Aaa and nLeu have an initial reaction rate of 0.07 mM/min which is about half the maximum reaction achieved (ca. 0.16 mM/min). It seems that Aaa (Figure B.2.g) has an induction period while nLeu does not (Figure B.2.c) and this cannot be explained by theory.

The induction period observed for some amino acids indicates that an activation time is necessary before the reaction conditions are suitable to reach the maximum reaction rate. $H_2O_2$ is continuously supplied during the reaction and therefore its initial concentration in solution is low. It can be speculated that the induction period could be caused by the low concentration of $H_2O_2$. However, the induction period is not observed for the acidic amino acids and not even for Asp, the amino acid which requires a higher concentration of NaBr (>2 mM) to be fully converted.

Similar to the amino acids with aliphatic and hydroxyl side chain, both MeGlu and MeAsp present an induction period of 10 min. Due to the lack of activation time for the acidic amino acids it can be hypothesised that the ω-carboxylic functionality is involved in a self-catalysis mechanism. This mechanism is presented further.

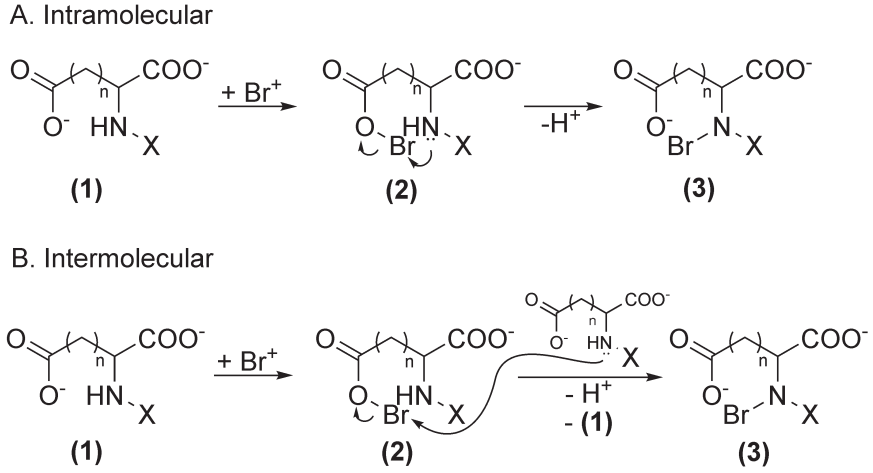
### 3.3.3 Reaction mechanism for acidic amino acids

The exact details of the reaction mechanism of oxidative decarboxylation of amino acids are not fully proven by experimental studies. However, a general reaction mechanism describes that two equivalents of $Br^+$ species will react with one equivalent of amino acid to generate one equivalent of nitrile. This mechanism is generally accepted independent of

the approach chosen for the generation of $X^+$ species.[65,81,82,117] The opinions regarding the exact pathway of the oxidative decarboxylation of amino acids are, however divided. In most of the cases, it is believed that the two $Br^+$ species reacts with the amino acid at the amino functionality and form a *N,N*-dibrominated amino acid (Scheme 1.5). The *N,N*-dibrominated amino acid will eliminate one bromide to form an iminium intermediate. This iminium intermediate will further eliminate the other bromide and the carboxyl functionality to form the nitrile.[65,81,82] Another study points towards the mechanism where the halogenation takes place at the α-carboxylic functionality rather than at the amine, suggesting that the exact mechanism could be dependent on the halogen source.[117]



Bromination at the ω-carboxylic functionality could explain the experimental observations in this thesis. For the oxidative decarboxylation of acidic amino acids, a reaction mechanism is proposed in Figure 3.3.




Scheme 3.3. Proposed mechanism for the self-catalysis of acidic amino acids.

It is proposed that in the first step a $Br^+$ species, produced by the enzyme VCPO, will react at the ω-carboxyl group of the amino acid or of the *N*-monobrominated amino acid **1** to form *in situ* a new brominated intermediate **2**. Next to the other $Br^+$ species present in the reaction, **2** can act as a brominating species as well. **2** could further react *via* an intramolecular reaction between the amino group and the bromide bound to the carboxyl group of the same molecule to yield the *N*-mono- or *N*-dibrominated amino acid **3**. Further, **3** could also be formed *via* an intermolecular reaction of **2** with another amino acid or an *N*-monobrominated amino acid **1**. During the intermolecular reaction **1** will be reformed.

In this reaction mechanism the new brominating species **2** is proposed to be formed next to those produced by the enzyme. The presence of more reactive brominating species such

as **2**, should lead to higher reaction rates and this is the case for acidic amino acids which do not present an induction period. More reactive brominating species will lead to higher conversion and less side product formation and therefore higher selectivity. In general the acidic amino acids present higher conversion and selectivity compared to the amino acids with the same carbon number but without carboxylic functionality at the side chain, which can be explained by the proposed reaction mechanism.

The reaction mechanism in solution of single amino acid can be different compared to mixtures of amino acids. As it was shown before in the mixture of Asp and Glu the reactivity of Glu was reduced by the presence of Asp.[116] However, when an oxidative decarboxylation reaction of complex mixture of amino acids from a proteic hydrolysate was performed, full conversion was achieved for most amino acids.[81] Probably the presence of Glu in high concentration can have a positive effect over the conversion of a mixture of amino acids. Amino acids such as Glu which have a beneficial influence on the reactivity could be used to improve the oxidative decarboxylation of mixtures of amino acids.

## 3.4 Conclusions

The aim of this chapter was to understand the influence of the side chain length and functionality of amino acids on the reactivity towards nitriles by oxidative decarboxylation reaction using VCPO as generator for $Br^+$ species. It was shown that the conversion can be modified as a function of the concentration of NaBr for all amino acids tested. Only two amino acids, Glu and Aaa, can be fully converted into nitriles with catalytic amounts of NaBr (0.2 mM). For all other amino acids tested a minimum requirement of NaBr present in the solution (≥ 2 mM) is needed to reach full conversion.

The length of the side chain does not make a significant difference for the selectivity, as previously proposed. However, the position of the functionality on the side chain (β-carbon) in relation to the bromination centre could hinder the production of nitriles by oxidative decarboxylation by reducing the reaction rate of the bromination.

It was shown that while functional groups like aliphatic, hydroxyl or methyl ester show no significant influence on the reactivity of amino acids, the carboxyl functionality has a positive effect during the oxidative decarboxylation reaction. An addition to the known reaction mechanism was proposed for the amino acids with carboxyl functionality at the side chain. It is proposed that the side chain carboxyl functionality is involved in a self-catalysis mechanism. The elucidation of the exact reaction mechanism can enable reactions of mixtures of amino acids at lower concentration of NaBr for the production of biobased nitriles.

# Chapter 4

# Enzymatic halogenation and oxidation using an alcohol oxidase-vanadium chloroperoxidase cascade



This chapter was published in adapted form as: Enzymatic halogenation and oxidation using an alcohol oxidase-vanadium chloroperoxidase cascade, A. But, A. van Noord, F. Poletto, J. P. M. Sanders, M. C. R. Franssen, E. L. Scott, *Journal of Molecular catalysis*, 443, **2017**, 92-100

## Abstract

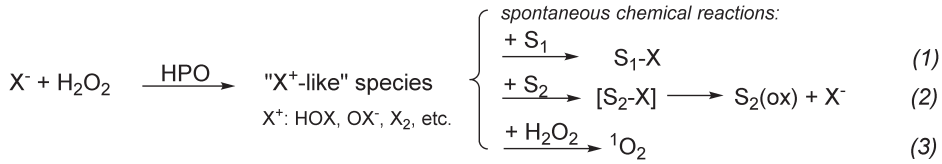
The chemo-enzymatic cascade which combines alcohol oxidase from *Hansenula polymorpha* ($AOX_{Hp}$) with vanadium chloroperoxidase (VCPO), for the production of biobased nitriles from amino acids was investigated. In the first reaction $H_2O_2$ (and acetaldehyde) are generated from ethanol and oxygen by $AOX_{Hp}$. $H_2O_2$ is subsequently used in the second reaction by VCPO to produce HOBr *in situ*. HOBr is required for the non-enzymatic oxidative decarboxylation of glutamic acid (Glu) to 3-cyanopropanoic acid (GluCN), an intermediate in the production of biobased acrylonitrile. It was found that during the one-pot conversion of Glu to GluCN by $AOX_{Hp}$-VCPO cascade, $AOX_{Hp}$ was deactivated by HOBr. To avoid deactivation, the two enzymes were separated in two fed-batch reactors. The deactivation of $AOX_{Hp}$ by HOBr appeared to depend on the substrate: an easily halogenated compound like monochlorodimedone (MCD) was significantly converted in one-pot by the cascade reaction of $AOX_{Hp}$ and VCPO, while conversion of Glu did not occur under those conditions. Apparently, MCD scavenges HOBr before it can inactivate $AOX_{Hp}$, while Glu reacts slower, leading to detrimental concentrations of HOBr. Enzymatically generated $H_2O_2$ was used in a cascade reaction involving halogenation steps to enable the co-production of biobased nitriles and acetaldehyde.

## 4.1 Introduction

Halogenation and oxidation reactions are key steps in the production of both commodity and fine chemicals. Halogen atoms are inserted because of their unique physicochemical and biological properties, in commodities such as vinyl chloride[119] but also in medicines like diclofenac.[120] The reagents involved are often molecular halogens: toxic, corrosive and reactive compounds that are produced in energy-intensive processes.[26] Oxidation reactions in bulk chemistry are usually carried out using molecular oxygen and a solid catalyst, but in fine chemical industry hypervalent heavy metal oxides are used, which is undesirable due to toxicity and low atom efficiency.[121] Environmental concerns are stimulating more sustainable production of chemicals.[122] There is an increasing amount of research dedicated to develop greener halogenation and oxidation processes. For example, the *in situ* generation of halogenating species, such as $X^+$ (where X: Cl, Br) is desired.[123] In addition to the typical halogenation reaction, $X^+$ species can also be used as oxidising reagents where a substrate is oxidised *via* an intermediate halogenation step.



The *in situ* production of $X^+$ species ($X^+$: HOX, $X_2$, etc.) by catalysis using haloperoxidases (HPO) has received a lot of attention due to the mild reaction conditions such as aqueous solutions at ambient temperature.[59,123,124] HPO use $H_2O_2$ in acidic conditions to oxidise halides to the corresponding hypohalous acid (HOX). In the presence of an organic halogen acceptor ($S_1$), HOX will react to give a halogenated product ($S_1$-X) according to Eq. 1.[70] When an unstable intermediate ($S_2$-X) is formed, hydrolysis and/or further oxidation to a more stable product will occur as shown in Eq. 2.




$S_1$: monochlorodimedone, barbituric acid, alkenes, etc.; $S_2$: amino acids, thiols, thioesters, etc..

The most investigated HPO is the Fe-heme containing chloroperoxidase from *C. fumago* (CPO).[71,84,125–127] Despite the broad application of CPO,[128] excess of $H_2O_2$ in the reaction mixture causes deactivation of the haloperoxidase by oxidation of its heme moiety.[129] A solution to overcome deactivation by $H_2O_2$, is to replace Fe-heme HPO with the more robust vanadium containing haloperoxidase (VHPO) which has been successfully used for halogenation reactions like the conversion of alkenes into halohydrins,[130] the halogenation of phenols[98,131] and oxidation reactions like the aza-Achmatowicz[124] or the oxidative decarboxylation reaction of amino acids.[59,116] It was shown that VCPO has superior resistance against $H_2O_2$.[98] However, with an excess of $H_2O_2$, HOBr may react with $H_2O_2$ to form singlet oxygen (Eq. 3)[98] which may lead to undesired oxidation products and enzyme inactivation.[129] Gradual addition of $H_2O_2$ could be an option to alleviate this problem.[59,84] As

well as this, the production of $H_2O_2$ itself is not sustainable due to energy use and safety issues.[132] Another option would be the *in situ* production of $H_2O_2$ by the use of oxidases. These enzymes can activate molecular oxygen for the production of $H_2O_2$ together with the oxidation of a substrate (Eq. 4).

$$\text{Substrate} + O_2 \longrightarrow \text{Substrate}_{ox} + H_2O_2 \qquad (4)$$

The majority of processes for the enzymatic generation of $H_2O_2$ use glucose oxidases (GOX).[126] GOXs are well characterised enzymes[133] which have flavin adenine dinucleotide (FAD) as a cofactor. To produce one equivalent of $H_2O_2$, GOX converts equimolar amounts of glucose to gluconic acid which results in the acidification of the reaction mixture which requires pH control and results in salt by-products. In addition, the atom efficiency of this system is poor.

Other FAD-containing oxidases could lead to the same performance as GOX for the *in situ* production of $H_2O_2$ but without the drawbacks related to gluconic acid. For example, alcohol oxidases (AOX)[86] convert short chain primary alcohols into (highly) volatile aldehydes. The substrate affinity of AOXs decreases with the length of the carbon chain of the alcohol, therefore, AOXs have the highest activity with methanol and ethanol as substrates.[87] Formaldehyde is the oxidation product of methanol, while ethanol is converted to acetaldehyde; as formaldehyde is more toxic compared to acetaldehyde,[88] ethanol is the preferred substrate. It was shown that acetaldehyde can be easily separated by pervaporation[89] or air stripping.[90] Deactivation of AOX by its own product $H_2O_2$ is possible due to oxidation of the -SH residues of AOX.[134] However, this can be prevented by *in situ* product removal techniques such as cascade reactions. Deactivation of AOX was prevented in a cascade reaction where $H_2O_2$ was immediately consumed by the next reaction with a catalase[134] or peroxidase.[135] Replacing GOX by AOX and glucose with ethanol for the *in situ* generation of $H_2O_2$ would avoid complex downstream processing. Furthermore, the cascade reaction of AOX with an HPO which will use $H_2O_2$ for the production of $X^+$ species would give access to more sustainable reactions involving halogenation reagents.

Examples for the cascade reactions of oxidases with HPO are known. The majority of these reactions use HPO for its so-called classical peroxidase activity. Some HPO, in addition to the haloperoxidase activity, catalyse a typical peroxidase reaction where a substrate is oxidised using $H_2O_2$ in the absence of halides.[128] For example, the cascade reaction of GOX with an HPO in the absence of halides was shown to be successful for the oxidation of thioanisole or indole.[126,127] However, only a few examples are known for the cascade reaction of oxidases with haloperoxidases for the production of HOX and the use of it as a halogenating agent (Eq. 4+1). GOX was coupled to CPO in presence of $Cl^-$ for microbial disinfection purposes of surface[136] and for chlorination reactions of model substrates such as monochlorodimedone[137]or barbituric acid.[138] Recently, the cascade GOX-CPO was also

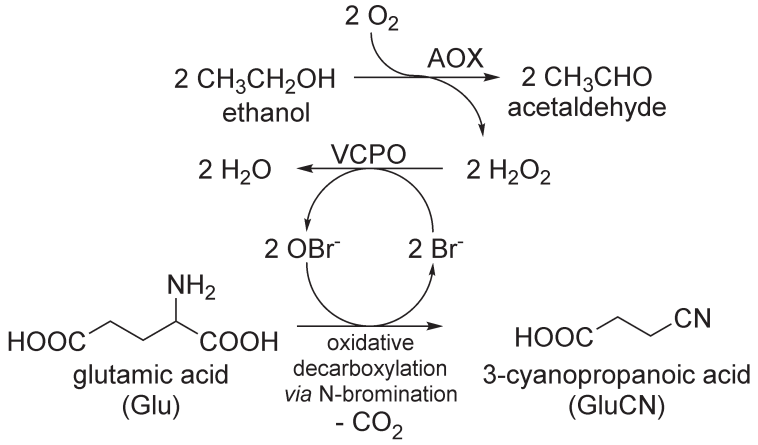
used in presence of $Br^-$ for the bromination of allenes.[139] An industrial example for the combination of an AOX using methanol with a HPO for a chlorination reaction was briefly mentioned for propylene chlorohydrin generation from propene for the Cetus process.[140] However, to the best of our knowledge no details are given about the reaction conditions or the results obtained.

The combination of AOX with HPO for the production of HOX and the use of it in a further chemical reaction as oxidising agent (Eq. 4+2) has not yet been investigated. Therefore, to have more sustainable oxidation reactions *via* halogenation more insight is required for the combination of oxidases with haloperoxidases in a chemo-enzymatic cascade reaction.

A recent publication reported the use of vanadium chloroperoxidase from *Curvularia inaequalis* (VCPO) for the oxidative decarboxylation of amino acids to nitriles. A reaction that occurs *via* brominated amines. When biomass-derived amino acids are used as a feedstock, a biobased alternative for the production of nitriles is obtained.[59] It was shown that glutamic acid (Glu) can give access to biobased acrylonitrile, an important building block in the polymer industry, *via* the intermediate 3-cyanopropanoic acid (GluCN).[42] To produce GluCN from Glu, two equivalents of an oxidised bromine species such as HOBr are required. For this, VCPO generates HOBr by oxidising $Br^-$ with $H_2O_2$. Subsequently HOBr brominates Glu to give *N,N*-dibrominated Glu which is further converted to GluCN, the corresponding nitrile.



This chapter describes the reaction configuration required to perform the chemo-enzymatic cascade that combines an AOX with VCPO for the production of nitriles from amino acids. The required HOBr is generated *in situ* by the VCPO-mediated oxidation of $Br^-$ with $H_2O_2$. The necessary $H_2O_2$ is generated *in situ* as well by an AOX which activates molecular oxygen. To do this AOX converts ethanol to acetaldehyde, a volatile product which can be readily removed (Scheme 4.1).




Scheme 4.1. Oxidative decarboxylation of glutamic acid to 3-cyanopropanoic acid by VCPO coupled to AOX for $H_2O_2$ production from ethanol and molecular oxygen.

## 4.2 Experimental

### 4.2.1 Materials

L-Glutamic acid (98.5% pure), NaBr (99% pure), $H_2O_2$ (35 wt-%), citric acid, $Na_3VO_4$ (99.98% pure), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (98% pure) and Trizma base were purchased from Sigma-Aldrich. HPLC grade ethanol, methanol were received from Actu-All chemicals. 3-Cyanopropanoic acid (95.9% pure) was provided by Interchim and monochlorodimedone by BioResourse Products. Oxygen was used as received from Linde-gas.

### 4.2.2 Enzymes

Vanadium chloroperoxidase was expressed in *Escherichia coli* cells containing the VCPO plasmid using a protocol described elsewhere.[141] The VCPO was isolated from the lysed *E. coli* cells by two purification steps. The first purification step was heat treatment at 70°C for ca. 10 min, followed by centrifugation. The second step was a batch purification of the supernatant using a DEAE resin and a stepwise elution with 100 mM Tris/$H_2SO_4$ buffer pH 8.2 and a 100 mM Tris/$H_2SO_4$ buffer pH 8.2 containing 1 M NaCl. The obtained partially purified VCPO was concentrated on 30 kDa membrane and stored in 100 mM Tris/$H_2SO_4$ buffer pH 8.2 containing 100 μM $Na_3VO_4$ at -20°C. The VCPO stock has an activity of 79 U/mL based on the *Standard MCD assay*[142] (see below) and a protein concentration of 4.7 mg/mL based on Bradford assay.

Alcohol oxidases from *Candida boidinii* (0.7 U/mg solid, 6.7 U/mg protein), *Pichia pastoris* (14 U/mg protein, 45 mg protein/mL) and *Hansenula polymorpha* (0.6 U/mg solid) and horseradish peroxidase type II (163 U/mg solid) were purchased from Sigma-Aldrich and used as received. The activity and protein concentration listed above are data from supplier. The activity of AOXs was determined based on *Standard ABTS assay* (see below) before each reaction was started. The activity of HRP was measured using the *Standard ABTS assay* with 1 mM $H_2O_2$ instead of ethanol and AOX. Typically, a stock solution of AOX or HRP was freshly prepared in an enzyme diluent (0.25%, w/v, bovine serum albumin in 20 mM sodium citrate buffer pH 5.6).

### 4.2.3 Standard ABTS assay

In a disposable cuvette for visible range a solution (1.1 mL) containing 2 mM ABTS in 20 mM sodium citrate pH 5.6 was saturated for 5 min with $O_2$. To this solution 100 mM ethanol and 1 U/mL of HRP were added and the absorbance was read at 405 nm, at 24°C. The reaction was started with the addition of AOX. The enzyme activity was calculated using an extinction coefficient for ABTS $\varepsilon$=36.8 $(mM \cdot cm)^{-1}$. Deviations from this standard procedure are presented in the caption of figures.

### 4.2.4 ABTS assay - AOX deactivation by denaturation (Figure 4.5)

In a 4 mL glass vials a solution (2 mL) containing 0.05 U/mL AOX (48 µg AOX/mL) in 20 mL sodium citrate pH 5.6 was supplied with $O_2$ at a flow rate of ca. 5 mL pure oxygen/min. Samples were taken at given time points. The AOX activity was measured by direct mixing of the sample in a standard ABTS assay solution.

### 4.2.5 ABTS assay - AOX inhibition by potential inhibitors (Figure C.3)

The same reaction conditions were used for the *standard ABTS assay* in presence of different inhibitors. To the ABTS solution the desired amount of inhibitor was added (Figure C.3). HOBr was produced by reacting excess of NaBr with the desired amount of NaOCl in a NaBr:NaOCl ratio of 1.5:1. For the HOBr inhibition test immobilised AOX on HFA epoxy beads according to a protocol described elsewhere[143] was used. The immobilised AOX was kept in HOBr solution for 2 min and after separation the activity of AOX was measured using the standard ABTS assay.

### 4.2.6 Standard MCD assay

In a disposable cuvette for UV range a solution (1 mL) containing 50 µM MCD, 0.5 mM NaBr, 1 mM $H_2O_2$, 100 µM $Na_3VO_4$ in 20 mM sodium citrate pH 5.6 was prepared. The absorbance of MCD solution was read at 290 nm, at 24°C. The reaction was started by addition of VCPO. The enzyme activity was calculated using an extinction coefficient for MCD ε=20 $(mM \cdot cm)^{-1}$. In Figure C.2, the first 0.5 min is not shown due to an observed lag phase. This lag phase is probably due to the change of pH of VCPO from storage pH (8.2) to reaction pH (5.6). Therefore, time 0 min in the graph represents 0.5 min in practice.

### 4.2.7 MCD assay – the ratio between AOX and VCPO (Figure 4.3)

The same reaction conditions were used as for the *Standard MCD assay* in presence of 100 mM ethanol and different ratios of VCPO to AOX. The amounts of enzymes are presented in Table 4.1. The reaction was started with AOX.

Table 4.1. Amounts of enzymes used to determine the ratio between AOX and VC PO.

| AOX:VCPO | 1:10 | 1:5 | 1:1 | 2:1 | 5:1 | 10:1 | 20:1 |
|---|---|---|---|---|---|---|---|
| VCPO (U/mL) | 0.1 | 0.05 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| AOX (U/mL) | 0.01 | 0.01 | 0.01 | 0.02 | 0.05 | 0.1 | 0.2 |

### 4.2.8 One-pot conversion of MCD by AOX and VCPO – the effect of oxygen supply on reactivity (Figure 4.4)

In two 4 mL glass vials a solution containing 2.5 mM MCD, 5 mM NaBr was saturated with $O_2$. To this solution ethanol was added to a concentration of 100 mM and 0.25 U/mL VCPO. The reaction in the first vial was performed with the cap open, allowing $O_2$ from air to diffuse in the liquid. To the second vial, $O_2$ was supplied directly in the solution with a continuous flow rate of ca. 5 mL pure $O_2$/min. The reaction was started with the addition of 0.05 U/mL AOX. The reaction was performed at room temperature without stirring and the

total volume of the reaction was 2 mL. Every 5 or 10 min samples were taken from the reaction mixture. Each sample was quenched with $Na_2S_2O_3$ and diluted 50 times in 20 mM citric acid pH 2. The absorbance of diluted MCD solution was immediately read at 290 nm. The concentration of MCD was calculated with $\varepsilon_{MCD}$ =20 $(mM \cdot cm)^{-1}$.

### 4.2.9 One-pot conversion of Glu to GluCN by AOX and VCPO (Figure 4.2)

The same procedure as the one from *One-pot conversion of MCD by AOX and VCPO – the effect of oxygen supply on reactivity* was used with a few exceptions. MCD was replaced by 5 mM Glu and 0.5 mM NaBr were used. Samples were taken and diluted ten times for Glu and two times for GluCN analysis. Glu and GluCN were analysed by HPLC.

### 4.2.10 Oxidative decarboxylation of Glu by physical separation of AOX from VCPO (Figure 4.6)

*Step 1.* 0.5 mL of solution A, containing 2 mM ethanol and 0.5 mM NaBr in 20 mM sodium citrate buffer pH 5.6 and saturated with oxygen, was added to an ultrafiltration tube with a cut-off of 30 kDa. Oxygen was supplied to solution A with a continuous flow rate of ca. 5 mL pure oxygen/min. The reaction was started by addition of 0.1 mL solution A in which was dissolved 1.1 mg AOX (0.8 U/mg based on standard ABTS assay). The AOX reaction was performed at room temperature without additional mixing. After 5 min of reaction the AOX reaction mixture was centrifuged for 3 min, 7832 rpm, 20°C. *Step 2.* Ca. 0.5 mL reaction solution separated from AOX was added over 0.5 mL of solution B. Solution B contained 10 mM glutamic acid, 0.5 mM NaBr and 4 U/mL VCPO (based on MCD assay) dissolved in 20 mM sodium citrate buffer pH 5.6. The VCPO reaction mixture was homogenised by mixing using a spatula. The reaction was performed at room temperature (24°C) without stirring. After 10 min step 1 was repeated and the resulting solution was added to the solution from step 2. Every 10 min this procedure was repeated (resulting in a dilution of solution B). Individual reactions were performed for each time point in Figure 4.6. The VCPO reaction was quenched by adding $Na_2S_2O_3$. The final volume of VCPO solution was measured and the conversion was calculated accordingly. Samples were taken for HPLC analysis.

### 4.2.11 Phenol red assay (qualitative)

VCPO activity: to a solution containing 50 µM Phenol red, 0.5 mM NaBr, 1 mM $H_2O_2$, 100 µM $Na_3VO_4$ in 50 mM sodium citrate pH 5.6, a sample containing VCPO was added.

AOX activity: to a solution containing 50 µM Phenol red, 0.5 mM NaBr, 100 mM ethanol, 100 µM $Na_3VO_4$ in 20 mM sodium citrate pH 5.6, VCPO (0.5 U/mL) was added followed by a sample containing AOX. The change in colour from yellow to purple-blue indicates that the enzymes are active.

### 4.2.12 HPLC

Glutamic acid was analysed by derivatisation as described before.[59] 3-Cyanopropanoic acid was analysed without dilution or derivatisation by HPLC using an UltiMate 3000 equipment

from Thermo Scientific. Detection was achieved using a RI-101 detector from Shodex set at 35°C. The column used was a Rezex ROA Organic acid H+ (8%) column (7.8 × 300 mm) from Phenomenex, at 35°C with an eluent flow of 0.5 mL/min of 12 mM $H_2SO_4$.

## 4.3 Results & discussion

To combine an AOX with VCPO in a chemo-enzymatic cascade for the oxidative decarboxylation of Glu several factors have been investigated: the kinetics of AOXs, the ratio between AOX and VCPO for optimal $H_2O_2$ production, the effect of $O_2$ supply on the enzyme activity, the effect of potential inhibitors on the activity of AOX and the reactor configuration.

### 4.3.1 AOX selection

The first step was to select the AOX which is the most active under the same reaction conditions as VCPO. It was shown that pH 5.6 and room temperature were the best reaction conditions for VCPO in the conversion of Glu to GluCN.[59] Using these reaction conditions and ethanol as a substrate, the AOX with the highest enzymatic activity was selected from: *Candida boidinii* ($AOX_{Cb}$), *Pichia pastoris* ($AOX_{Pp}$) and *Hansenula polymorpha* ($AOX_{Hp}$). The AOX selection was based on the standard 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay which couples an (alcohol) oxidase (Eq. 4) with horseradish peroxidase (HRP) (Eq. 5).

$$ABTS + H_2O_2 \rightarrow ABTS_{ox} + H_2O \qquad (5)$$

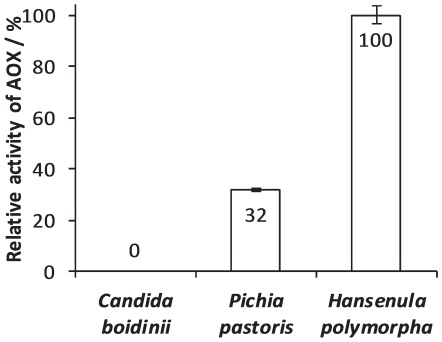



Figure 4.1. The relative activity of AOXs from *Candida boidinii*, *Pichia pastoris* and *Hansenula polymorpha*. Relative activity is based on the activity of *Hansenula polymorpha* (0.011 U/mL) being set to 100%. Reaction conditions: 20 mM sodium citrate buffer pH 5.6 containing dissolved $O_2$, 100 mM ethanol, 2 mM ABTS, 1 U/mL HRP, 0.005 U/mL AOX based on ABTS assay using methanol as substrate (5 mM methanol in 20 mM sodium citrate buffer pH 5.6), total reaction volume of 1.1 mL. The results represent the average of triplicate experiments.

In Figure 4.1, the relative activity of different AOXs can be seen. At pH 5.6 and room temperature the $AOX_{Pp}$ has a low activity while the $AOX_{Cb}$ has no activity. The enzyme with the highest activity was $AOX_{Hp}$ and it was selected to be used in further experiments. AOXs typically have an optimal pH around 8 with $AOX_{Hp}$ exhibiting a broad optimal pH range (5.5-11). Therefore, these results are in accordance with literature.[87]

The kinetics of $AOX_{Hp}$ for the oxidation reaction of ethanol were studied (Figure C.1). $AOX_{Hp}$ follows Michaelis-Menten kinetics without substrate inhibition. The highest activity was reached at ca. 100 mM ethanol with a $K_m$ value for ethanol of 15.9 mM. The $K_m$ value found for ethanol is somewhat higher than that previously found (4.4 mM)[144] which is attributed to deviation from the non-optimal pH used in this research (pH 5.6).

### 4.3.2 One-pot conversion of Glu to GluCN by AOX-VCPO cascade

The aim of this study was to combine an AOX with VCPO in a chemo-enzymatic cascade for the conversion of Glu to GluCN. Here AOX uses molecular oxygen to produce $H_2O_2$ *in situ* which is immediately utilised by VCPO to produce the species required for the oxidative decarboxylation of Glu, Scheme 4.1. To achieve this, the cascade $AOX_{Hp}$-VCPO was performed with an ethanol concentration of 100 mM, ensuring a maximum velocity for $AOX_{Hp}$. In Figure 4.2 the concentration of Glu and GluCN as a function of time is depicted. Surprisingly, after 55 min of reaction, the concentration of Glu and GluCN remains unchanged (Figure 4.2.A). This shows that performing the $AOX_{Hp}$-VCPO cascade under these reaction conditions, conversion does not occur. Several factors could be responsible for this. One of these could be the presence of ethanol leading to the inhibition of VCPO.

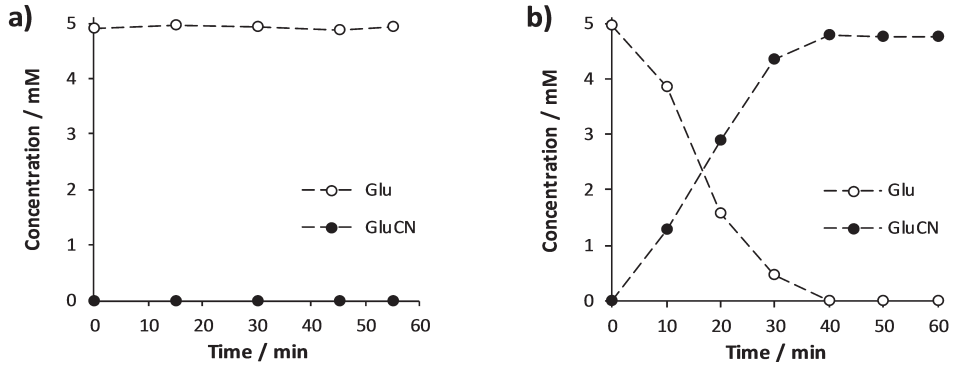



Figure 4.2. The conversion of glutamic acid (Glu) to 3-cyanopropanoic acid (GluCN) in time using VCPO and $H_2O_2$. **a)** $H_2O_2$ was produced *in situ* by $AOX_{Hp}$ by using a $AOX_{Hp}$:VCPO ratio of 1:1.2 (0.25 U/mL VCPO) and air was continuously administrated to the reaction mixture; **b)** $H_2O_2$ was added externally with a rate of 18 mM/h. Each data point represents the result of a single and distinct experiment. Dashed lines (---) are only drawn as visual aids.

It is known that vanadium haloperoxidases are stable in the presence of organic solvents,[69] and VCPO is active in presence of ethanol.[124] To confirm that Glu can be converted to GluCN by VCPO in presence of ethanol, this reaction was performed using an external source of $H_2O_2$ as opposed to the proposed cascade reaction (Figure 4.2.B). Here it is observed that Glu is fully converted to GluCN in 40 min. When ethanol was not present in the reaction, similar results were obtained.[59,116] This shows that during the conversion of Glu to GluCN, VCPO is equally active in presence and absence of ethanol. It also indicates that during the $AOX_{Hp}$-VCPO cascade reaction (Figure 4.2.A) $AOX_{Hp}$ does not produce $H_2O_2$ and therefore, the second reaction of the cascade catalysed by VCPO cannot proceed.

It was shown that when $AOX_{Hp}$ was coupled to HRP during the ABTS assay (Figure 4.1), $AOX_{Hp}$ is producing $H_2O_2$. Therefore, other factors must interfere with the $AOX_{Hp}$-mediated production of $H_2O_2$. These factors were studied further using a specific assay for VCPO – the monochlorodimedone (MCD) assay.

### 4.3.3 One-pot conversion of MCD by AOX-VCPO cascade

To investigate whether $AOX_{Hp}$ is able to produce $H_2O_2$ for the $AOX_{Hp}$-VCPO cascade reaction, Glu was replaced by MCD, a common substrate which allows rapid activity measurements for haloperoxidases. Typically VCPO catalyses the oxidation of halides, in this case $Br^-$, by $H_2O_2$ to produce HOBr, which subsequently brominates MCD (Scheme 1.4, equation 1 and 2, $S_1$ =MCD).

For this experiment the required $H_2O_2$ was produced *in situ* from ethanol and oxygen by $AOX_{Hp}$, using a ratio of $AOX_{Hp}$:VCPO of 1:1. A control experiment where $H_2O_2$ was supplied externally was also performed (Figure C.2). It was observed that in the reaction where $H_2O_2$ was produced by $AOX_{Hp}$, the concentration of MCD is decreasing and full conversion is achieved after ca. 12 min (reaction rate 0.0038 mM/min). This shows that the $AOX_{Hp}$ is producing $H_2O_2$ *in situ*. This experiment also demonstrates that the chemo-enzymatic cascade $AOX_{Hp}$-VCPO for the bromination reaction of MCD is possible in a one-pot reaction.

When $H_2O_2$ was supplied externally, a full conversion of MCD was obtained within ca. 7 min (reaction rate 0.0065 mM/min). This is approximately twice as fast compared to the reaction using $AOX_{Hp}$. This would suggest that when sufficient $H_2O_2$ is supplied to the reaction, VCPO can reach higher reaction rates. Using the cascade $AOX_{Hp}$-VCPO in a ratio of 1:1, higher reaction rates could not be achieved. Thus indicating that the production of $H_2O_2$ by $AOX_{Hp}$ is rate determining in the cascade and thus requires an alternative $AOX_{Hp}$/VCPO ratio.

### 4.3.4 The ratio between $AOX_{Hp}$ and VCPO

In haloperoxidase reactions, excess of $H_2O_2$ can react with the enzymatically generated HOBr to produce singlet oxygen[98] leading to by-products. In addition, a high concentration of $H_2O_2$ will deactivate AOX[134,144] by reacting with cysteine residues of the enzyme.[145] This can be prevented by using *in situ* removal of $H_2O_2$ by a second enzyme in a cascade reaction. Accumulation of $H_2O_2$ is detrimental for both enzymes used in this research, so to avoid deactivation in a cascade reaction and to ensure maximal conversion rate, the rate of production of the deactivator and that of its consumption must be balanced.[144] This can be achieved by determining the optimal ratio between $AOX_{Hp}$ and VCPO, using the MCD assay. The conversion of MCD was compared between different ratios of $AOX_{Hp}$:VCPO after 5 min of reaction (Figure 4.3).

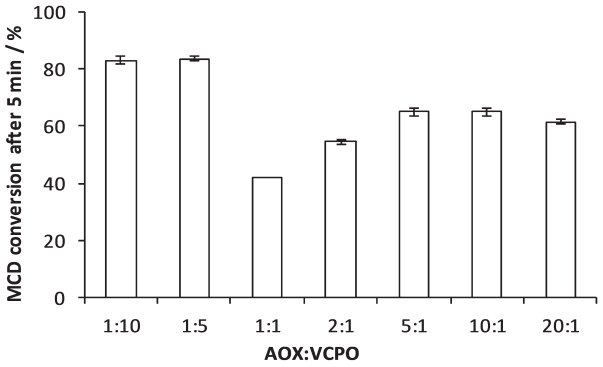



Figure 4.3. The conversion of MCD (50 µM) after 5 min of reaction as a function of time at different ratios between $AOX_{Hp}$:VCPO, at 0.5 mM NaBr. The error bars represent the range of duplicate experiments.

A minimum in MCD conversion can be observed at an $AOX_{Hp}$:VCPO ratio of 1:1. With an increasing $AOX_{Hp}$ concentration there is an increase in the reaction rate but the highest conversion of MCD was found when an excess of VCPO was used (83%). From this it is concluded that an $AOX_{Hp}$:VCPO ratio of 1:5 is optimal. Pereira *et al.*[127] have used a cascade of GOX and CPO for the oxidation of thioanisole. For this reaction an optimal ratio of GOX:CPO of 5:1 was found, which is contrary to our findings. This is probably due to the much lower resistance against $H_2O_2$ of CPO compared to VCPO.

### 4.3.5 The effect of oxygen supply on reactivity

To produce $H_2O_2$ $AOX_{Hp}$ requires dissolved molecular oxygen. However, the solubility of oxygen in water is about 0.26 mM at 25°C and 1 atm.[146] Therefore, for larger scale reactions it is necessary to supply oxygen to the reaction mixture in order to avoid the reaction rate to be limited by the diffusion rate of oxygen.

The effect of oxygen supply to the reaction was investigated for the bromination of MCD using $AOX_{Hp}$ coupled to VCPO by supplying oxygen to the reaction by two methods: by diffusion from air at the surface of the reaction mixture and by a continuous stream of pure oxygen directly in the reaction mixture. For both methods of oxygen supply the solution containing all reagents was saturated with oxygen at time zero. The results are depicted in Figure 4.4.

In the first experiment where oxygen was supplied by diffusion a decrease in MCD concentration from 2.55 to 1.95 mM is noticed in the first 15 min (Figure 4.4, ●). As expected, after 15 min almost no conversion was observed and this is likely due to the lack of dissolved oxygen in the reaction mixture. Additional oxygen was supplied at 107 min by administering a stream of pure oxygen for 1 min and as a result, MCD was further converted from 1.78 to 1.64 mM. Apparently, the diffusion of oxygen in the reaction mixture is a limiting factor therefore a more efficient method to supply oxygen is required.

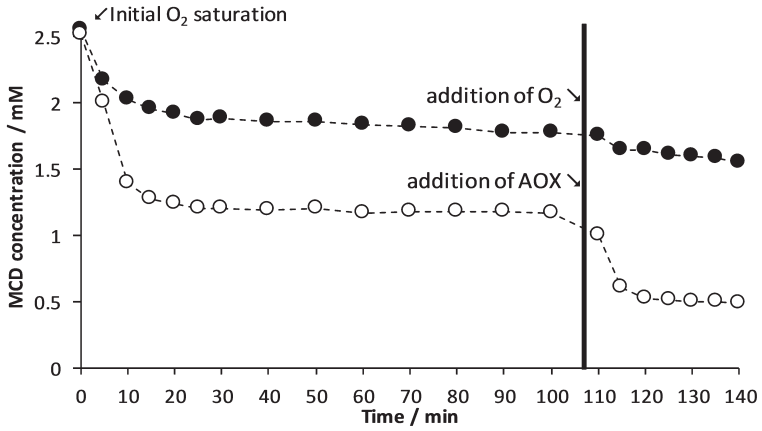



Figure 4.4. Consumption of MCD in the bromination reaction catalysed by the cascade $AOX_{Hp}$-VCPO as a function of time. Reaction conditions: 2.5 mM MCD, 5 mM NaBr, 100 mM ethanol, $AOX_{Hp}$:VCPO = 1:5 (0.25 U/mL VCPO), 20 mM sodium citrate buffer pH 5.6, room temperature. Reactions were started by $AOX_{Hp}$ addition. Oxygen diffusion (●): oxygen saturation at t=0 min, reaction under air atmosphere; oxygen stream (○): continuous oxygen supply at ca. 5 mL/min; at 107 min (|) oxygen was supplied to ● for 1 min with a flow rate of ca. 5 mL/min and 0.05 U/mL $AOX_{Hp}$ was added to ○. The data represent the average of duplicate experiments. Dashed lines (---) are only drawn as visual aids.



The second experiment was performed using a continuous stream of pure oxygen directly in the reaction mixture (Figure 4.4, ○). In this case a decrease in concentration of MCD from 2.51 to 1.28 mM was observed in the first 15 min which is equivalent to twice the conversion obtained in the diffusion experiment. After 15 min this reaction does not proceed further as was observed earlier in the diffusion experiment. In the second experiment, oxygen was supplied continuously so oxygen cannot be the limiting factor here.

During this second experimental setup, excessive foam formation was observed at the surface of the solution. Foam formation could indicate enzyme deactivation by protein denaturation at the gas-liquid interface. To determine if one of the enzymes were deactivated, a qualitative test based on the phenol red assay[69] was performed (see Experimental, 4.2.11). The phenol red test showed that VCPO is still active while $AOX_{Hp}$ is not. This was further confirmed by addition of fresh $AOX_{Hp}$ to the reaction at 107 min. On addition of $AOX_{Hp}$ the concentration of MCD decreased from 1.17 to 0.53 mM. However, after 13 min from the second addition of $AOX_{Hp}$ no additional conversion is observed. This confirms the above hypothesis that $AOX_{Hp}$ is deactivated during reaction. Deactivation of $AOX_{Hp}$ by $H_2O_2$ can be ruled out due to the use of 5-fold excess of VCPO which will immediately consume $H_2O_2$.

To conclude, these experiments show once more that is possible to perform a typical bromination reaction by combining an $AOX_{Hp}$ for the *in situ* production of $H_2O_2$ with a VCPO. Moreover, this is possible at concentrations in the mM range where a stream of pure oxygen supplied directly in the reaction mixture is required. This creates the possibility of

converting other substrate such as Glu using the cascade $AOX_{Hp}$-VCPO. However, $AOX_{Hp}$ is deactivated by an unknown mechanism which needs to be elucidated next. For this, the $AOX_{Hp}$ activity was investigated by following the enzymatic activity of an $AOX_{Hp}$ solution in the presence and absence of a continuous stream of oxygen over a period of one hour (Figure 4.5).

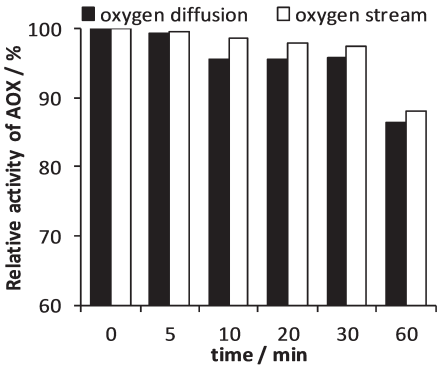



Figure 4.5. Influence of oxygen supply on $AOX_{Hp}$ activity as a function of time. Reaction conditions: 0.05 U/mL $AOX_{Hp}$, 20 mM sodium citrate buffer pH 5.6, room temperature; the activity of $AOX_{Hp}$ was based on ABTS assay; oxygen diffusion (■): reaction under air atmosphere without oxygen saturation at t=0 min, slope = -0.1903; oxygen stream (□): continuous oxygen supply at ca. 5 mL/min, slope = -0.2095. For each time point the $AOX_{Hp}$ activity was determined by ABTS assay. The results represent the value of single experiment.

When oxygen was supplied to the reaction by diffusion, a decrease of the relative activity of $AOX_{Hp}$ from 100% to 86% was observed after 1 hour. When oxygen was supplied by a continuous stream of pure oxygen, no significant difference in activity was observed compared to the diffusion experiment. Hence the deactivation of $AOX_{Hp}$ is not caused by protein denaturation at the gas-liquid interface but by another mechanism.

### 4.3.6 The effect of potential inhibitors on $AOX_{Hp}$ activity

(Haloper)oxidases are known to be inhibited by their products, this is especially true for oxidases which form $H_2O_2$ as a product.[144,145] However, this may be overcome in a cascade reaction between oxidases and (halo)peroxidases where $H_2O_2$ is removed *in situ* by the coupled enzyme. Moreover, in the conversion of Glu, a small excess of VCPO compared to $AOX_{Hp}$ was used, therefore, inhibition by $H_2O_2$ should have been prevented.

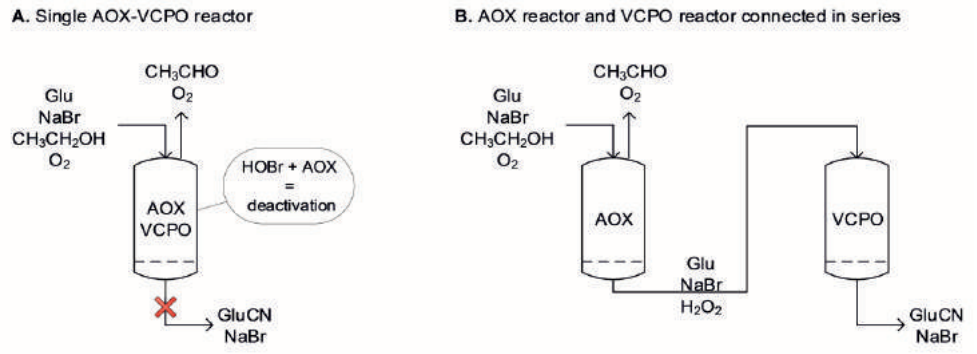
To investigate the possible inhibition by components present or formed in the reaction, the activity of $AOX_{Hp}$ was checked based on ABTS assay in presence of different potential inhibitors and compared to the control reaction where no inhibitor was present. These potential inhibitors were either reaction components (Glu, NaBr, $Na_3VO_4$) or products (acetaldehyde, GluCN, HOBr) (Figure C.3). Compared with the control reaction, only limited decrease in activity was registered in presence of Glu, GluCN, NaBr, $Na_3VO_4$ or acetaldehyde. However, in presence of HOBr the relative activity of $AOX_{Hp}$ is significantly

lower. This indicates that in presence of HOBr $AOX_{Hp}$ seems to be inhibited or deactivated therefore further investigation was necessary.

### 4.3.7 Reactor configuration for the cascade $AOX_{Hp}$-VCPO

Hypohalous acids (HOX) are able to halogenate or oxidise amino acid residues in proteins, such as cysteine, cystine and tyrosine.[147–149] Modification of amino acids which play a role in the catalysis or in maintaining the quaternary or tertiary structure could lead to enzyme deactivation. This issue can be overcome by selecting a suitable reactor configuration.[150]

To avoid the deactivation of $AOX_{Hp}$ in the cascade $AOX_{Hp}$-VCPO the reactor configuration was changed from a single reactor containing both enzymes (Scheme 4.2.A) to a series of two reactors where the two enzymes are physically separated (Scheme 4.2.B). The configuration presented in Scheme 2-B involves the use of two different reactors. The first one is an air-lift membrane reactor for AOX reaction. The membrane will keep AOX inside the reactor and allow recycling of the enzyme. The air-lift will mix the solution and remove acetaldehyde by gas stripping. To avoid deactivation of $AOX_{Hp}$, $H_2O_2$ is transferred immediately to the second reactor. The second reactor is a fed-batch chemo-enzymatic reactor for the VCPO reaction and the oxidative decarboxylation of Glu. Ideally this second reactor would be equipped with a membrane to allow recycling of VCPO. This reactor configuration allows the physical separation of the two enzymes which prevents the direct contact between HOBr and $AOX_{Hp}$.




Scheme 4.2. Reactor configurations for the conversion of Glu to GluCN.

The new reactor configuration was tested for the oxidative decarboxylation of Glu to GluCN (see Experimental, 4.2.10). The results of this experiment are plotted in Figure 4.6, where the conversion of Glu and formation of GluCN is presented as a function of time. The concentration of Glu decreased linearly with time. After 90 min of reaction about 3 mM Glu was converted to GluCN, which corresponds to 29% conversion. This relatively low conversion was expected due to the non-optimised conditions used for AOXHp. To avoid accumulation of $H_2O_2$, low substrate concentration (2 mM ethanol) was used which resulted in reaction rate below $V_{max}$. This could be solved or avoided by use of other reactor configuration, for example a plug-flow reactor combined with enzyme immobilisation.[150,151]

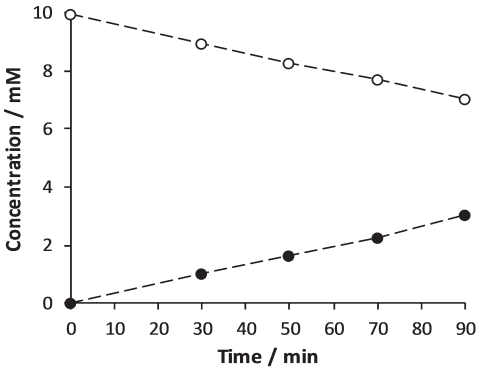



Figure 4.6. Conversion of glutamic acid (○) to 3-cyanopropanoic acid (●) by the coupled $AOX_{Hp}$ with VCPO in separate reactors as a function of time. Reaction rate: 0.033 mM/min. Reaction conditions: AOX reactor contained 2 mM ethanol, 0.5 mM NaBr, 1.5 U/mL $AOX_{Hp}$ in 0.6 mL of 20 mM sodium citrate buffer pH 5.6 saturated with oxygen, oxygen flow rate was ca. 5 mL pure oxygen/min; every 10 min the reaction mixture was separated from AOX and transferred to VCPO reactor then the AOX reactor was supplied with fresh reagents; VCPO reactor (before the addition of reaction mixture from AOX reactor) contained 10 mM glutamic acid, 0.5 mM NaBr, 4 U/mL VCPO in 0.5 mL of 20 mM sodium citrate buffer pH 5.6. Dashed lines (---) are only drawn as visual aids.

Despite the low conversion obtained this experiment proves that it is possible to perform the oxidative decarboxylation of Glu with HOBr produced *in situ* by the coupled reaction between VCPO and $AOX_{Hp}$. For this reaction to proceed, physical separation of the two enzymes appeared to be essential.

As mentioned in the introduction, only a few examples are known in literature regarding the combination of an oxidase with a haloperoxidase for the production of HOX for a typical halogenation reaction. These examples use either GOX or AOX with CPO and substrates that are easily susceptible to halogenation reactions, namely MCD,[136] barbituric acid[138] or propene.[140] Here it was shown that using the property of MCD as a good scavenger for HOBr[66] allowed full conversion of MCD in a single reactor for $AOX_{Hp}$ and VCPO. This is most likely due to the chemical reactivity of the substrate. MCD has a highly acidic proton at the reaction centre due to the two adjacent carbonyl groups which promotes fast halogenation reactions. Therefore, the conversion of MCD was detectable before the complete deactivation of $AOX_{Hp}$.

In contrast, the halogenation-oxidation of Glu seems to be slow, leading to accumulation of HOBr which inactivates $AOX_{Hp}$. However, it should be noted that another inactivation mechanism could be present: it has been reported[70] that bromoamines are unstable in the presence of $H_2O_2$ and decompose to give $^1O_2$. As bromoamines are intermediates in the VCPO-mediated oxidative decarboxylation of Glu, this reaction is likely to play a role. As inactivation of HPO by $^1O_2$ has been shown before,[98] this compound could also inactivate $AOX_{Hp}$ in our system.

Deactivation of GOX by HOX has not been reported before. This could be due to the low concentrations of substrate used in case of MCD[136] or the physical separation of GOX and CPO in the two chambers of the electrochemical cell used for barbituric acid.[138] Another reason could be that GOX compared to AOX is more stable in presence of HOX.

## 4.4 Conclusions

The aim of this chapter was to show the reaction setup required to perform the chemo-enzymatic cascade that combines an $AOX_{Hp}$ with VCPO for its ability to produce halogenating species which will be used in a later step for an oxidation reaction. In particular, it was shown that the cascade $AOX_{Hp}$-VCPO can be used for the oxidative decarboxylation of glutamic acid (Glu) to 3-cyanopropanoic acid (GluCN). The reagents required for this reaction were produced *in situ*. VCPO generated HOBr from $Br^-$ and $H_2O_2$ and $AOX_{Hp}$ generated $H_2O_2$ by activation of molecular oxygen and by oxidation of ethanol to volatile acetaldehyde. It was found that during the one-pot conversion of Glu to GluCN by the cascade $AOX_{Hp}$-VCPO, $AOX_{Hp}$ was deactivated by HOBr or by HOBr-derived $^1O_2$. Therefore, only when the two enzymes were physically separated in two fed-batch reactors the conversion of Glu to GluCN was possible. The deactivation of $AOX_{Hp}$ by HOBr is not so severe when HOBr is in presence of substrates which can be easily halogenated. This was the case of monochlorodimedone which was converted in one-pot by the cascade reaction of $AOX_{Hp}$ and VCPO. The chemo-enzymatic cascade $AOX_{Hp}$-VCPO using a suitable type of reactor can enable the generation of GluCN, an essential intermediate in the production of biobased acrylonitrile. The selection of easily separable co-products (e.g. volatile aldehydes) for cascade reactions could improve the sustainability of the biobased nitriles production but also of other oxidation reactions involving halogenating reagents. No doubt, further insight is required to elucidate the mechanism of deactivation of $AOX_{Hp}$ by HOBr. The selection of appropriate reactors and reaction conditions are necessary to bring this process to larger scale.

# Chapter 5

# Techno-economic assessment of the production of biobased nitriles from glutamic acid



The co-author for this chapter is: J.P.M. Sanders

## Abstract

Biobased acrylonitrile can be obtained from biomass derived glutamic acid (Glu) *via* an oxidative decarboxylation reaction forming the intermediate 3-cyanopropanoic acid (GluCN). Previously it was found that the oxidative decarboxylation step is detrimental from both economic and environmental standpoints due to the use of sodium hypochlorite (NaOCl). To improve the feasibility of this process, NaOCl was replaced with hydrogen peroxide and the enzyme vanadium chloroperoxidase (VCPO). The conversion of Glu to GluCN by VCPO-$H_2O_2$ was evaluated from a technical and economic perspective and was compared to the NaOCl process. Two other processes that make direct use of oxygen from air for GluCN production from Glu were also evaluated. Besides the conversion of Glu to GluCN, the process includes the purification of the nitrile by extraction and crystallisation. This work is aimed at studying the effects of different process parameters on the production costs of biobased nitriles as a guideline for future improvements.

It was found that by replacing NaOCl with VCPO-$H_2O_2$ the energy requirement of the process is reduced by approx. 40%. This is mainly a result from performing the reaction at 25°C, eliminating the need for cooling below room temperature. The mass balance is improved as selectivity close to 100% can be achieved by the VCPO-$H_2O_2$ system. The direct use of oxygen instead of NaOCl in the production of GluCN from Glu is the most beneficial from economic, energy use and waste generation perspective. The process that activates oxygen by an alcohol oxidase in a cascade reaction with VCPO was found to have the highest cost-benefit margin (194 €/t GluCN), mainly owed to the co-production of acetaldehyde which is a valuable product.

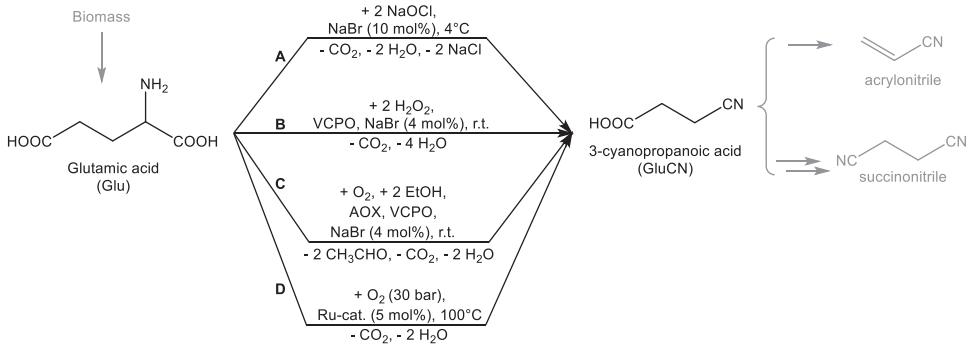
The price of GluCN and of Glu was identified as a bottleneck in this assessment however, the price of Glu (500 €/t) is already a best case scenario. Therefore, constraints attached to oil exploitation and $CO_2$ emissions must be applied to make the biobased nitriles competitive with the fossil-based nitriles.

## 5.1 Introduction

Nitriles, e.g. acrylonitrile, succinonitrile, are produced at large scale *via* the ammoxidation process.[26] However, the ammoxidation process is consuming high amounts of energy and relies on fossil resources. Environmental concerns are stimulating industry to find alternatives to the petro-chemical processes. Biobased alternatives have been proposed to produce nitriles from amino acids originated from biomass (Scheme 5.1).[42,44,59,81,93,94] It was shown that acrylonitrile can be produced in a two-step reaction from glutamic acid *via* an intermediate, 3-cyanopropanoic acid (GluCN).[42] For this the oxidative decarboxylation of glutamic acid to GluCN sodium hypochlorite and catalytic amounts of NaBr was used to form *in situ* the active species, HOBr (Scheme 5.1, route A).

The oxidative decarboxylation step for the production of biobased acrylonitrile is however, detrimental from both economic and environmental perspectives.[64,92] This is due to the use of sodium hypochlorite (NaOCl) which generates equimolar amounts of salt (NaCl) and the necessity of cooling below room temperature required to maintain high selectivity. The same bottlenecks were found for the production of biobased succinonitrile which can be produced in 3 steps from the glutamic acid.[64] Therefore, alternatives to the use of NaOCl to produce GluCN could bring significant improvements to the production of biobased nitriles originated from biomass.






Scheme 5.1. The conversion of glutamic acid (Glu) originated from biomass by routes A-D to the intermediate 3-cyanopropanoic acid (GluCN) and further to bulk nitriles such as acrylonitrile or succinonitrile. VCPO = vanadium chloroperoxidase, EtOH = ethanol, AOX = alcohol oxidase, r.t. = room temperature.

NaOCl can be replaced by other oxidants such as hydrogen peroxide or oxygen. To perform the oxidative decarboxylation of Glu, HOBr is required.[42] Haloperoxidases, a class of oxidative enzymes, are able to oxidise NaBr with hydrogen peroxide to produce HOBr.[74] In previous research it was demonstrated that glutamic acid can be converted to GluCN in a chemo-enzymatic reaction catalysed by vanadium chloroperoxidase (VCPO).[59] VCPO is active at room temperature and is using a cheaper and relatively clean oxidant ($H_2O_2$). Therefore, the necessity of cooling below room temperature and the use of NaOCl are eliminated from the process. The replacement of NaOCl with VCPO and $H_2O_2$ seems to

eliminate the two main bottlenecks identified previously in the production of acrylonitrile from biomass. However, other unfavourable aspects could arise in the new VCPO-$H_2O_2$ route. In order to make a rational evaluation a techno-economic assessment is performed. For this, the VCPO-$H_2O_2$ route is compared with the NaOCl route.

This work is aimed at studying the effects of different process parameters on the production costs of biobased nitriles as a guideline for future improvements and is not aimed at determining the absolute cost of 3-cyanopropanoic acid nor at an economic optimisation of the production process. The selection of the process conditions was based on laboratory data and previous research. The scale up was performed linearly following the guidelines of Piccinno *et al.*[152] The mass and energy balance was performed for different scenarios. A one at a time sensitivity analysis was performed to evaluate the robustness of the data selected in this chapter. The scenarios for producing GluCN from Glu are presented in Table 5.1.

Table 5.1. Overview of scenarios analysed for the production of biobased 3-cyanopropanoic acid from glutamic acid. Abbreviations: VCPO = vanadium chloroperoxidase, AOX = alcohol oxidase.

| Scenario | Name | Based on | Reaction route in Scheme 1 |
|---|---|---|---|
| 1 | NaOCl process | literature data[42] | A |
| 2 | VCPO-$H_2O_2$ process | laboratory data[116] | B |
| 3 | Optimised VCPO-$H_2O_2$ process | Scenario 2 upscale | B |
| 4 | AOX-VCPO process | laboratory data[153] | C |
| 5 | Ru-catalysed aerobic halide-free process | literature data[94] | D |

## 5.2 Process description

### 5.2.1 Scenario 1. NaOCl process

Scenario 1 represents the starting point of this techno-economic assessment. Although this process was already evaluated by Lammens *et al.*,[64] for the clarity of the assessment the key features and the key assumptions of this process are shown here as well. A detailed list of assumptions and the mass balance is available under Appendix D (Table D.1-6) and in the original assessment.[64]

In Figure 5.1 the process flow diagram is presented for the production of acrylonitrile. Previously for this process the main bottlenecks were identified in the first reactor as cooling below room temperature due to the use of NaOCl. Therefore, the focus of this chapter starts in reactor 1 and ends with the purification of GluCN before reactor 2, where the boundary (Figure 5.1, dashed line) of the process was set. The same boundary is used for the other scenarios.

Due to the boundary selected it was assumed that the starting material, glutamic acid is available from biomass, e.g. biorefinery rest streams such as sugar beet vinasse or distiller's grains with solubles, both rich in glutamic acid.[45]

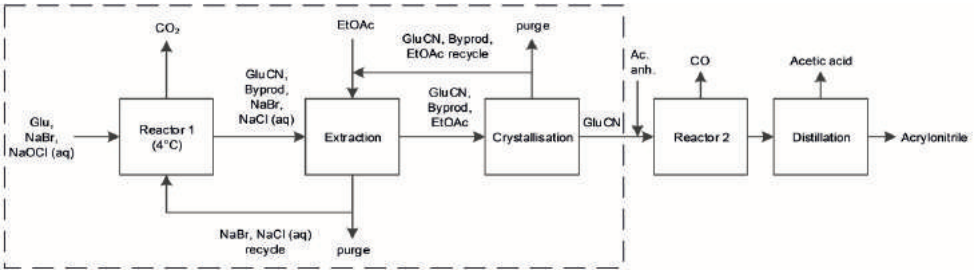



Figure 5.1. Process flow diagram for biobased acrylonitrile production *via* 3-cyanopropanoic acid. Abbreviations: Glu = glutamic acid, NaOCl = sodium hypochlorite, aq. = aqueous solution, GluCN = 3-cyanopropanoic acid, Byprod = by-products, EtOAc = ethyl acetate, Ac. anh. = acetic anhydride.

In reactor 1 the oxidative decarboxylation of Glu by NaOCl and catalytic amounts of NaBr (10 mol%) to GluCN and by-products (Scheme 5.1, route A) is conducted at low temperature (4°C). NaOCl needs to be added gradually to the solution to maintain a high selectivity.[42] Reactor 1 was considered a continuous stirred tank reactor (CSTR) or a plug flow reactor (PFR). The conversion of Glu to GluCN was considered 100% and the selectivity towards the nitrile was 90% which can be achieved only in a PFR. The extraction takes place in an extraction column in a continuous counter current with ethyl acetate. In a counter current flow the use of extraction solvent is significantly reduced however, a worst case scenario was assumed here where the volume of ethyl acetate was considered double the aqueous phase. To decrease the costs due to loss of ethyl acetate dissolved in the aqueous phase (ca. 8 wt-%), ethyl acetate is recovered by evaporation. In reactor 2, GluCN undergoes a decarbonylation-elimination reaction using acetic anhydride and a palladium catalyst. The product acrylonitrile and the valuable by-product acetic acid are removed from the reaction mixture by distillation. This reaction was already assessed previously[64] therefore this step is not analysed in the present study. The mass balance for Scenario 1 is presented in Table D.2.

### 5.2.2 Scenario 2. VCPO-$H_2O_2$ process

The production of GluCN *via* VCPO-$H_2O_2$ reaction[59,116] is the focus point of this techno-economic assessment. The chemo-enzymatic oxidative decarboxylation of Glu to GluCN by $H_2O_2$, is performed under VCPO catalysis, at room temperature (Scheme 5.1, route B). $H_2O_2$ is added gradually to the solution to avoid the formation of singlet oxygen, a reactive form of oxygen that would lead to side reactions.[116]

Based on previous findings at lab scale the conversion of Glu to GluCN in reactor 1 (Figure 5.2) is considered to be 100% and the selectivity close to 100%. The type of reactor is dependent on the formulation of the enzyme. If the enzyme is free in solution this reactor can be a fed-batch reactor (FBR),[154] in which case the enzyme will be returned to reactor 1 after the extraction step. However, continuous processes have a higher overall process efficiency.[155] Therefore, reactor 1 can be a continuous stirred tank reactor (CSTR) equipped

with a membrane with the pore size suitable to recover the free enzyme[156] or with a coarse mesh to recover the enzyme if it is immobilised. Continuous processes using immobilised enzymes are preferred over the batch reactors also due to lower size of production facilities (10 to 100 times smaller) which translates to lower capital costs.[155] If the enzyme is immobilised, reactor 1 can be a packed bed reactor (PBR). A PBR could provide a higher conversion per weight of catalyst compared to CSTR but it might be unsuitable as the $CO_2$ formed as by-product can be trapped in the packing disrupting the homogeneity of the flow.

To minimise the costs associated with enzyme purification, VCPO was only partially purified and it is considered to be 50% pure. From experimental data at lab scale the concentration of Glu was 5 mM and with 0.36 U/mL VCPO.

The assumptions made for Scenario 1 for extraction and crystallisation steps are also valid for Scenario 2. The mass balance for Scenario 2 is presented in
Table D.3.

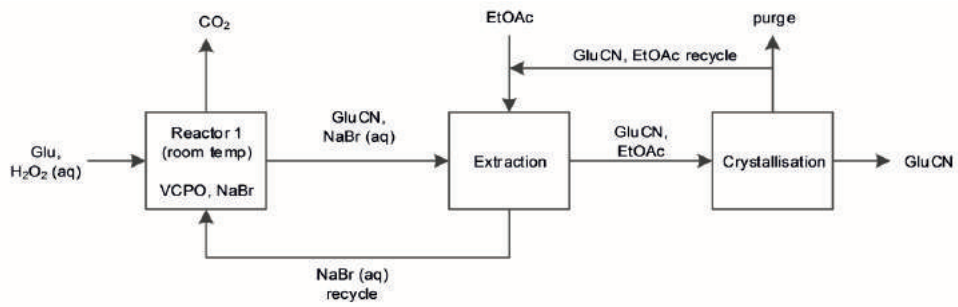



Figure 5.2. Process flow diagram for 3-cyanopropanoic acid production from glutamic acid by vanadium chloroperoxidase. Abbreviations: Glu = glutamic acid, aq. = aqueous solution, VCPO = vanadium chloroperoxidase, GluCN = 3-cyanopropanoic acid, EtOAc = ethyl acetate.

### 5.2.3 Scenario 3. Optimised VCPO-$H_2O_2$ process

The concentration of 5 mM (= 0.1 wt%) for Glu in Scenario 2 is not a realistic value for industrial scale and as a result the total reaction volume is above 2000 $m^3$ which results in large reactor volume and high amounts of extraction solvent. This would lead to high capital and operational expenses and therefore the concentration of the starting material needs to be scaled up towards industrial values. Although higher amounts of Glu were not tested, the concentration of Glu for Scenario 3 was set at 1 M (= 15 wt%), in the range of concentration used in Scenario 1, which makes it suitable for comparison. Due to the relatively low solubility of Glu in water, 7.5 g/L (that is 0.75 wt%), significant changes in the kinetics of the reaction are not expected. In a CSTR, the solution needs to be well mixed so that as soon as Glu is consumed in the reaction, more Glu will become soluble. However, a mixing tank should be included before a PBR. The energy consumption required for mixing was not considered as it is significantly lower compared to the energy required by the chemical reaction.

Next to the concentration of Glu, the enzyme was assumed to be reused for 50 runs without any loss in activity which allows a lower use of catalyst per run. A best case scenario is assumed here for the reusability of the enzyme and this will be further assessed under the sensitivity analysis. The mass balance for Scenario 3 is presented in Table D.4.

### 5.2.4 Scenario 4. AOX-VCPO process

The replacement of hypochlorite (Scenario 1) with hydrogen peroxide and VCPO (Scenario 2 and 3) has the benefit of reducing the amount of salt waste, the need of cooling below room temperature and an increase in the selectivity of the reaction. To further improve the conversion of amino acids to nitriles it was recommended to replace the hypochlorite with oxygen (Scenario 4 and 5). For example, oxygen can be converted *in situ* to hydrogen peroxide by oxidases.[127] These enzymes reduce oxygen to hydrogen peroxide with the help of an electron donor as a substrate (Scheme 5.2).

$$\text{Substrate} + O_2 \xrightarrow{\text{oxidase}} \text{Substrate}_{ox} + H_2O_2$$

Scheme 5.2. General reaction scheme of oxidases which reduce molecular oxygen to hydrogen peroxide by a substrate.



It was shown that using a cascade of alcohol oxidase (AOX) and VCPO it was possible to convert glutamic acid to 3-cyanopropanoic acid using ethanol as an electron donor (Scheme 5.1, Route C).[153] The separation of the two enzymes in two reactors (Figure 5.3) was necessary in order to avoid inactivation of AOX by HOBr species produced *in situ* by VCPO.[153] In reactor 1 oxygen gas from air is supplied to an ethanol solution which is converted by AOX to acetaldehyde and $H_2O_2$. For mass transfer intensification from gas to liquid phase reactor 1 is a column reactor equipped with a suitable sparger.[157] Using ethanol enables a relatively easy downstream processing by separating the by-product, i.e. acetaldehyde by pervaporation before the hydrogen peroxide stream enters reactor 2. In reactor 2 the oxidative decarboxylation reaction takes place as described in Scenario 3. Naturally, one-pot cascade reactions are preferred due to lower capital costs, nevertheless if the process economics are beneficial the capital costs could be recovered. The mass balance for Scenario 4 is presented in Table D.5.

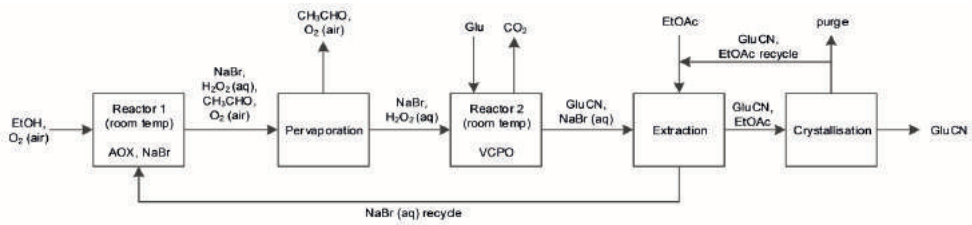



Figure 5.3. Process flow diagram for biobased 3-cyanopropanoic acid production by enzymatic cascade reactions. Abbreviations: EtOH = ethanol, AOX = alcohol oxidase, Glu = glutamic acid, aq. = aqueous solution, VCPO = vanadium chloroperoxidase, GluCN = 3-cyanopropanoic acid, EtOAc = ethyl acetate.

### 5.2.5 Scenario 5. Ruthenium-catalysed aerobic halide-free process

The use of hypochlorite in oxidative decarboxylation can be replaced by direct use of oxygen. This was shown to be possible under catalytic conditions using a heterogeneous ruthenium catalyst in water, without the use of halides (Scheme 5.1, Route D).[94] This process which minimises waste significantly, has a high potential to become a good alternative for the production of nitriles.

Figure 5.4 shows the flow scheme for the production of GluCN from Glu by Ru aerobic catalysis. The conversion of Glu to GluCN takes place at 100°C and 30 bar $O_2$ in reactor 1 which is a pressure stainless steel reactor. After 24 h, 80% conversion of Glu was achieved with a selectivity of 85%.[94] For the conversion and the reuse of the catalyst a best case scenario is assumed. Thus, a 100% conversion and a 100 runs reuse of the catalyst without loss in activity is assumed. The reaction mixture is then extracted with ethyl acetate and GluCN is crystallised similar to the other scenarios. The mass balance for Scenario 5 is presented in Table D.6. The concentration of the starting material was scaled up (from 0.1 M to 1 M) to reach a concentration in the range of Scenario 1, 3 and 4 which allows a more fair comparison.

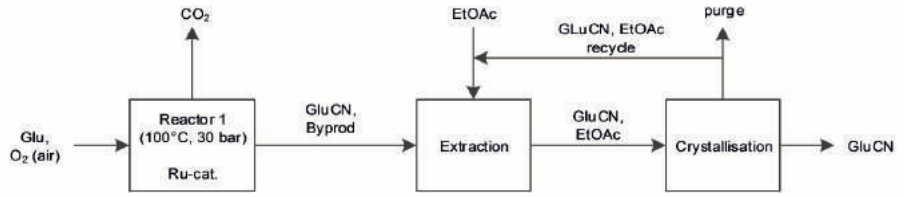



Figure 5.4. Process flow diagram for biobased 3-cyanopropanoic acid production by aerobic Ru-catalysis. Abbreviations: Ru-cat. = ruthenium catalyst, Glu = glutamic acid, aq. = aqueous solution, GluCN = 3-cyanopropanoic acid, Byprod = by-products, EtOAc = ethyl acetate.

## 5.3 Energy requirements of the process

For energy calculations the approach of Lammens *et al.*[64] was followed and for clarity the main assumptions are indicated here. The Gibbs free energy of the reaction was determined by computational calculations (details at Appendix D) for the oxidative decarboxylation with NaOCl (-807.9 kJ/mol) and for the VCPO-$H_2O_2$ reaction (-850.6 kJ/mol). The Gibbs free energy for the oxidative decarboxylation with a Ru catalyst was estimated to be in the exothermic range (-800 kJ/mol) and for the hydrogen peroxide production by AOX was calculated with data from literature[158] (-72.1 kJ/mol). All reactions are exothermic therefore the heat generated during the reaction needs to be removed by cooling. The energy required for cooling associated with the conversion of 1000 kg of Glu (6.8 kmol) for different scenarios is presented in Table 5.2.

Table 5.2. Energy requirements of the process for the production of 1000 kg Glu or GluCN for different scenarios. Data for Scenario 2 are available at Appendix D (Table D.7).

| Process unit | Scenario 1 | | Scenario 3 | | Scenario 4 | | Scenario 5 | |
|---|---|---|---|---|---|---|---|---|
| | T | Process energy | T | Process energy | T | Process energy | T | Process energy |
| | °C | MJ | °C | MJ | °C | MJ | °C | MJ |
| **Reactor 1** | 4 | 8236[a] | 25 | 5781 | 25 | 490 | 100 | 3307[b] |
| **Pervaporation** | - | - | - | - | 25 | nc | - | - |
| **Reactor 2** | - | - | - | - | 25 | 5781 | - | - |
| **Extraction** | 25 | nc | 25 | nc | 25 | nc | 25 | nc |
| **Crystallisation** | 4 | 618[a] | 4 | 783[a] | 4 | 783[a] | 4 | 857[a] |
| **Total for 1000 kg Glu** | | **8854** | | **6564** | | **7054** | | **4164** |
| **Total for 1000 kg GluCN[c]** | | **14610** | | **9877** | | **10604** | | **7606** |

T = operational temperature, [a]contains a multiple of 1.5 due to cooling necessity below room temperature, [b]reactor 1 benefits from the exothermic reaction that is released above 100°C (see Appendix D for details), [c]energy per process unit is not presented, nc = not considered.

The Gibbs free energy for the NaOCl reaction is lower than previously reported[64] and this is probably due to an updated version of the software (see Appendix D) which is more accurate. Next to this, the computations were performed in water as solvent compared to vacuum as performed previously.

For the extraction of GluCN from the aqueous phase it was assumed that the volume of ethyl acetate is double compared to that of the aqueous phase. Further in the crystallisation unit the ethyl acetate needs to be cooled from room temperature (25°C) to 4°C.[64] In Table 5.2 the energy requirement for the crystallisation unit is presented. Cooling below room temperature (4°C) is required for reactor 1 in Scenario 1 and in the crystallisation units in all scenarios. This energy which is supplied as electricity is assumed to be 1.5 fold the heat to be removed from the system.[64] Other energy requirements e.g. stirring, extraction, pumping fluids or filtration are not considered as it would be insignificant compared to the energy required to cool a reactor for relatively high exothermic reaction.

From the energy requirements (Table 5.2) it can be concluded that the main energy consumption in Scenario 1 is associated with cooling of reactor 1 from 25°C to 4°C which accounts for 93% of the total energy of the process. This result validates the previous assessment[64] where it was suggested that performing the reaction at higher temperature would benefit the energy balance.

In Scenario 2 the reaction takes place at room temperature without temperature control, however the reaction is exothermic. The temperature increase of the total water in reactor 1 (1223147 kg $H_2O$, Cp= 4186 J/kg·K) for the conversion of 1000 kg of Glu is 1°C (from 25°C to 26°C). This small increase in temperature of the reaction mixture is due to the dilute solution used in reactor 1 (5 mM Glu). In a batch process the temperature increase can be disregarded compared to the energy required for cooling for the crystallisation unit. Due to the high energy associated with cooling the large amount of ethyl acetate, this scenario requires optimisation with respects to the concentration of Glu.

In Scenario 3, by optimisation of the starting concentration of Glu, the amount of water as solvent is reduced and as a result the amount of ethyl acetate that requires cooling is reduced as well. Scenario 3 is comparable with Scenario 1 in terms of Glu concentration and energy requirement. However, for Scenario 3 the energy consumption associated for the production of 1 t GluCN is by a factor of 1.5 lower compared to Scenario 1. This difference in energy consumption is due to the following: reactor 1 is operated at room temperature and the selectivity of VCPO-$H_2O_2$ reaction (100%) is slightly higher compared to NaOCl reaction (90%). In Scenario 3 cooling can be performed using water from e.g. rivers and the heat gained could be used in another step where heating is required e.g. in the distillation step of acrylonitrile.

Performing the reaction at temperatures above room temperature could be possible as the enzyme VCPO is relatively thermostable[66] and this would further benefit the energy balance. Preliminary results of the oxidative decarboxylation reaction at 20, 30, 40, 50, 60 and 70°C (Figure D.1) show that the conversion of Glu towards GluCN has a peak at 40°C. The increase in conversion of Glu is by a factor of 2 from 20°C to 40°C. Above 40°C the conversion is decreasing with the increase in temperature of the reaction. Although VCPO is stable at higher temperatures the decrease in conversion of Glu is probably due to thermal degradation of $H_2O_2$ and at a non-optimal pH of 5.6 for $H_2O_2$.[159] It is not clear from the data if the maximum reaction rate was reached for each temperature, it might be that $H_2O_2$ was the limitative reactant and the use of a higher excess of $H_2O_2$ will be necessary to compensate for the losses by degradation at high temperatures. However, if the catalyst has high affinity for $H_2O_2$ and $H_2O_2$ is added gradually the degradation of $H_2O_2$ could be minimised.

A possibility on performing the reaction above 40°C is the oxidative decarboxylation of Glu by $H_2O_2$ and W-based heterogeneous catalyst.[81] Although this reaction was performed at room temperature it is generally known that heterogeneous catalysts can stand and perform better at higher temperature.[160] Due to the similar reaction conditions and reaction performance in terms of conversion (100%) and selectivity (>99%),[81] comparable results are expected as in Scenario 2 and 3 regarding the energy requirements and the economics. This is, however, based on the assumption that oxidant efficiency of the W-based catalyst (6-10 equivalents $H_2O_2$) can be optimised. Although the W-based catalysis approach was not evaluated in this techno-economic assessment it is recommended to further investigate this reaction at higher temperatures.

The energy consumption for scenario 4 is slightly higher (10.6 GJ) compared to that for Scenario 3 (9.8 GJ) and this is mainly due to the additional energy generated by the AOX reaction. The reaction conditions selected for the AOX could be further improved, one parameter is the reaction temperature. The optimal reaction temperature for AOX from *H. polymorpha* is around 40°C, using methanol as a substrate.[87] Operating the cascade reaction

at the same temperature would avoid intermediate cooling/heating steps. Furthermore, operating the cascade reactions at higher temperature would increase the reaction rate which in turn would reduce the residence time in the reactor and as a result the residual heat could be used further upstream.

The lowest energy requirement was found for Scenario 5 at 7.6 GJ for the production of 1 t GluCN. Although the reaction is exothermic the reaction energy is released above 100°C which is the reaction temperature. Therefore, this reaction can actually benefit from heat integration (see details at Appendix D). It can be concluded that in order to reduce the energy requirements the oxidative decarboxylation reaction should be operated above room temperature so the heat generated by the reaction can be integrated up/down stream in the process.

## 5.4 Cost-benefit analysis

The costs of all inputs of the process are calculated based on the mass and energy balances for 1000 kg GluCN. The prices for chemicals and utilities were taken from available market prices, from the previous assessment by Lammens *et al.*[64] or estimated. The investment costs are estimated by a method provided by Lange[161] based on a linear correlation of the so-called investment recovery provision and the overall energy losses in a chemical process. The total fixed costs were obtained by increasing the value of the provision by 33% (see details at Appendix D, Table D.8-D.11). The costs of operation of the factory were not calculated in detail in this assessment however, similar costs are expected for all scenarios. In Figure 5.5 the costs of inputs, of the goods sold and cost-benefit margin for the production of 1000 kg of GluCN are shown.



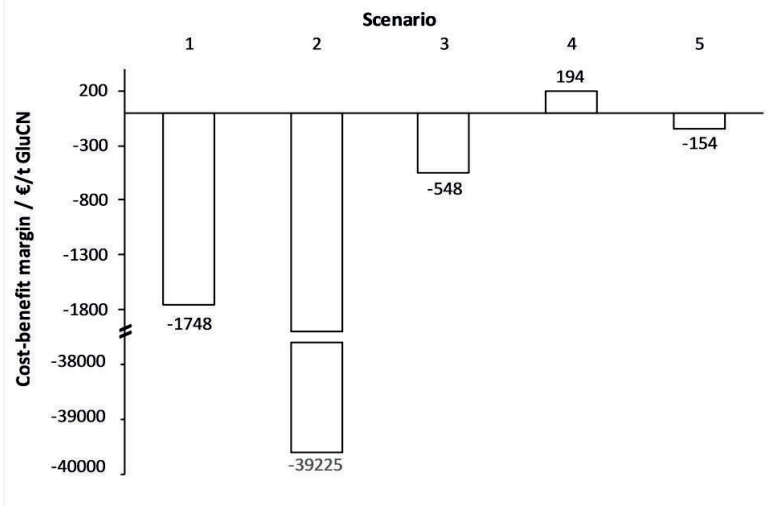



Figure 5.5. Cost-benefit margin for Scenario 1 to 5 as it results from Table D.12. Cost-benefit margin = goods sold – variable costs. For full size scale see Figure D.2.

### 5.4.1 Scenario 1

The mass balance of Scenario 1 (Table D.2) shows that each 1 t of GluCN requires 1.65 t of Glu and results in 0.99 t of waste ($CO_2$, water and other by-products). Also 2.18 t of NaCl are produced which from economic perspective is considered a valuable product and not waste. The cost-benefit margin found for Scenario 1 is -1748 €/t GluCN (Figure 5.5) and a negative value means that the production costs are higher than the value of the goods that are sold (Table D.8) therefore, the process is not profitable. This confirms the negative cost-benefit margin previously found for the production of acrylonitrile *via* the oxidative decarboxylation using NaOCl.[64] It was suggested that NaOCl needs to be replaced by cheaper sources of oxidants, such as hydrogen peroxide or oxygen.

### 5.4.2 Scenario 2 & 3

In Scenario 2 $H_2O_2$ and VCPO was used instead of NaOCl to produce GluCN from Glu. The cost-benefit margin found for Scenario 2 (-39225 €/t GluCN) is significantly lower compared to that of Scenario 1. The negative cost-benefit margin of Scenario 2 is due to large amounts of ethyl acetate needed for extraction and the energy required to cool ethyl acetate during crystallisation. Due to these detrimental factors in Scenario 3 the concentration of Glu in reaction mixture was increased which leads to a lower consumption of extraction solvent.

The mass balance in Scenario 3 and 2 is slightly improved as 100% selectivity can be achieved in the VCPO-$H_2O_2$ reaction. From 1.49 t of Glu, 1 t of GluCN and 1.17 t waste ($CO_2$ and water) are produced (Table 5.3). The cost-benefit margin found for Scenario 3 (-548 €/t GluCN) is improved compared to Scenario 1 (-1748 €/t GluCN) and Scenario 2 (-39225 €/t GluCN). The cost-benefit margin is negative and this is mainly due to the high costs of Glu and $H_2O_2$ (Table 5.3).

From these two inputs $H_2O_2$ can be still replaced by a cheaper oxidant i.e. oxygen from air, while the price of Glu remains an issue for the future. In Scenario 3 the amount of non-valuable products is higher compared to the value obtained in Scenario 1 (Table D.8). This is because NaCl produced in Scenario 1 is sold as a valuable product. However, the price of NaCl is relatively low compared to the mass that needs to be processed and is also an unfavourable product from environmental perspective. As a result of replacing NaOCl by $H_2O_2$ and VCPO in Scenario 2 and 3 the formation of NaCl as by-product is completely eliminated. Although the cost-benefit margin is negative it can be concluded that the changes made to the process (NaOCl replaced by $H_2O_2$ and VCPO, reaction at 25°C instead of 4°C) contributed to an overall improvement towards an economically feasible process.

Table 5.3. Economic assessment for Scenario 3.

| Scenario 3 | | | |
|---|---|---|---|
| **Compounds** | **Amount (kg)** | **Price (€/kg)** | **Value (€/t GluCN)** |
| ***Starting materials*** | | | |
| Glutamic acid | 1485 | 0.50[64] | 743 |
| Hydrogen peroxide | 687 | 0.62[162] | 426 |
| ***Catalysts & solvents*** | | | |
| Sodium bromide | 42 | 1.13[163] | 47 |
| Vanadium chloroperoxidase | 0.16 | 100[64] | 16 |
| Ethyl acetate* | 213 | 0.81[163] | 172 |
| Water as solvent* | 9899 | 0.00077[164] | 8 |
| **Energy & Fixed costs** | | | |
| Electricity (kWh) | 2744** | 0.055[64] | 151 |
| Fixed costs | | | 295◊ |
| ***Non-valuable products*** | | | |
| Carbon dioxide | 444 | - | |
| Water as by-product | 727 | - | |
| **Total costs** | | | **1858** |
| ***Valuable product*** | | | |
| 3-Cyanopropanoic acid | 1000 | 1.31Δ | 1310 |
| | | | |
| **Cost-benefit margin** | | | **- 548** |

* represents 1% losses; ** includes electricity required for cooling reactor 1 and for crystallisation unit; ΔGluCN is not a commercially available product, therefore the price is estimated to be a generic price for intermediate chemicals based on Szmant;[165] ◊ Investment costs are estimated by a method provided by Lange[161] based on a linear correlation of investment recovery provision and the overall energy losses in a chemical process. 9.9 GJ/t product corresponds to 150 $/t product in 1993 (value corrected for inflation). The total fixed costs were obtained by adding 1/3 to the provision; Cost-benefit margin = goods sold – variable costs, goods sold = 3-cyanopropanoic acid, variable costs = starting materials costs + catalysts & solvents costs + energy and fixed costs.

### 5.4.3 Scenario 4

In Scenario 4 according to the mass balance (Table D.5) 1.49 t Glu, 0.93 t EtOH and 0.65 t $O_2$ are required to produce 1 t GluCN and 0.9 t acetaldehyde. 1.17 t of waste are generated as $CO_2$ and water. In Figure 5.5 the cost-benefit margin is 194 €/t GluCN produced which means the process is profitable (for details see Table D.10). A one at the time sensitivity analysis (Figure 5.6) was performed for Scenario 4 to evaluate the robustness of the cost-benefit margin with variations in the prices of main compounds of the process (i.e. Glu, ethanol, ethyl acetate, alcohol oxidase, GluCN and acetaldehyde) and the fixed costs. The cost-benefit margin was recalculated first with the value of each price set to 75% and then to 125% of the nominal value of the price provided in Scenario 4. As seen in Figure 5.6, the lower the slope of the line between the value at 75% and 125% the less influence a parameter has on the cost-benefit margin. It can be observed that the major parameters influencing the cost-benefit margin are the price of Glu, acetaldehyde and GluCN. The major influencer is by far the price of the main product, GluCN. The other parameters such as the price of the enzymes, extraction solvent, ethanol and the fixed costs do not influence significantly the cost-benefit margin.

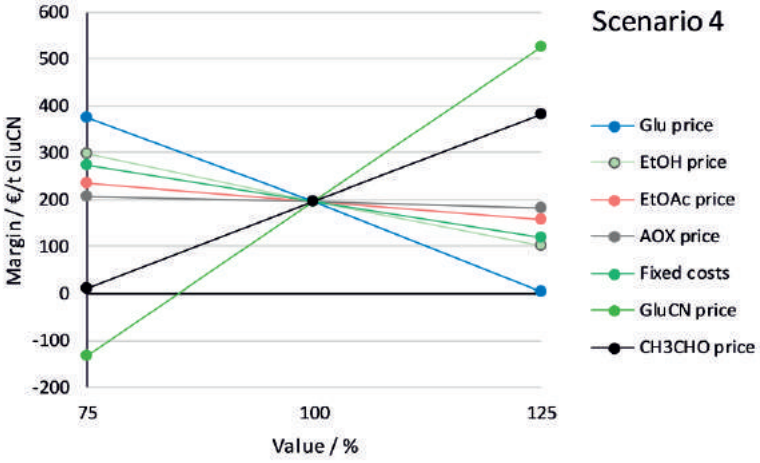



Figure 5.6. One at the time sensitivity analysis for Scenario 4.

### 5.4.4 Scenario 5

To produce 1 t GluCN using Ru catalysis in Scenario 5, 1.75 t Glu and 0.32 t $O_2$ are used and 1.1 t waste ($CO_2$, water and by-products) are generated (Table D.6). According to Figure 5.5 the cost-benefit margin is 154 €/t GluCN, which means the production costs for GluCN are higher than the benefits of selling GluCN (for details see Table D.11). To find which parameters influence the cost-benefit margin a one at time sensitivity analysis was performed in the same way as described in Scenario 4 (Figure 5.7). From the sensitivity analysis it can be observed that the price of Glu and of GluCN have a major influence on the cost-benefit margin.

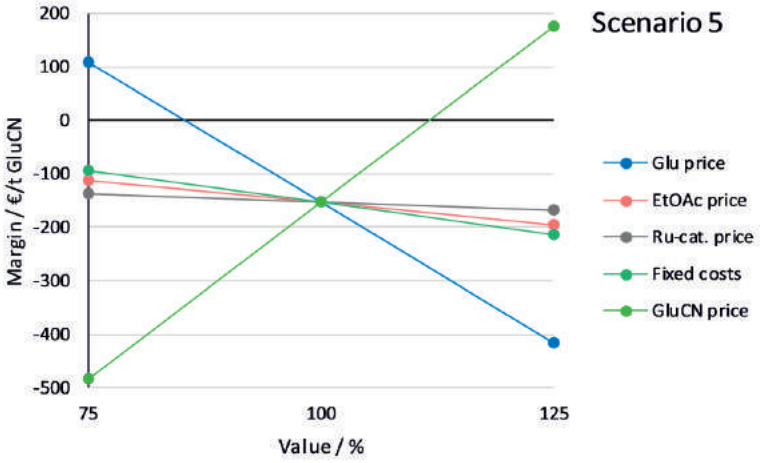



Figure 5.7. One at the time sensitivity analysis for Scenario 5.

Additionally, the influence of the selectivity of the reaction on the cost-benefit margin was evaluated in Figure 5.8. The selectivity was set to 70% and 100% compared to the value

reported in literature (85%). In Figure 5.8, it can be observed that the when the selectivity is set at 100% the cost-benefit margin is increasing from -154 €/t GluCN to -23 €/t GluCN, therefore optimisation towards achieving higher selectivity is recommended.

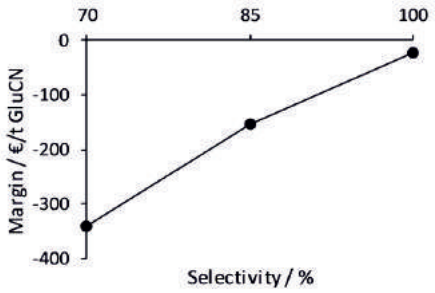



Figure 5.8. The influence of the selectivity value on the cost-benefit margin for Scenario 5.

The replacement of hydrogen peroxide by oxygen increased significantly the cost-benefit margin in Scenario 4 and 5. It can be concluded that from an economic perspective the most beneficial process is Scenario 4 closely followed by Scenario 5. The main cost contributors to the process of these 2 scenarios are the price of Glu (and of ethanol for Scenario 4), the investment recovery provision, followed by auxiliary materials (electricity and extraction solvent). From the sensitivity analysis it is clear that changes in the price of Glu and GluCN are the most influential on the cost-benefit margin. Additionally for Scenario 4 is the change in the price of acetaldehyde, and for Scenario 5 is the value of the selectivity of the oxidative decarboxylation reaction.



### 5.4.5 Other costs

Costs considerations that are not evident from the cost-benefit analysis are for example, the type of reactor. A detailed analysis for choosing the type of the reactor will give a better idea on how the type of reactor influences the production costs. This is especially true for Scenario 4 where two reactors are required. The capital cost could be reduced by designing a packed bed reactor where both enzymes reside one after another so that the AOX does not come in contact with the HOBr produced by VCPO which avoids inhibition of AOX.

Furthermore, the use of a crude enzyme can cause foaming due to high protein concentration which could increase the size of the reactor and therefore the capital costs. Immobilisation of the enzyme on a solid support could avoid the foam formation as well as it allows an easy separation and reuse of enzyme. The use of immobilised enzyme(s) could bring other advantages: an increased rate of catalysis, prolonged duration of catalysis, greater operational stability to extremes of pH, temperature, etc.[155] The reactor type and the possibility to immobilise the enzymes should be further investigated.

### 5.4.6 The price of Glu

The price of Glu is one of the main contributors to the costs of the process. The price of Glu (500 €/t) was previously estimated for pure amino acids originating from biomass[64] and it is already a best case scenario. The relatively high costs of amino acids are due to the difficulty to separate compounds with very similar properties. Therefore, the decrease in the price of Glu even with improvements in biorefinery technology is quite unlikely. However, the use of un-purified streams of amino acids could reduce the price of Glu and other amino acids. The conversion of amino acids from protein hydrolysates[81] or synthetic mixtures of amino acids[59,93,116] into their corresponding nitriles was demonstrated by different catalysis methods. However, further investigation to optimise these conversions and to test different protein hydrolysates is yet necessary.

The use of cheaper sources of un-purified amino acids would only transfer the purification step from upstream – purification of amino acids, to downstream – purification of nitriles. Therefore, to decrease the costs, the separation and purification of the nitriles has to be easier and cheaper than the purification of amino acids. For example, the nitriles originating from amino acids can be separated by liquid-liquid extraction into polar and non-polar nitriles. The non-polar nitriles could be further separated by distillation while the polar ones by a succession of various techniques such as electrodialysis, crystallisation. However, further research is necessary to conclude whether the separation of nitriles is more feasible than the separation of amino acids.

### 5.4.7 How does the cost-benefit margin change with the end-application of GluCN?

GluCN is an intermediate compound which is not commercially available and in practice GluCN would be used directly in the next step therefore, the value of GluCN might have been underestimated. In this case an increase in the GluCN price by 25% would give a cost-benefit margin slightly above 500 €/t GluCN. If the oxidative decarboxylation of Glu to GluCN *via* VCPO-$H_2O_2$ is implemented in the production of acrylonitrile – a commodity chemical with a quite low price (~1400 €/t[163,166]) then the cost-benefit margin would decrease towards negative values.

The oxidative decarboxylation reaction is also used in the production of succinonitrile *via* the ω-methylated Glu (MeGlu). This process was previously found to have higher potential to be profitable compared to acrylonitrile process but only if the NaOCl is replaced by a cheaper and cleaner source of oxidant and the oxidative decarboxylation is performed at higher temperatures. The oxidative decarboxylation of MeGlu to MeGluCN was shown to be successful at lab scale with the VCPO-$H_2O_2$ system at room temperature.[116] It can be concluded that if MeGluCN is implemented in the production of succinonitrile the cost-benefit margin will be positive. This is due to the higher price of succinonitrile (~2700 €/t[64]) compared to acrylonitrile as well as a more efficient mass balance of the overall process.

With increasing environmental concerns related to the use of fossil resources and with significant constraints applied for their exploitation or to $CO_2$ emissions, the cost of fossil resources will increase in the future. Consequently, this will increase the price of chemicals produced from fossil resources and the biobased alternatives can become competitive or even cheaper. To produce nitriles such as acrylonitrile from biomass the price of amino acids needs to decrease by using raw mixtures of amino acids or most sensibly, constraints attached to oil exploitation and $CO_2$ emissions need to be applied.

In conclusion, by replacing NaOCl with catalysts such as VCPO, the bottleneck is directed up and downstream from the oxidative decarboxylation reaction.

## 5.5 Waste considerations

A fast method to evaluate the environmental impact of the production process of chemicals is the E-factor.[167,168] This is calculated based on the mass balance as the total amount of waste which is everything but the desired product divided by the amount of the desired product. Water is not considered as waste therefore, is completely excluded from the calculation. The higher the E-factor the more waste is produced and consequently, the greater the environmental impact is.



Table 5.4. The values of the E-factor for Scenarios 1-5. [a]only GluCN is considered the desired product, [b]both GluCN and acetaldehyde are considered the desired products.

| Scenario | E-factor (kg waste/kg product[a]) |
|---|---|
| 1 | 3.0 |
| 2 | 40.9 |
| 3 | 0.7 |
| 4 | 0.4[b] (1.6) |
| 5 | 0.9 |

The values of the E-factor are in the low range of bulk chemicals, i.e. <1-5 kg waste/kg product,[167] except for the non-optimised Scenario 2 (Table 5.4). The lowest E-factor (0.4) was found for Scenario 4 where acetaldehyde is considered a valuable product and not waste. When acetaldehyde cannot be sold the E-factor increases to 1.6 but still this is lower in comparison with Scenario 1.

The E-factor for Scenario 5 is quite low (0.9) however, this value should be further considered due to the use of ruthenium catalyst which requires special disposal. Next to the amount of waste produced, the nature of the waste, the ease of recycling should be considered through an in-depth environmental analysis such as life cycle assessment.

## 5.6 Conclusions

The biobased conversion of glutamic acid to 3-cyanopropanoic acid by VCPO-$H_2O_2$ was evaluated from a technical and economic perspective (Scenario 2 & 3). This process was compared to the previous process where NaOCl was used as oxidant (Scenario 1) and with two new processes that use oxygen. One process produces hydrogen peroxide from oxygen and ethanol by an alcohol oxidase (Scenario 4) and the other is using oxygen under Ru catalysis as direct oxidation source (Scenario 5).

It was found that by replacing NaOCl with VCPO-$H_2O_2$ the energy requirements of the process is reduced by a factor of 1.5 for 1 t GluCN. This is mainly as a result of performing the reaction at 25°C, eliminating the need of cooling below room temperature (4°C) as in the case of NaOCl. The mass balance is slightly improved as selectivity close to 100% can be achieved by VCPO-$H_2O_2$ system. Scaling up the initial concentration of Glu in the VCPO-$H_2O_2$ reaction reduces dramatically the amount of extraction solvent required and reduces the production costs.

By further replacing NaOCl with oxygen in Scenario 4 and 5 the cost-benefit margin was increased significantly. Based on the cost-benefit analysis the only scenario with a positive margin of 194 €/t GluCN is Scenario 4, owed to the co-production of acetaldehyde which is a valuable product. The sensitivity analysis of Scenario 4 and 5 where the price of different compounds was changed, shows that the price of Glu and GluCN are the parameters that influence the most the economics of the process.

It can be concluded that replacing NaOCl in the production of GluCN from Glu by a process that makes direct use of oxygen, is the most beneficial from economic, energy use and waste generation perspective. Furthermore, to produce biobased industrial nitriles such as acrylonitrile from amino acids, the cost of amino acids needs to decrease by using raw mixtures of amino acids or constraints attached to oil exploitation and $CO_2$ emissions need to be applied.

# Chapter 6

# General discussion

In this thesis the enzyme vanadium chloroperoxidase was used as bio-catalyst to produce biobased nitriles from amino acids *via* the oxidative decarboxylation reaction.

The aim of this chapter is to integrate the main findings of this research in a general discussion. Relevant aspects regarding the oxidative decarboxylation of amino acids that were not addressed in Chapters 1-5 will be pointed out here. The discussion is conducted based on two key factors that influence the production of biobased nitriles from amino acids by oxidative decarboxylation:

1. *The technology available to produce biobased nitriles from amino acids*, which is related to the developments in catalysis and the reaction conditions associated to the use of a certain catalyst.

2. *The availability and the costs of nitrogen containing biomass*, which is related to the biomass sources that contain relevant amounts of protein and/or amino acids not required as food or feed, the technology available to extract and separate protein and amino acids from biomass and, the price of amino acids.

## 6.1 The technology available to produce biobased nitriles

The technology available to produce biobased nitriles is dependent on the catalyst used for the oxidative decarboxylation reaction. As a result of the chosen catalyst, other parameters will further influence the production of biobased nitriles: the availability of the catalyst, the requirement for other reagents, the reaction conditions, etc.

### 6.1.1 The availability of the enzyme

Enzymes are specific catalysts which work under mild reaction conditions: water as solvent, room temperature and pH close to neutral values. These characteristics comply with environmental sustainability targets and low capital investments which promote the use of enzymes as catalysts in industrial applications.[169]

Despite the consensus that enzymes are expensive to produce,[170] enzymes are used in the bulk industrial applications. For example, one time use enzymes are incorporated in detergents and these range from large classes of enzymes like proteases, lipases, amylases and cellulases[170] to very specific such as mannanases.[171] Another enzyme used for the production of bulk chemicals is the nitrile hydratase from *Rhodococcus* sp. or mutants of *Pseudomonas chloraphis* by the company Mitsubishi Rayon. The immobilised cells containing the nitrile hydratase are used for the conversion of acrylonitrile to acrylamide at an industrial scale exceeding 30000 t/y.[172]

In this thesis the production of nitriles from amino acids was conducted with VCPO, which is produced at laboratory scale in expression hosts such as *Escherichia coli* or *Saccharomyces cerevisiae*. This involves cell breaking and isolation of the enzyme from the cell material next to further purification steps including chromatography. At industrial scale expression hosts such as *Aspergillus niger*, *Bacillus subtilis, Pichia pastoris* which allow the harvest of the enzyme from the fermentation media[173] are commonly used. In the past, the expression of VCPO in *A. niger* was tested at industrial pilot scale however, the activity of VCPO turned out to be low.[174] This was probably due to the simultaneous production of catalase which consumes hydrogen peroxide faster than VCPO. There was at that time little incentive to further investigate the cause of low VCPO activity so the upscaling process was stopped.[174]

The main cost of enzyme production is the purification step[175] however, cost reduction can be achieved by optimisation of the manufacturing process by using e.g. genetic engineering and low-tech isolation and purification methods.[170,175] Based on the previously reported thermostability (up to 70°C)[101] the purification of VCPO expressed in *Escherichia coli* in this thesis (Chapter 2, see 2.2.2) involved a thermal treatment step.[116] Thermolabile proteins or enzymes such as catalases and proteases that may interfere with the VCPO activity can be removed also at industrial scale in a relatively cheap and low tech purification step.

Despite the robustness of VCPO[130] some reaction conditions should be avoided when converting amino acids to nitriles. For example, the use of phosphate buffers is not advised

as phosphate can replace vanadate and reduce the enzymatic activity by half.[176] Therefore, biomass treated by phytases to hydrolyse phytic acid – an anti-nutritional factor in plants, and storage of VCPO in phosphate buffers should be avoided. Based on laboratory practice, VCPO can be stored for months in suitable conditions[177] and can withstand multiple freeze-thaw cycles without loss in activity.[174] The high storage stability can be a great advantage for the industrial use of VCPO.

In comparison with chemical catalysts enzymes generally have, amongst other, lower stability and higher sensitivity to inhibitors, but this can be overcome by applying control of the reaction conditions. For example, high concentration of halides decreases the VCPO activity by substrate inhibition mechanisms. However, it was shown that the use of halide concentration above the $K_i$ value improved the conversion and the nitrile selectivity in the case of some amino acids (Chapter 2 & 3, Table 2.1, Figure 3.1). Compared to other haloperoxidases VCPO is resistant to higher concentration of oxidants and can withstand organic solvents[130] such as methanol, ethanol and 2-propanol[66] which enables better solubility of substrates. Also ethyl acetate was used in combination with VCPO which improved the selectivity by continuous extraction of products.[59]

Furthermore, by immobilisation of enzymes some characteristics e.g. stability, thermal resistance, use of organic solvents can be improved[175] which brings economic benefits next to the reuse of the enzyme. Although, VCPO was not yet immobilised, many immobilisation techniques are available.[178] The use of a charged support which would attract the amino acid in the proximity of the enzyme as in the case of W-based heterogeneous[81] catalyst can be a promising option.

### 6.1.2 Other catalytic methods

Alternatives to the production of biobased nitriles using the chemo-enzymatic catalysis by VCPO have been developed.

A heterogeneous catalysis approach based on a W-catalyst, $H_2O_2$ as oxidant and $NH_4Br$ as bromide source can be used to produce nitriles from amino acids.[81] High yield (91-99%) and selectivity (99%) were achieved for the majority of amino acids. It was shown that this approach can be applied to a mixture of amino acids obtained by protein hydrolysis. An advantage of this approach is the possibility to recycle the catalyst. This was tested for three consecutive runs and yields above 81% were achieved. The results obtained with the W-catalyst are similar to those of the VCPO however, the oxidant efficiency is 2- to 4-folds lower than with VCPO. Next to this, the load of the catalyst is higher compared to the VCPO process and it is not shown how feasible is to prepare the catalyst at industrial scale.

Another possibility to produce nitriles from amino acids is an electrochemical approach.[93] By two electron oxidation, $Br^-$ is oxidised at a Pt anode to hypobromite ($OBr^-$). The *in situ* generated $OBr^-$ is further reacting with the amino acids to form the corresponding nitriles.

The protons generated by the oxidative decarboxylation of amino acids are reduced to $H_2$ with the electrons that are transmitted to a Ni cathode. Good yields (70-94%) for the nitriles were obtained for most of amino acids. Some nitriles (5-aminopentanenitrile, LysCN) are further converted at the Ni cathode to the corresponding amides and amines, which are suitable building blocks for polymers applications. The charge applied and the current density are critical parameters of this approach as well as the regeneration of the Pt and Ni electrodes at large scale.

A halide-free approach for the production of biobased nitriles is based on the use of a Ru-catalyst and oxygen as oxidator.[94] Ruthenium promotes the aerobic oxidation by direct interaction with the amino acid. Next to the nitrile a hydroperoxide or hydrogen peroxide are produced which is rapidly decomposed by the catalyst itself to water and oxygen. High yield (89-99%) and fairly high selectivity (75-83%) was obtained for the aliphatic amino acids. Though, for the amino acids with OH (threonine) and COOH (aspartic acid) side functionality, significantly lower values were found partially due to aerobic alcohol oxidation at the Ru-catalyst. After three consecutive runs the activity of the Ru catalyst was 50% which is much lower compared to that of the W-based catalyst.[81] In Chapter 5 it was shown that this method can be economically feasible if the catalyst could be reused up to 100 runs and if the price of fossil based-nitriles will increase offering a fair competition for the biobased processes.

Today, biobased nitriles can be produced using a multitude of catalytic methods. A techno-economic assessment as performed in Chapter 5 can show which method should be selected in order to have a profitable process. It is important to further supplement the techno-economic assessment by an environmental assessment such as a life cycle assessment. This will indeed show the impact of each process on environment and if the biobased nitriles will be more environmentally friendly than the fossil-based equivalents.



### 6.1.3 The influence of reaction conditions on nitrile selectivity

In this thesis a better understanding of the parameters that influence the selectivity towards nitriles has been achieved. It was found that the type and the concentration of the halide is important as well as the structure of the amino acid and its side chain functionality.

In Chapter 3 (see 3.3.3) a self-catalytic reaction mechanism for the acidic amino acids was proposed. It is necessary to further confirm the reaction mechanism proposed for the acidic amino acids. It is yet unclear whether the bromination occurs at nitrogen of the amino group or at the oxygen of the carboxylic group(s). Isolation of intermediates would be also difficult as the brominated amino acids are not expected to have high stability. However, larger scale reactions using chlorination agents could allow the isolation of the chlorinated intermediates which are more stable than the brominated ones. The size difference between the chlorine and the bromine atoms could influence the reactivity of the amino acid. Larger scale reactions would also allow to verify the possibility of side bromination

reaction of malonic acid or other by-products from the oxidative decarboxylation of Asp. Quenching the reaction using thiosulphate should be avoided as it might reduce the active halogenated intermediate and not only the hypohalite.

The influence of the side chain functionality and its position on the carbon chain (Chapter 3) should be further investigated with amino acids carrying functionalities at the β carbon such as threonine and β-methyl aspartic acid (Maa). New preliminary results at Biobased Chemistry and Technology department suggest that due to the extra methyl group in Maa the side chain carboxyl functionality would be forced in a sterically favourable position which promotes the conversion towards the nitrile even at low concentrations of NaBr (0.5 mM).[179] Further investigation at larger scale to better identify all side-products combined with computational methods could also be used to confirm this hypothesis.

Amino acids with basic side chain functionalities (arginine, ornithine, lysine) could provide further insight in the reaction mechanism involving the bromination of the side chain as it is expected that the nitrogen from amino group would be easier brominated than the oxygen from the carboxyl. Other amino acids with amide side chain functionality (glutamine and asparagine) could provide further confirmation of the results obtained with the ω-methyl esters of Glu and Asp.

Results on mixtures of amino acids such as Asp and Glu (Chapter 2, Figure 2.3) but also on complex mixtures (results not presented) show that there is a competition between amino acids when bromide is not provided in sufficient amounts. In Chapter 5 it was concluded that cheaper sources of amino acids, such as mixtures of amino acids, could improve the economics of the process. Therefore, the use of rest streams of proteic hydrolysates need to be tested. The demonstration of larger scale reactions and in crude mixtures of amino acids would provide more incentive to produce nitriles from amino acids at industrial scale.

### 6.1.4 The source of bromide

Next to the use of halides for oxidation reactions such as the production of nitriles from amino acids (this thesis), halides are used in numerous applications: hard plastics (polyvinylchloride-PVC), water treatment, medicines, pesticides, cleaning agents, flame retardants, etc. Due to these applications there is a relatively high demand to produce active halides i.e. $X_2$. Some halides, such as NaCl can be mined as almost pure NaCl and converted by electrolysis to $Cl_2$ which is further used as chlorinating agent.

Less abundant halides, such as bromide, are available in sea water and brine wells. Their direct crystallisation from sea water is however, challenging due to the low concentration of bromide i.e. 0.065 g/L in sea water and 6.5 g/L in the southern basin of the Dead Sea,[180] compared to other salts like chloride i.e. 20 g/L in sea water.[181] The extraction of halides and bromide in particular is driven at the moment by the so-called 'bromine-based drilling fluids' market which is fluctuating according to the demand for fossil gas and oil.[180] However, it is

expected that in the future with the increasing demands for sustainable energy (EU directive) the fossil oil exploitation will decrease and along with this the incentives for extracting bromide from sea water will decrease as well. Next to this, the low selling price of NaBr also encourages the industry to convert bromide to more profitable products such as $Br_2$, HBr, fine brominated chemicals, etc.[180] However the production of halogenated organic compounds which uses stoichiometric reagents is associated with environmental issues such as toxic products and energy-intensive processes.[123]

Therefore, alternative methods that activate halides *in situ* by catalysis in sea water will become more popular. For example, the direct use of sea water as a source of bromide and as reaction media was demonstrated for the Aza-Achmatowicz reaction using the VCPO from *C. inaequalis*.[124] However, in sea water lower conversion was obtained than in buffered solution with only bromide. This is due to the broader range of substrate of VCPO which includes chloride and bromide both present in sea water.

In the presence of a mixture of $Cl^-$ and $Br^-$, Glu was fully converted into the corresponding nitrile while Asp had low conversion and nitrile selectivity. Due to the higher ratio of bromide to chloride in the Dead Sea compared to other sea waters[25] and the higher affinity of VCPO for bromide[80] the conversion of amino acids to nitriles could give promising results using water from the Dead Sea. It was shown that using bromide instead of chloride as a source of oxidising reagent results in higher selectivity for nitriles in the case of Phe[59] and Asp (Chapter 2, Figure 2.2). The use of a bromoperoxidase such as the vanadium bromoperoxidase from *A. nodosum*[182] that can convert bromide but not chloride would be likely the most viable option when sea water is used as a reaction medium for the nitrile production.



### 6.1.5 Alternatives to $H_2O_2$

VCPO requires $H_2O_2$ to oxidise bromide *in situ* to hypobromite which is then used for the oxidative decarboxylation of amino acids to nitriles. In the absence of a good scavenger, the active bromine can react in a side reaction with $H_2O_2$ to form singlet oxygen which can further react with the organic compounds present in the reaction. It was shown that continuous dosing of $H_2O_2$ by a syringe pump gives higher nitrile selectivity in case of Glu, compared to addition of $H_2O_2$ in small portions (Chapter 2, Figure A.2). To avoid undesired side reactions and an inefficient use of the peroxide, continuous addition of $H_2O_2$ is required in the conversion of amino acids to nitriles. In this thesis the continuous dosing of $H_2O_2$ was by a syringe pump (Chapter 2 and 3) and by *in situ* generation of $H_2O_2$ by an alcohol oxidase (Chapter 4) but other alternatives are available as well. A few examples are detailed further.

Direct synthesis of $H_2O_2$ presents considerable industrial interest as an alternative to the anthraquinone process.[85] This method involves the direct use of hydrogen and oxygen in the presence of a nanoparticle catalyst containing a noble metal such as Au, Pd, Pt or bi-metallic combination supported on carbon or on metal oxides. Many challenges still need

to be overcome such as the explosive nature of oxygen and hydrogen mixtures, competitive reactions which are thermodynamically favourable ($H_2O_2$ decomposition or the formation of water), mass transport barriers, etc. Direct synthesis would align with the requirements of most oxidation reactions (3-5 wt-%)[183] and dilution of streams would not be necessary. Due to the partial decomposition of $H_2O_2$ at the metal surface into $H_2$ and water, *in situ* production and immediate consumption is desired. For example, the production of propylene oxide from propylene is carried out with *in situ* production of $H_2O_2$ by direct synthesis from hydrogen and oxygen.[183]

Another alternative for *in situ* production of $H_2O_2$ is electrolysis. Hydrogen peroxide is produced in the concentration range of 1-2% (wt-%) at the cathode which is a graphite electrode coated by different materials.[184,185] Electroreduction of oxygen at the cathode combined with a ion-exchange membrane was also reported for the *in situ* production of $H_2O_2$.[184]

Photocatalytic reactions over semiconductor oxide ($TiO_2$) particles have been proposed for the *in situ* $H_2O_2$ production.[132] Also UV irradiated flavin mononucleotide is used in cascade reactions involving (halo)peroxidases.[186,187] For example, the light driven bromination of thymol by a vanadium bromoperoxidase was demonstrated.[187]

It would be interesting to evaluate the performance of different methods for the *in situ* production of hydrogen peroxide in combination with the nitrile production. This will allow a suitable selection for the pathway towards biobased nitriles.

### 6.1.6 The AOX-VCPO cascade reaction

In Chapter 4 the cascade reaction AOX-VCPO was used to produce nitriles from amino acids. For this the operational window of the cascade – where the operational windows of both enzymes overlap – had to be determined.[188]

VCPO has a pH range among the acidic values (4-6)[78] and an optimum pH for the nitrile production at pH 5.6.[59] The AOX enzymes however, have a higher and broader operational pH range (5-10) depending on the source of the enzyme.[87] The alcohol oxidase from *H. polymorpha* ($AOX_{Hp}$) was selected to be used in the cascade $AOX_{Hp}$-VCPO due to its high activity at pH 5.6 (Chapter 4, Figure 4.1). It is known that the activity of $AOX_{Hp}$ at pH 5.6 at 30°C is about 70% of the activity at the optimal pH (8-10).[189] Therefore, by performing the reaction at higher pH values, the AOX performance could increase and the production of the nitrile by the cascade AOX-VCPO would increase as well. The use of haloperoxidases active at higher pH values (> 5.6) such as mutants of VCPO[78,79] or bromoperoxidases[190,191] should be tested as well.

In Chapter 5 it was concluded that performing the reaction at higher temperature is beneficial due to the possibility of heat integration. The optimal temperature of the $AOX_{Hp}$ is around 40°C[189] and VCPO is known to be thermostable.[101] Also based on an empirical rule

the activity of the enzymes doubles with every 10°C increase in reaction temperature.[192] The cascade reaction should be further investigated at higher temperature but keeping in mind the stability of $H_2O_2$ with increase in temperature.[159]

The *in situ* production of hydrogen peroxide is dependent on the availability of dissolved oxygen in aqueous media which limits the oxygen mass transfer. The concentration of oxygen in water at 25°C and 1 atm is about 0.26 mM and the solubility decreases with increase in temperature and salt concentration.[146] By increasing the stirring rate the oxygen solubility in reaction media can be increased however, when a stirring rate of 400 rpm (used for VCPO) was applied to AOX reaction, the activity of AOX decreased (results not shown). The magnetic stirring was replaced by increase in oxygen supply rate but this resulted in excessive foaming for AOX leading towards denaturation of the protein at the gas-liquid interface (Chapter 4, Figure 4.5). To avoid denaturation of AOX due to foaming the use of immobilised enzyme[193] should be tested.

Other process intensification methods such as pressurised reactors,[194] use of spargers or oxygen vectors[195] can be employed to increase the availability of oxygen in the aqueous solution. The so-called 'oxygen vectors'[195] are commonly used to enhance the oxygen mass transfer in fermentation processes.[196] These oxygen vectors are organic liquids e.g. ethanol, octanol, *n*-hexane, *n*-dodecane or toluene, in which the solubility of oxygen is significantly higher.[197] By emulsification of the reaction media with an inert organic liquid the oxygen mass transfer is enhanced due to high interfacial area and chemical potential gradient.[195] The use of ethanol in higher amounts should be further investigated for its double role as substrate for AOX and as oxygen vector.



To apply the AOX-VCPO cascade reaction at industrial scale accurate kinetic data need to be collected. However, due to the complex enzymatic kinetics, this is rather challenging especially for reactions with multiple substrates.[192] Due to the chemical nature of the compounds (not UV active), chromatography methods in combination with universal detectors e.g. refractive index (nitriles, ethanol), flame detection ionisation (ethanol, acetaldehyde) may be used. Derivatisation of amino acids with *ortho*-phthalaldehyde reagent[97] or acetaldehyde with 2,4-dinitrophenylhydrazine[198] followed by UV detection can be used as well. For high accuracy, however, direct and in-line measurements of the reactants and products are preferred.[192] Reactions in $D_2O$ were shown to promote the production of singlet oxygen[98] which prevents the use of NMR in combination with bromination reactions of VCPO. Furthermore, Raman measurements are not suitable for amino acids due to low solubility of amino acids in aqueous solutions which generates a low signal but Raman analysis could be suitable for other components.

## 6.2 The availability of nitrogen containing biomass

### 6.2.1 The costs of amino acids

Currently, the costs of the starting material (i.e. glutamic acid) for the production of acrylonitrile is too high to achieve a positive cost-benefit margin as shown in Chapter 5 and by Lammens *et al*.[64]

A first possibility to lower the costs of amino acids is by deriving more value from waste as shown previously[21,45] and by using alternative sources of proteins to extract amino acids. These alternative sources are biomass grown for bioremediation of soil contaminated with heavy metals,[199] biomass containing toxins[200,201] or insects grown on plastic waste[202] which according to FDA is not considered food-grade quality. Today, about one-third of the food produced for human consumption is lost or wasted,[203] this includes also unhygienic and expired animal or plant based protein which due to liability issues ends up on landfills or is incinerated. The incineration of biomass for electricity gives the lowest value for the product at 60-150 $/t biomass, while landfill was estimated to cost 400 $/t biomass.[21] However, if these sources of proteins would be used for chemical applications their intrinsic value as starting material could be upgraded significantly.

Currently, food waste (decayed, unusable parts, etc.) end up in the best case in anaerobic digesters for biogas production or on composting facilities where some value (biogas, fertiliser) can be made out of waste. But in the worst case food waste ends up on landfills where biomass is anaerobically degraded producing high amounts of GHG. Due to aerobic decomposition of biomass composting is producing less GHG than landfill and due to the beneficial properties as organic fertiliser in agriculture composting is preferred over landfill. As a consequence from the degradation process of these methods the nitrogen functionality in amino acids which could be used to produce chemicals is lost to a certain extent. The extraction of proteins and amino acids before composting or anaerobic digestion could reduce further the GHG emissions and enhance the value of rest materials. In Figure 6.1 the nitrogen cycle includes biorefinery steps where the nitrogen containing components are separated from rests and converted to nitrogen containing chemicals, which at the end of their life are returned in the nitrogen cycle.

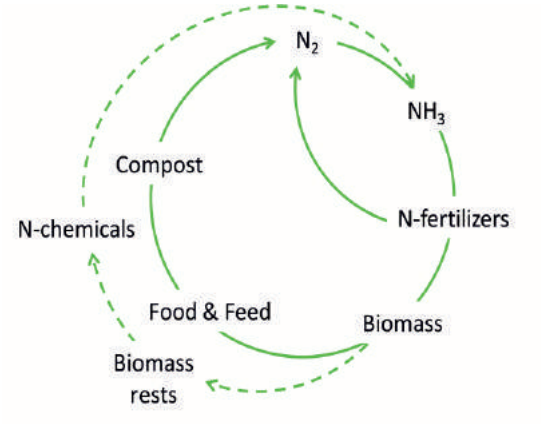



Figure 6.1. Nitrogen cycle (continuous line) with integrated biorefinery steps (dashed line) without landfill practices.

Another possibility to lower the costs is to further improve the processes that generate amino acids, e.g. biorefinery techniques for extraction of proteins or separation systems for amino acids. A bottleneck in converting amino acids to nitriles represents the separation of amino acids from mixtures. As explained in Chapter 5, the conversion of amino acids to nitriles without the prior separation could facilitate the production of biobased nitriles by saving separation costs. This can be achieved only on the premises that the separation of the nitriles becomes cheaper than the separation of the amino acids due to better differentiation of the physical properties of the nitriles.



### 6.2.2 Biomass sources for amino acids

The availability of the biomass is yet the primary factor that needs to be fulfilled for the production of biobased nitriles. Biomass growth is limited by the availability in the growing media of potassium, phosphorus and nitrogen. In agriculture, nitrogen is applied either as a derivative of the synthetic ammonia, as animal manure, a by-product from food industry or can be introduced in soil by legumes. An analysis on nitrogen distribution from its production as synthetic fertiliser to human consumption (Figure 6.2) shows that only 4% of the nitrogen produced is actually consumed in a carnivorous diet and 14% in a vegetarian diet, the rest is lost or wasted along the chain.[204] While this study underlines how wasteful the agriculture and the food chain is regarding nitrogen, it also reveals opportunities for remediation.

For example, animals (including humans) require a certain amount of essential amino acids to grow however, they are fed an oversupply of some amino acids by supplying full proteins. Overdose of protein in animal feed (non-essential amino acids) is converted to energy while excess of nitrogen is excreted as manure or urea. Although manure is used as fertiliser, in agriculture around 50% of nitrogen is lost between application of the fertiliser and the uptake by the crops (Figure 6.2). This loss of nitrogen is mainly due to volatilisation, denitrification or leaching leading to increase GHG, land degradation or eutrophication of

aquatic systems.[205] According to FAO, the incorporation of one t of synthetic L-lysine in feed could prevent the use of 33 t of soybean meal which saves water, nitrogen, and land use.[206] Therefore, the application of protein biorefinery and separation in essential amino acids used for food and feed, and non-essential amino acids used for nitrogen containing chemicals gains more incentives.

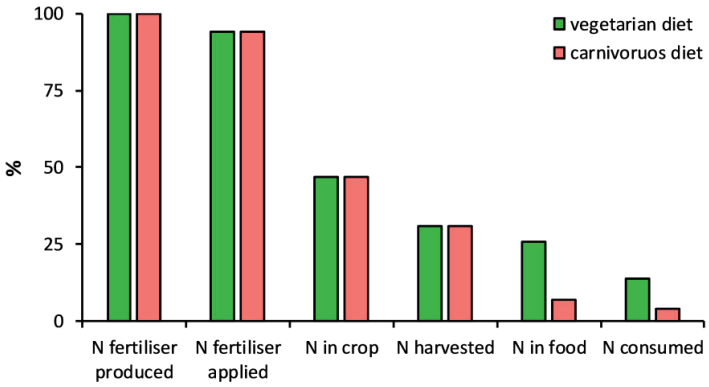



Figure 6.2. The nitrogen chain from the synthetic nitrogen fertiliser to human consumption in a vegetarian and carnivorous diet, adapted from Galloway and Cowling.[204]

Due to climate change the increasing amounts of $CO_2$ in the atmosphere will have a detrimental effect on the uptake of nitrogen in biomass. For different varieties of rice it was shown that higher concentrations of $CO_2$ in the atmosphere reduce significantly the integration of proteins, minerals (Fe, Zn) and nitrogen containing vitamins ($B_1$, $B_2$, $B_5$ and $B_9$) in the rice grains.[207] While this has a direct impact on human health by nutritional deficit in rice-dependent countries, it will also affect the availability of nitrogen containing biomass for chemical production.

The nitrogen cycle along with climate change and biodiversity loss are the three planetary boundaries for a safe operating space for humanity that are substantially exceeded.[208] Therefore, alternatives that do not rely exclusively on ammonia fertilisation (synthetic or manure) must be developed. One alternative could be the intensification of the symbiosis between plants and nitrogen-fixing bacteria[205] by e.g. genetic modification or the extension of the symbiosis or associative nitrogen-fixing bacteria to other crops besides the usual ones (legumes, some cereals and trees). Another alternative, perhaps futuristic, could be to design enzymes that use molecular nitrogen to introduce it to carbon substrates, in a similar fashion as oxygenases introduce molecular oxygen to carbon substrates. All these alternatives should be assessed for their potential in reducing the nitrogen losses in the chain.

Alternatives that do not rely on amino acids for the production of nitrogen containing chemicals are being developed. For example, acrylonitrile can be made available from glycerol derived from biodiesel[34] or 3-hydroxypropanoic acid derived from sugars.[35] These

processes however, are dependent on the energy-intensive Haber-Bosch process for the production of ammonia.[39] The production of nitriles in cascade reactions that generate ammonia *in situ* from air and water in ambient reaction conditions, e.g. by electrochemical synthesis[209] or nitrogen-fixing bacteria,[205] have not been investigated yet.

## 6.3 Concluding remarks

In this thesis it was shown that nitrogen containing chemicals such as nitriles can be produced from amino acids by chemo-enzymatic catalysis.

Advancements in understanding the oxidative decarboxylation of amino acids were made. A range of amino acids with different side chain functionalities can be converted in nitriles by VCPO catalysis in certain concentrations of NaBr. The side chain functionality plays an important role in the reactivity towards nitriles. The carboxylic side chain promotes the production of nitriles only when the carboxyl is situated at two carbons away from the α-functionalities (glutamic acid, aminoadipic acid), even at low concentrations of bromide. The poor reactivity of Asp is attributed to the steric arrangements of the atoms around the α-functionalities. It was shown that by increasing the bromide concentration the selectivity and conversion in case of Asp and other amino acids increases as well. The exact mechanism is still to be elucidated.

The direct use of oxygen by AOX was investigated as alternative to the hydrogen peroxide originated from the energy-intensive anthraquinone process. The conversion of ethanol to the volatile acetaldehyde was selected for the half redox reaction of AOX due to the easiness of the downstream processing, e.g. by pervaporation of acetaldehyde. The cascade AOX-VCPO was used for *in situ* production of hydrogen peroxide for fast halogenation reactions and oxidation reactions *via* halogenation. For the first time, the oxidative decarboxylation of glutamic acid - an oxidation reaction *via* halogenation - was shown to be possible using the cascade AOX-VCPO. For this reaction, the two enzymes had to be separated in two reactors due to inhibition of AOX caused by HOBr – the product of VCPO. However, the fast halogenation reactions such as the bromination of monochlorodimedone, using the cascade AOX-VCPO was possible in one reactor. Oxygen availability in aqueous solutions, scaling up as well as the reaction kinetics need to be further addressed.

The bottlenecks of producing biobased nitriles were identified in a techno-economic assessment. It was shown that the replacement of NaOCl with VCPO and $H_2O_2$ improved the economics and the mass balance, and reduced significantly the waste generation. The availability of the enzyme at industrial scale together with its immobilisation is not expected to be a bottleneck as enzyme production at industrial scale was shown to be cost effective. The most profitable scenario is the AOX-VCPO cascade mainly due to the co-production of acetaldehyde which was considered a valuable product. At the moment, the price of the substrate, Glu, and the price of the product, GluCN – the intermediate in the production of

acrylonitrile or succinonitrile – are too high to be competitive with the fossil based nitriles. As the price of Glu is already a best case scenario the use of cheaper sources of amino acids, e.g. crude mixtures of amino acids, should be tested. It was concluded that sanctions applied to polluting industries will increase the price of fossil-based nitriles and as a result it can make the biobased nitriles more competitive.

Increasing $CO_2$ levels in atmosphere, due to the exploitation of fossil resources, cause a reduction in the inclusion of nitrogen in plants. This has an effect not only on the food security due to lower availability of proteins in the future, but also on the possibility of producing biobased nitrogen containing chemicals such as nitriles.

In order to produce biobased nitriles, the use of nitrogen containing biomass along the food chain can be made more efficient by including biorefinery techniques which separate the essential amino acids, required in food and feed, from the non-essential amino acids, suitable for nitrogen containing chemicals. This approach allows a more efficient use of biomass by using each component at its highest value.

Today, biobased chemicals have to compete with already optimised processes of conventional fossil-based chemicals. As the biobased technology is developing, pressure from the society and the policy makers are key factors in catalysing the transition from the fossil-based to biobased sustainable economy.

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# Appendices

# Appendix A. Supplementary information to Chapter 2

Based on previously reported research[59] the reaction conditions were optimised in order to obtain shorter reaction times (see Experimental).

In this research the enzyme used, vanadium chloroperoxidase (VCPO), was expressed in *E. coli*[141] ($VCPO_{E.\ coli}$). A validation was performed to investigate whether the partially purified $VCPO_{E.\ coli}$ has the same reactivity towards the amino acids as the highly purified VCPO produced in *S. cerevisiae* ($VCPO_{S.\ cerevisiae}$), which was used in previous research.[59] Validation was performed for the conversion of glutamic acid (Glu) into 3-cyanopropanoic acid (GluCN) (Scheme 2, n=2). For this validation the same units of VCPO from the two organisms were used. The units of VCPO were determined based on the monochlorodimedone activity assay (see Experimental). The validation test confirmed that the changes performed did not affect the conversion of Glu to GluCN (Figure A.1).

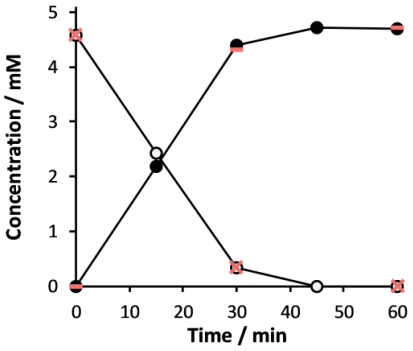


Figure A.1. Glutamic acid (Glu) conversion into 3-cyanopropanoic acid (GluCN) by vanadium chloroperoxidase (VCPO) at 0.5 mM NaBr for the validation of $VCPO_{E.\ coli}$. ○ concentration of Glu in reaction with VCPO produced in *E. coli*; ● concentration of GluCN Glu in reaction with VCPO produced in *E. coli*; ✕ concentration of Glu in reaction with VCPO produced in *S. cerevisiae*; — concentration of GluCN in reaction with VCPO produced in *S. cerevisiae*.

The addition of $H_2O_2$ was tested on the conversion of Glu to GluCN (Figure A.2). The addition of $H_2O_2$ was performed using different amounts of aliquots of $H_2O_2$. It can be observed that the more concentrated the aliquot of $H_2O_2$ the less the conversion of Glu. Only when 1.2 mM aliquots were used full conversion was achieved. The unidentified products corresponding to about 20% of the starting Glu could be due to the use of a crude extract of the VCPO which could have other enzymatic activities besides the VCPO. A continuous addition of $H_2O_2$ and a purified VCPO ensured full conversion and no side reactions.

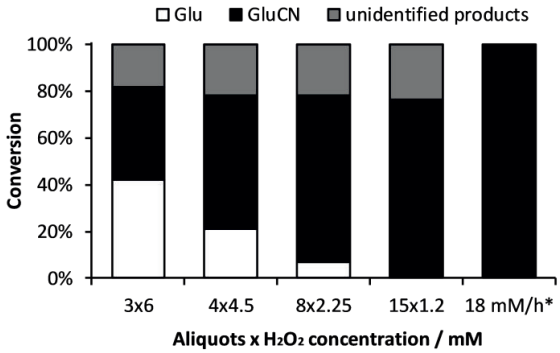


Figure A.2. Conversion of Glu to GluCN after 1 h as a function of addition of $H_2O_2$ at 2 mM NaBr. The unidentified products were calculated based on the molar balance of each reaction. *$H_2O_2$ was added continuously using a syringe pump.

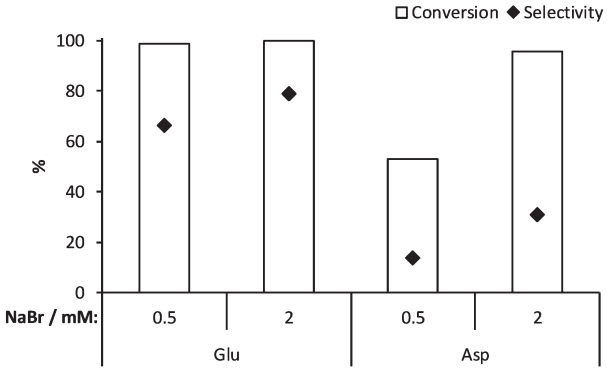


Figure A.3. Conversion of Glu and Asp and the selectivity to GluCN and AspCN after 1 h as a function of NaBr concentration using NaOCl as oxidant at a constant addition rate (18 mM/h), 5 mM amino acid was used as starting concentration.

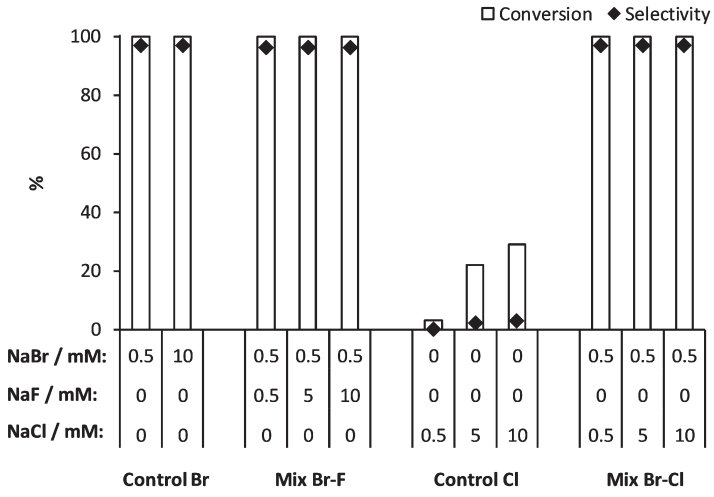


Figure A.4. Conversion of glutamic acid (Glu) and selectivity towards 3-cyanopropanoic acid (GluCN) as function of halides concentration. The data represent results from single experiments.

It can be observed (Figure A.4) that the reactivity of Glu is not influenced by the concentration of NaBr nor by the additional presence and concentration of NaF and NaCl. Even when only chloride was present the conversion of Glu and selectivity to GluCN is low. From these results it can be concluded that the ionic strength does not influence the reactivity of Glu.

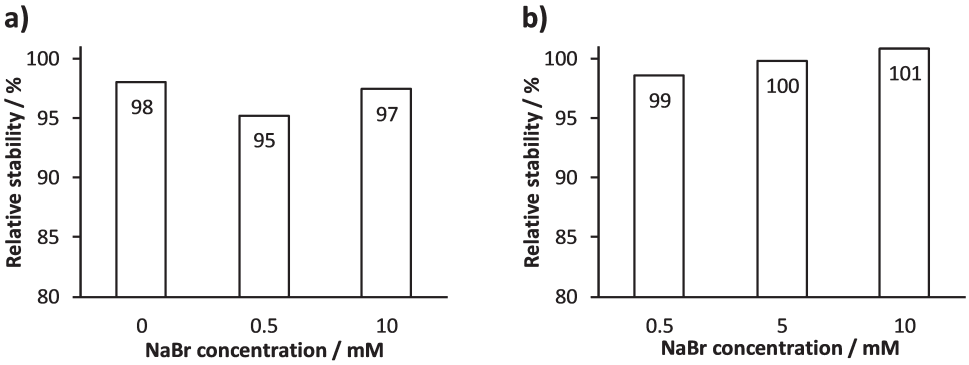


Figure A.5. Stability test of **a)** AspCN and **b)** malonic acid after 1 h under oxidative decarboxylation reaction conditions at different concentrations of NaBr. 5 mM of AspCN or malonic acid were used as initial concentrations and 18 mM/h of $H_2O_2$ were added continuously.

# Appendix B. Supplementary information to Chapter 3

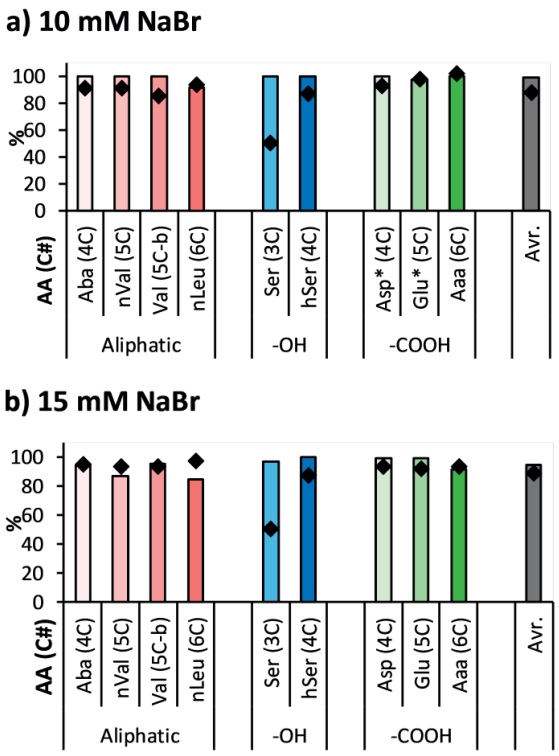


Figure B.1. Amino acids conversion into nitriles as a function of the concentration of NaBr after 1 hour reaction time. The concentration of NaBr was **a)** 10 mM, **b)** 15 mM. Bars represent conversion, ◆ selectivity, and C# carbon number. The data represents the average of at least two individual experiments except for nVal and hSer at 15 mM NaBr which are single data points. *data replicated according to [116]. AA = amino acid, C# = carbon number.

Table B.1. Overview of conversion, selectivity and reaction rate at 2 mM NaBr for amino acids tested.

| Amino acid code | Amino acid category (electronic effect) | Number of carbon atoms | Functionality present at β carbon | Initial* reaction rate [mM/min] | Maximum reaction rate [mM/min] | Average initial (maximum) reaction rate [mM/min] | Maximum / Initial reaction rate |
|---|---|---|---|---|---|---|---|
| Aba | Aliphatic (electron donating) | 4 | - | 0.020 | 0.148 | 0.036 (0.156) | 4.3 |
| nVal | | 5 | - | 0.030 | 0.155 | | |
| Val | | 5 (b**) | $-(CH_3)_2$ | 0.023 | 0.154 | | |
| nLeu | | 6 | - | 0.070 | 0.165 | | |
| Ser | Hydroxyl (electron donating) | 3 | -OH | 0.060 | 0.155 | 0.045 (0.160) | 3.5 |
| hSer | | 4 | - | 0.030 | 0.165 | | |
| Asp | Carboxylic (electron withdrawing) | 4 | -COOH | 0.125 | 0.139 | 0.101 (0.144) | 1.4 |
| Glu | | 5 | - | 0.108 | 0.137 | | |
| Aaa | | 6 | - | 0.069 | 0.157 | | |
| MeAsp | ω-Methyl ester (electron withdrawing) | 4+1 | $-COOCH_3$ | 0.016 | 0.093 | 0.010 (0.121) | 12.1 |
| MeGlu | | 5+1 | - | 0.004 | 0.149 | | |

*initial=the first 10 min, **b = branched. The data are the average of experiments from the NaBr screening and the time course after 60 min of reaction time.

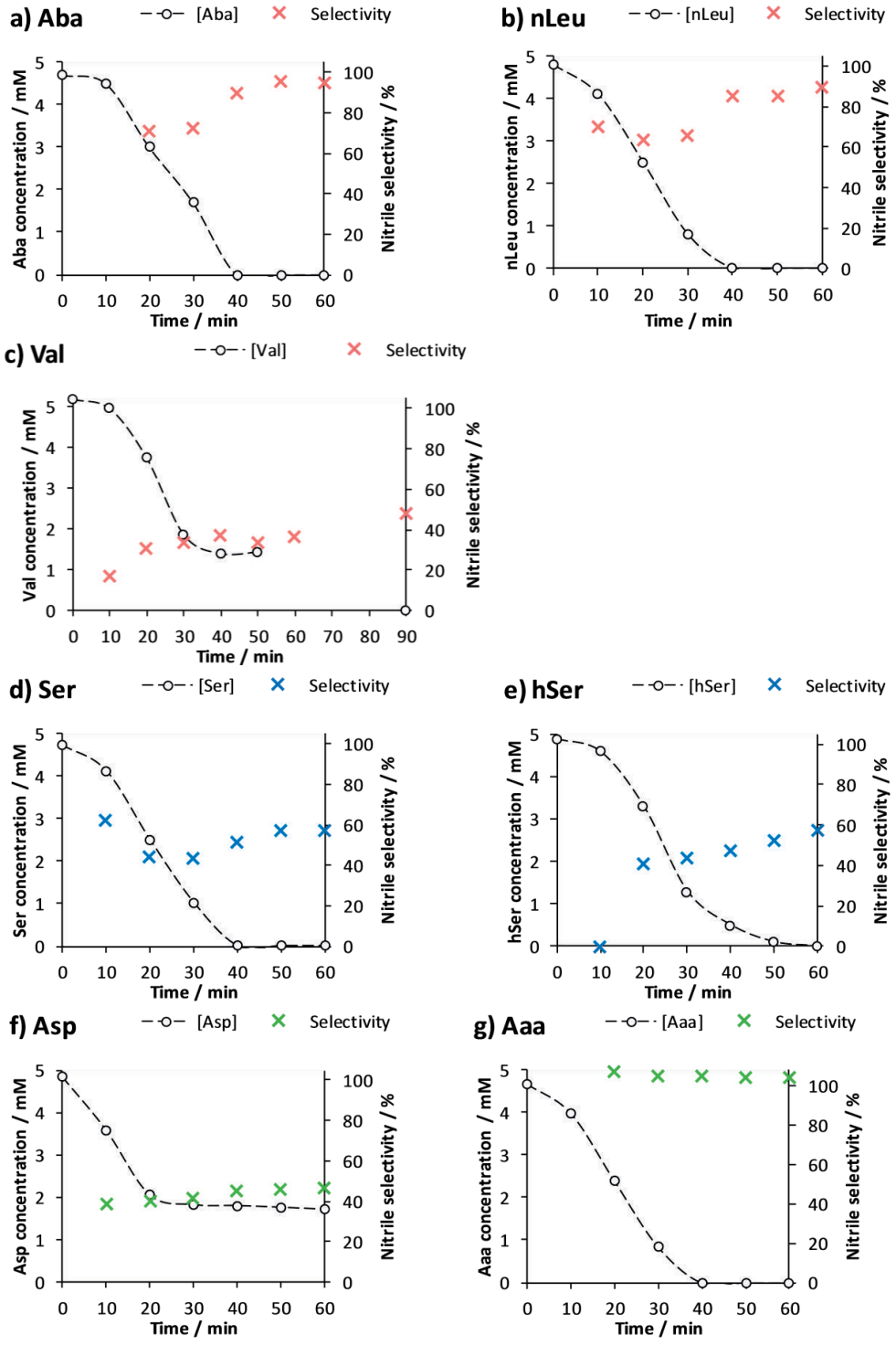


Figure B.2. Time course reactions of amino acids at 2 mM NaBr. **a)** Aba, **b)** nLeu, **c)** Val, **d)** Ser, **e)** hSer, **f)** Asp, **g)** Aaa. The value for Val (c) at 60 min not available. a, b, c, and e - single experiment data, d, f and g average of duplicate experiments.

# Appendix C. Supplementary information to Chapter 4

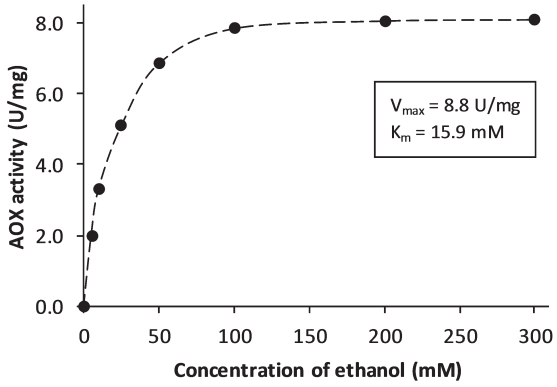


Figure C.1. AOX activity at different ethanol concentrations. Reaction conditions: 20 mM sodium citrate pH 5.6 containing dissolved $O_2$, 2 mM ABTS, 1 U/mL HRP, 0.0073 mg/mL AOX. The results represent the average of at least duplicate experiments.

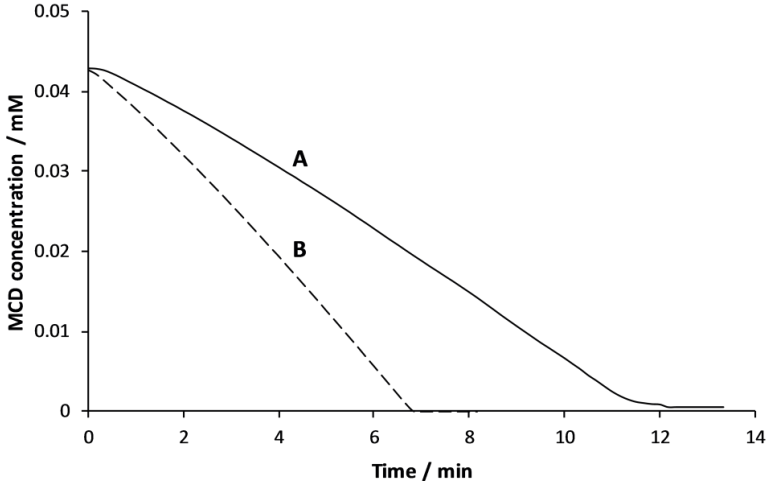


Figure C.2. Bromination of MCD by VCPO as a function of time, in presence of 5 mM NaBr. $H_2O_2$ was **A.** produced by $AOX_{Hp}$ from ethanol (100 mM) and oxygen (saturated aqueous solution), $AOX_{Hp}$:VCPO ratio of 1:1; or **B.** added externally (1 mM $H_2O_2$). The results represent the average of duplicate experiments.

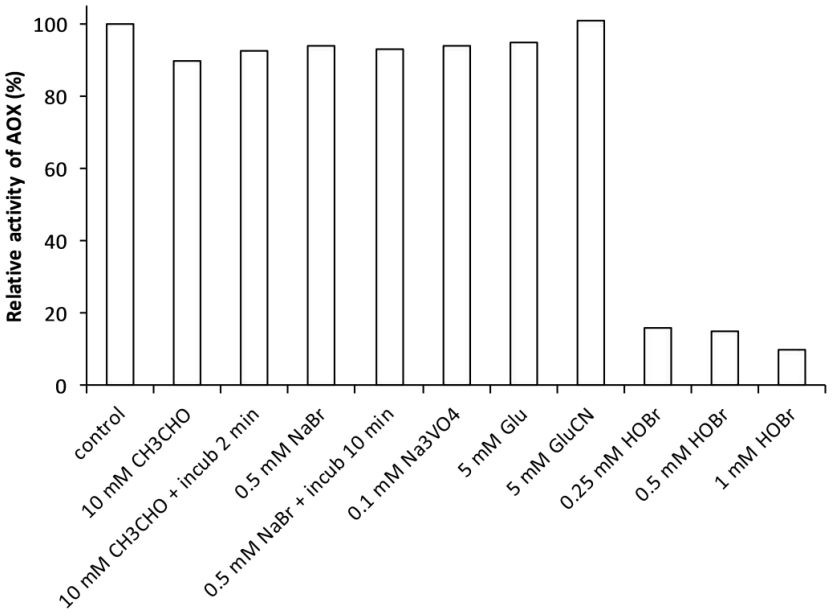


Figure C.3. Relative activity of AOX in presence of different potential inhibitors.

# Appendix D. Supplementary information to Chapter 5

Table D.1. Assumptions

| General | value | Unit | Notes |
|---|---|---|---|
| GluCN | 1000 | kg | Starting point |
| Extraction yield | 100 | mol% | |
| Extraction purge | 25 | mol% | |
| Crystallisation yield | 100 | mol% | |
| EtOAc loss | 1 | wt% | Due to solubility in water |
| EtOAc volume | 200 | % | of the volume of aqueous solution |
| **Scenario 1** | | | |
| Conversion | 100 | mol% | mol% in 1h |
| Selectivity GluCN | 90 | mol% | Lammens *et al.*[64] |
| NaBr catalyst | 10 | mol% | mol% of initial Glu |
| NaOCl | 15 | wt% | |
| **Scenario 2** | | | |
| Conversion | 100 | mol% | mol% in 40 min |
| Selectivity GluCN | 100 | mol% | A.But *et al.*[116] |
| Glu concentration | 0.005 | M | mol/L = mol/kg (= 5mM) |
| NaBr catalyst | 4 | mol% | mol% of initial Glu (0.2 mM NaBr) |
| H2O2 | 35 | wt% | |
| VCPO purity | 50 | wt% | protein based |
| VCPO activity | 0.36 | U/mL | Based on MCD assay using bromide |
| VCPO conc. | 60 | nM | nmol/L |
| VCPO catalyst | 0.0012 | mol% | mol% of initial Glu in 1 h conversion |
| **Scenario 3** | | | |
| Glu concentration | 1.02* | M | 15 wt% |
| VCPO reuse | 50 | times | Without loss in activity |
| **Scenario 4** | | | |
| Glu concentration | 1.02 | M | 15 wt% |
| VCPO reuse | 50 | times | without loss in activity |
| AOX:VCPO | 2:1 | | Based on enzymatic activity |
| AOX reuse | 50 | times | without loss in activity |
| **Scenario 5** | | | |
| Glu concentration | 1 | M | 15 wt% |
| Ru catalyst reuse | 100 | times | without loss in activity |
| Ru catalyst concentration | 5 | mol% | Based on the Ru metal |

*due to solubility limit of Glu (7.5 g/L) the maximum concentration in solution (water at 20°C) of Glu is 0.051 M (0.75 wt%).

## Mass balance

Table D.2. The mass balance for 1000 kg of 3-cyanopropanoic acid for Scenario 1.

| Scenario 1 | | | |
|---|---|---|---|
| **Compounds** | **Abbreviation** | **IN (kg)** | **OUT (kg)** |
| **Starting materials** | | | |
| **Glutamic acid** | Glu | 1650 | 0 |
| **Sodium hypochlorite** | NaOCl | 1586 | 0 |
| **Catalysts & solvents** | | | |
| **Sodium bromide** | NaBr | 29 | 29 |
| **Water as solvent (from NaOCl)** | $H_2O^s$ | 8987 | 8987 |
| **Ethyl acetate*** | EtOAc | 168 | 168 |
| **Valuable product** | | | |
| **3-Cyanopropanoic acid** | GluCN | 0 | 1000 |
| **Non-valuable products** | | | |
| **Sodium chloride** | NaCl | 934** | 2179 |
| **Carbon dioxide** | $CO_2$ | 0 | 493 |
| **By-product** | bp | 0 | 114 |
| **Ammonia** | $NH_3$ | 0 | 19 |
| **Water as by-product** | $H2O^{bp}$ | 0 | 364 |
| **Total (kg)** | | 13 353 | 13 353 |

*represents 1% losses, **from recycle, s=solvent, bp=by-product.

Table D.3. The mass balance for 1000 kg of 3-cyanopropanoic acid for Scenario 2.

| Scenario 2 | | | |
|---|---|---|---|
| **Compounds** | **Abbreviation** | **IN (kg)** | **OUT (kg)** |
| **Starting materials** | | | |
| **Glutamic acid** | Glu | 1485 | 0 |
| **Hydrogen peroxide** | $H_2O_2$ | 687 | 0 |
| **Catalysts & solvents** | | | |
| **Sodium bromide**** | NaBr | 42 | 42 |
| **Vanadium chloroperoxidase***** | VCPO | 8 | 8 |
| **Water as solvent** | $H_2O^s$ | 2018394 | 2018394 |
| **Ethyl acetate** | EtOAc | 40382 | 40382 |
| **Valuable product** | | | |
| **3-Cyanopropanoic acid** | GluCN | 0 | 1000 |
| **Non-valuable products** | | | |
| **Carbon dioxide** | $CO_2$ | 0 | 444 |
| **Water as by-product** | $H2O^{bp}$ | 0 | 727 |
| **Total (kg)** | | 2 060 997 | 2 060 997 |

*represents 1% losses, ** 100% recycled, *** 100% recycled, 1 time used, s=solvent, bp=by-product.

Table D.4. The mass balance for 1000 kg of 3-cyanopropanoic acid for Scenario 3.

| Scenario 3 | | | |
|---|---|---|---|
| **Compounds** | **Abbreviation** | **IN (kg)** | **OUT (kg)** |
| **Starting materials** | | | |
| **Glutamic acid** | Glu | 1485 | 0 |
| **Hydrogen peroxide** | $H_2O_2$ | 687 | 0 |
| **Catalysts & solvents** | | | |
| **Sodium bromide**** | NaBr | 42 | 42 |
| **Vanadium chloroperoxidase***** | VCPO | 0.16 | 0.16 |
| **Water as solvent** | $H_2O^s$ | 9899 | 9899 |
| **Ethyl acetate*** | EtOAc | 213 | 213 |
| **Valuable product** | | | |
| **3-Cyanopropanoic acid** | GluCN | 0 | 1000 |
| **Non-valuable products** | | | |
| **Carbon dioxide** | $CO_2$ | 0 | 444 |
| **Water as by-product** | $H_2O^{bp}$ | 0 | 727 |
| **Total (kg)** | | 12 324 | 12 324 |

*represents 1% losses, ** 100% recycled, *** 100% recycled, 50 times reused, s=solvent, bp=by-product.

Table D.5. The mass balance for 1000 kg of 3-cyanopropanoic acid for Scenario 4.

| Scenario 4 | | | |
|---|---|---|---|
| **Compounds** | Abbreviation | IN (kg) | OUT (kg) |
| **Starting materials** | | | |
| **Glutamic acid** | Glu | 1485 | 0 |
| **Ethanol** | EtOH | 930 | 0 |
| **Oxygen (air)** | $O_2$ | 646 | 0 |
| **Catalysts & solvents** | | | |
| **Sodium bromide**** | NaBr | 42 | 42 |
| **Vanadium chloroperoxidase**** | VCPO | 0.16 | 0.16 |
| **Alcohol oxidase**** | AOX | 0.46 | 0.46 |
| **Water as solvent** | $H_2O^s$ | 191 | 191 |
| **Ethyl acetate*** | EtOAc | 9899 | 9899 |
| **Valuable product** | | | |
| **3-Cyanopropanoic acid** | GluCN | 0 | 1000 |
| **Acetaldehyde** | $CH_3CHO$ | 0 | 889 |
| **Non-valuable products** | | | |
| **Carbon dioxide** | $CO_2$ | 0 | 444 |
| **Water as by-product** | $H_2O^{bp}$ | 0 | 727 |
| **Total (kg)** | | 13 192 | 13 192 |

*represents 1% losses, ** 100% recycled, s=solvent, bp=by-product.

Table D.6. The mass balance for 1000 kg of 3-cyanopropanoic acid for Scenario 5.

| Scenario 5 | | | |
|---|---|---|---|
| **Compounds** | Abbreviation | IN (kg) | OUT (kg) |
| **Starting materials** | | | |
| **Glutamic acid** | Glu | 1747 | 0 |
| **Oxygen** | $O_2$ | 323 | 0 |
| **Catalysts & solvents** | | | |
| **Ruthenium catalyst**** | Ru-cat | 0.09 | 0.09 |
| **Water as solvent** | $H_2O^s$ | 10092 | 10092 |
| **Ethyl acetate*** | EtOAc | 209 | 209 |
| **Valuable product** | | | |
| **3-Cyanopropanoic acid** | GluCN | 0 | 1000 |
| **Non-valuable products** | | | |
| **By-products** | bp | 0 | 262 |
| **Carbon dioxide** | $CO_2$ | 0 | 444 |
| **Water as by-product** | $H_2O^{bp}$ | 0 | 364 |
| **Total (kg)** | | 12 371 | 12 371 |

*represents 1% losses, ** 100% recycled and 100 x reused, s=solvent, bp=by-product.

**Energy requirements of the process**

The computational calculations were performed with the second order MØller-Plesset perturbation theory method, with basis set 6-311+G(2d,2p) and water as solvent, using the software package Gaussian 16.

**The energy requirement for Scenario 2**

Table D.7. Energy requirements of the process for Scenario 2.

| Process unit | Scenario 2 | |
|---|---|---|
| | T | Process energy[◊] |
| | °C | MJ |
| **Reactor 1** | 25 | 5781 |
| **Extraction** | 25 | nc |
| **Crystallisation** | 4 | 148730* |
| **Total for 1000 kg Glu** | - | **154511** |
| **Total for 1000 kg GluCN** | - | **254015** |

[◊]for 1000 kg Glu, na = not applicable, nc = not considered, *contains a multiple of 1.5 due to cooling necessity below room temperature, T = operational temperature.

**The energy requirement for reactor 1 (Scenario 5)**

The oxidative decarboxylation reaction takes place at 100°C in a pressure reactor under 30 bar oxygen pressure. For this reaction the Gibbs free energy was assumed to be -800 kJ/mol in the exothermic range as the reactions in Scenario 1-4. The energy required for cooling the reaction mixture for the conversion of 1747 kg Glu (11.87 kmol) to 1000 kg GluCN (10.09 kmol) is 800 × 11.87 = 9499 MJ. This energy is released only at 100°C therefore it can be integrated in the energy exchange further upstream e.g. in a distillation unit (see Figure 5.1). Reactor 1 needs to be heated from room temperature (25°C) to 100°C. The energy required to heat 10092 kg water to 100°C for the production of 1000 kg GluCN from 1747 kg Glu is 3164 MJ and was calculated as follows: $Q = m \times C_p \times \Delta T = 10092\ kg \times 4.18\ kJ/kg{\cdot}K \times (373\ K - 298\ K)$ = 3164 MJ. The final energy required for cooling reactor 1 is 9499 – 3164 = 6335 MJ. The energy required to compress the oxygen/air to reach 30 bar was not included in the energy balance.

**Oxidative decarboxylation of Glu by VCPO as a function of reaction temperature**

**Experimental procedure:** In 2 mL of 20 mM citrate buffer pH 5.6 containing 5 mM Glu, 0.5 mM NaBr, 0.72 U of VCPO is added 16 mM $H_2O_2$/h with a syringe pump to start the reaction. The reaction mixture except $H_2O_2$ was incubated for ~10 min at desired temperatures: 20, 30, 40, 50, 60, and 70°C (+/- 1°C). After 5 min reaction time (which ensured low conversion of Glu), the reaction was stopped by addition of $Na_2S_2O_3$. The reaction mixture was analysed by HPLC.[116] No duplicates were performed.

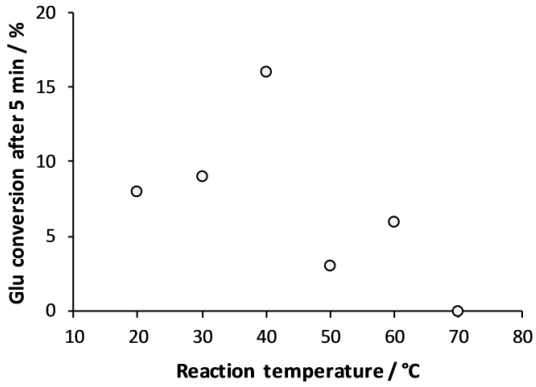


Figure D.1. Oxidative decarboxylation of glutamic acid (Glu) as a function of reaction temperature.

## Economic assessment

Table D.8. Economic assessment for Scenario 1.

| Scenario 1 | | | |
|---|---|---|---|
| **Compounds** | **Amount (kg)** | **Price (€/kg)** | **Value (€/t GluCN)** |
| ***Starting materials*** | | | |
| Glutamic acid | 1650 | 0.50[a] | 825 |
| Sodium hypochlorite | 1586 | 1.01[b] | 1602 |
| ***Catalysts & solvents*** | | | |
| Sodium bromide | 29 | 1.13[b] | 33 |
| Ethyl acetate* | 168 | 0.81[b] | 136 |
| **Energy & Fixed costs** | | | |
| Electricity (kWh) | 4058** | 0.055[a] | 223 |
| Fixed costs | | | 413[◊] |
| ***Non-valuable products*** | | | |
| Carbon dioxide | 493 | | |
| By-product | 114 | | |
| Ammonia | 19 | | |
| Water as by-product | 364 | | |
| **Total costs** | | | **3232** |
| ***Valuable products*** | | | |
| 3-Cyanopropanoic acid | 1000 | 1.31[c] | 1310 |
| Sodium chloride | 2179 | 0.08[a] | 174 |
| **MARGIN** | | | **- 1748** |

* represents 1% losses,

** includes electricity required for cooling reactor 1 below room temperature and for crystallisation unit,

a) based on [64],

b) based on [163],

c) GluCN is not a commercially available product, therefore the price is estimated to be a generic price for intermediate chemicals based on Szmant,[165]

◊ Investment costs are estimated by a method provided by Lange[161] based on a linear correlation of investment recovery provision and the overall energy losses in a chemical process. 14.6 GJ/t product corresponds to 210 $/t product in 1993 (value corrected for inflation). The total fixed costs were obtained by adding 1/3 to the provision.

Table D.9. Economic assessment for Scenario 2 (does not include fixed costs).

| Scenario 2 | | | |
|---|---|---|---|
| **Compounds** | **Amount (kg)** | **Price (€/kg)** | **Value (€/t GluCN)** |
| ***Starting materials*** | | | |
| Glutamic acid | 1485 | 0.50[a] | 743 |
| Hydrogen peroxide | 687 | 0.62[d] | 426 |
| ***Catalysts & solvents*** | | | |
| Sodium bromide | 42 | 1.13[b] | 47 |
| Vanadium chloroperoxidase | 8 | 100[a] | 800 |
| Ethyl acetate* | 40382 | 0.81[b] | 32709 |
| Water as solvent** | 2018394 | 0.00077[e] | 1554 |
| **Energy input** | | | |
| Electricity (kWh) | 70560** | 0.055[a] | 3881 |
| ***Non-valuable products*** | | | |
| Carbon dioxide | 493 | - | |
| Water as by-product | 727 | - | |
| **Total costs** | | | **40161** |
| ***Valuable product*** | | | |
| 3-Cyanopropanoic acid | 1000 | 1.31[c] | 1310 |
| **MARGIN** | | | **- 38851** |

* represents 1% losses,
** includes electricity required for cooling the crystallisation unit below room temperature,
a) based on [64],
b) based on [163],
c) GluCN is not a commercially available product, therefore the price is estimated to be a generic price for intermediate chemicals based on Szmant,[165]
d) based on [162],
e) based on [164].

Table D.10. Economic assessment for Scenario 4.

| Scenario 4 | | | |
|---|---|---|---|
| **Compounds** | **Amount (kg)** | **Price (€/kg)** | **Value (€/t GluCN)** |
| ***Starting materials*** | | | |
| Glutamic acid | 1485 | 0.50[a] | 743 |
| Ethanol | 930 | 0.42[f] | 388 |
| ***Catalysts & solvents*** | | | |
| Sodium bromide | 42 | 1.13[b] | 47 |
| Vanadium chloroperoxidase | 0.16 | 100[a] | 16 |
| Alcohol oxidase | 0.46 | 100[a] | 46 |
| Ethyl acetate* | 191 | 0.81[b] | 155 |
| Water as solvent* | 9899 | 0.00077[e] | 8 |
| **Energy & Fixed costs** | | | |
| Electricity (kWh) | 2946** | 0.055[a] | 162 |
| Fixed costs | | | 305[◊] |
| ***Non-valuable products*** | | | |
| Carbon dioxide | 444 | - | |
| Water as by-product | 727 | - | |
| **Total costs** | | | **1872** |
| ***Valuable products*** | | | |
| 3-Cyanopropanoic acid | 1000 | 1.31[c] | 1310 |
| Acetaldehyde | 889 | 0.85[b] | 756 |
| **MARGIN** | | | **194** |

* represents 1% losses,
** includes electricity required for cooling crystallisation unit below room temperature, does not include energy for pervaporation unit or compressing air,
a) based on [64],
b) based on [163],
c) GluCN is not a commercially available product, therefore the price is estimated to be a generic price for intermediate chemicals based on Szmant,[165]
d) based on [162],
e) based on [164],
f) based on [210],

◊ Investment costs are estimated by a method provided by Lange[161] based on a linear correlation of investment recovery provision and the overall energy losses in a chemical process. 10.6 GJ/t product corresponds to 155 $/t product in 1993 (value corrected for inflation). The total fixed costs were obtained by adding 1/3 to the provision.

Table D.11. Economic assessment for Scenario 5.

| Scenario 5 | | | |
|---|---|---|---|
| **Compounds** | **Amount (kg)** | **Price (€/kg)** | **Value (€/t GluCN)** |
| ***Starting materials*** | | | |
| Glutamic acid | 1747 | 0.50[a] | 874 |
| ***Catalysts & solvents*** | | | |
| Ruthenium catalyst | 0.09♦ | 687[g] | 62 |
| Ethyl acetate* | 209 | 0.81[b] | 169 |
| Water as solvent* | 10092 | 0.00077[e] | 8 |
| **Energy & Fixed costs** | | | |
| Electricity (kWh) | 2113** | 0.055[a] | 116 |
| Fixed costs | | | 235◊ |
| ***Non-valuable products*** | | | |
| Carbon dioxide | 444 | - | |
| Water as by-product | 364 | - | |
| By-products | 262 | - | |
| **Total costs** | | | **1464** |
| ***Valuable product*** | | | |
| 3-Cyanopropanoic acid | 1000 | 1.31[c] | 1310 |
| **MARGIN** | | | **-154** |

* 1% losses,
** *includes electricity required for cooling the crystallisation unit below room temperature,*
♦Ru-cat was assumed to be reused 100 times,
◊ Investment costs are estimated by a method provided by Lange[161] based on a linear correlation of investment recovery provision and the overall energy losses in a chemical process. 10.6 GJ/t product corresponds to 155 $/t product in 1993 (value corrected for inflation). The total fixed costs were obtained by adding 1/3 to the provision,
a) based on [64],
b) based on [163],
c) GluCN is not a commercially available product, therefore the price is estimated to be a generic price for intermediate chemicals based on Szmant,[165]
d) based on [162],
e) based on [164],
f) based on [210],
g) based on [211].

Table D.12. Cost-benefit analysis for Scenario 1 to 5. The total costs and benefits and the cost-benefit margin for each scenario are resulting from Table D.8-D.11 and Table 5.3.

| **Scenario** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| **Total costs[a] (€/t GluCN)** | 3232 | 40161[b] | 1858 | 1872 | 1464 |
| **Total benefits[c] (€/t GluCN)** | 1484 | 936 | 1310 | 2066 | 1310 |
| **Cost-benefit margin[d] (€/t GluCN)** | -1748 | -39224 | -548 | 194 | -154 |
| **Benefit-cost ratio[e]** | 0.46 | 0.02 | 0.71 | 1.10 | 0.89 |

[a] contains all the variable and fixed costs, [b] does not include fixed costs, [c] The total benefits are the goods sold, [d] cost-benefit margin = goods sold – variable costs, [e] benefit-cost ratio = goods sold/variable costs.

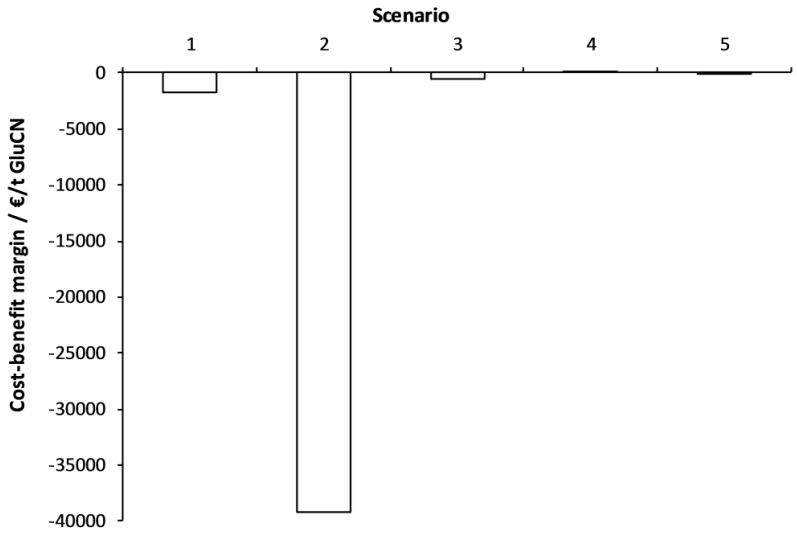


Figure D.2. Cost-benefit margin for Scenario 1 to 5 as it results from Table D.12. Cost-benefit margin = goods sold – variable costs.

# Summary

Today, nitriles are produced by introducing the nitrogen functionality in hydrocarbons originated from fossil resources under high pressure and temperature. Environmental concerns associated with the use of fossil resources, as shown in **chapter 1**, stimulate the need to produce chemicals in a more sustainable way. Renewable resources such as amino acids from biomass, that already contain nitrogen in their molecule, are investigated as alternative starting materials to produce biobased nitriles. In this thesis the enzyme vanadium chloroperoxidase (VCPO) was used as catalyst to produce biobased nitriles from amino acids *via* the oxidative decarboxylation reaction. Industrially relevant nitriles such as acrylonitrile and succinonitrile, can be prepared starting from amino acids. For example, glutamic acid (Glu) – the most abundant non-essential amino acid in biomass – can be converted to acrylonitrile *via* the intermediate 3-cyanopropanoic acid (GluCN). The oxidative decarboxylation reaction of other biomass derived amino acids was investigated as well.

Glu can be fully converted into GluCN with high selectivity using the enzyme VCPO, $H_2O_2$ and catalytic amounts of NaBr. In contrast, under the same reaction conditions the oxidative decarboxylation of aspartic acid (Asp) resulted in low conversion and selectivity towards the nitrile. In **chapter 2**, it was investigated how two chemically similar amino acids, Glu and Asp, react differently towards the oxidative decarboxylation. For this, the conversion of Glu and Asp was investigated as a function of bromide concentration. In presence of catalytic amount of bromide (0.1 equiv.), Glu resulted in full conversion and high selectivity. It was shown that by increasing the amount of bromide present in the reaction mixture to 2 equiv., the conversion of Asp was increased from 15% to 100% and its selectivity towards 2-cyanoacetic acid (AspCN) from 45% to 80%.

It was concluded that the difference in reactivity must be due to the difference of one carbon atom in the side chain between Asp and Glu and the proximity of the side chain to the reactive alpha functionalities of the amino acids. It was hypothesised that the alpha functionalities in Asp are stabilised in intra- or intermolecular interactions with the side chain carboxyl functionality which prevents Asp to react in a similar manner as Glu.

The influence of the side chain functionality and the side chain length of amino acids towards the reactivity of alpha functionalities with respect to oxidative decarboxylation was further investigated for different amino acids (**chapter 3**). It was shown that the conversion can be modified as a function of the concentration of NaBr for all amino acids tested. Only two amino acids, Glu and aminoadipic acid, can be fully converted into nitriles with catalytic amounts of NaBr (0.04 equiv.). For all other amino acids with aliphatic, hydroxy, carboxyl and methyl ester functionalities tested, a minimum amount of NaBr present in the solution (≥ 0.4 equiv.) is required to reach full conversion.

It was concluded that the length of the side chain does not make a significant difference for the selectivity, as previously proposed. However, the position of the functionality on

the side chain (β-carbon) in relation to the bromination centre could hinder the production of nitriles by oxidative decarboxylation by reducing the reaction rate of the bromination.

It was shown that while functional groups like aliphatic, hydroxyl or methyl ester show no significant influence on the reactivity of amino acids, the carboxyl functionality has a positive effect during the oxidative decarboxylation reaction. An addition to the known reaction mechanism was proposed for the amino acids with carboxyl functionality at the side chain. It is proposed that the side chain carboxyl functionality is involved in a self-catalysis mechanism. The elucidation of the exact reaction mechanism could enable reactions of mixtures of amino acids at lower concentration of NaBr for the production of biobased nitriles.

To further enhance the sustainability of the oxidative decarboxylation of amino acids, the *in situ* production of $H_2O_2$ was explored in **chapter 4**. The direct use of oxygen by alcohol oxidase (AOX) was investigated as alternative to the hydrogen peroxide originated from the energy-intensive anthraquinone process. The conversion of ethanol to the volatile acetaldehyde was selected for the half redox reaction of AOX due to the easiness of the downstream processing, e.g. by pervaporation of acetaldehyde. The cascade AOX-VCPO was used for *in situ* production of hydrogen peroxide for fast halogenation reactions and oxidation reactions *via* halogenation. For the first time, the oxidative decarboxylation of glutamic acid - an oxidation reaction *via* halogenation - was shown to be possible using the cascade AOX-VCPO. For this reaction, the two enzymes had to be separated in two reactors due to inhibition of AOX caused by HOBr – the product of VCPO. However, the fast halogenation reactions such as the bromination of monochlorodimedone, using the cascade AOX-VCPO was possible in one reactor. Oxygen availability in aqueous solutions, scaling up as well as the reaction kinetics need to be further addressed.

The feasibility of the conversion of Glu into GluCN – an intermediate in the production of biobased acrylonitrile, was evaluated in **chapter 5**. The production of GluCN by VCPO and $H_2O_2$ (Scenario 3) was compared in a techno-economic assessment with other alternative biobased routes i.e. the production of GluCN by NaOCl (Scenario 1), by the cascade AOX-VCPO (Scenario 4) and by oxygen with a Ru catalyst (Scenario 5).

It was found that by replacing NaOCl with VCPO-$H_2O_2$ the energy requirements of the process is reduced by a factor of 1.5 for the production of 1 t GluCN. This is mainly as a result of performing the reaction at 25°C, eliminating the need of cooling below room temperature (4°C) as in the case of NaOCl. The mass balance is slightly improved as selectivity close to 100% can be achieved by VCPO-$H_2O_2$ system and a significant reduction in waste was achieved.

By further replacing NaOCl with oxygen in Scenario 4 and 5 the cost-benefit margin was increased significantly. Based on the cost-benefit analysis the only scenario with a positive

cost-benefit margin of 194 €/t GluCN is Scenario 4, owed to the co-production of acetaldehyde which is a valuable product. The sensitivity analysis of Scenario 4 and 5 where the price of different compounds was changed, shows that the price of Glu and GluCN are the parameters that influence the economics of the process the most.

At the moment, the price of the substrate, Glu, and the price of the product, GluCN – the intermediate in the production of acrylonitrile or succinonitrile – are too high to be competitive with the fossil based nitriles. As the price of Glu is already a best case scenario the use of cheaper sources of amino acids, e.g. crude mixtures of amino acids, should be tested. To produce the biobased nitriles, constraints should be applied to polluting industries to increase the price of fossil-based nitriles and as a result make the biobased nitriles more competitive.

In **chapter 6**, the results presented in chapters 2-5 and their implications are discussed. Suggestions for future research and concluding remarks are also provided.

# Acknowledgements

A PhD thesis is not only the work of the author. Other people contributed and helped in different ways to the realisation of this thesis. I would like to acknowledge these people for their efforts and I would like to express my gratitude to them.

First, I would like to acknowledge my supervisors. **Johan**, you inspired me with your passion for the biobased economy and with your lifestyle based on sustainability. Few people live as they preach, yet you are one of them. I am grateful for your support along the years and specially in the last part of my PhD. **Elinor**, thank you for your support during my PhD and specially for setting up the project that allowed me to combine two of my passions: enzymes and biobased chemistry. **Harry**, although you were not officially my supervisor, you had a lot of interest in my work and you always asked challenging questions which helped me to develop as a scientist. Thank you for your input on writing skills, I always write now with the thought '*facts before fiction*' in my mind. **Jérôme**, you were at BCH (now BCT) only for the first year of my PhD, however, your professional way of supervision had a tremendous impact on my own style of supervision. I always tried to follow the way you guided me during my master thesis and in the first year of my PhD. Thank you so much for all the lessons.

Second, I would like to acknowledge the students I supervised during my PhD: **Maria**, **Jean-Paul**, **Evie**, **Francesca**, **Marc**, **Francesc** and **Aster**. The research you performed helped me to advance my PhD and working with you developed my supervision skills.

Third, I would like to acknowledge my other co-authors and contributors to my thesis. **Ron**, thank you for providing the VCPO enzyme for my PhD, for the training on expression and purification of VCPO and for the input on Chapter 2. **Maurice**, I had the pleasure to work with you in the last part of my PhD. Thank you for clear comments and the critical input on writing the manuscript for Chapter 4 and thanks for all the encouragements.

**Tijs**, thanks for the full disclosure of your results on the techno-economic assessment. **Ellen**, I would like to thank you for the useful discussions regarding the techno-economic assessment and LCA. **Marieke**, thanks for the critical input on Chapter 5 and for the fun during coffee breaks and during the PhD trip to Brazil. I am very grateful to **Annemieke** from ORC department for performing the computational modelling for Chapter 5. **Han**, thank you for useful discussion on Chapter 2. **Ruud**, **Astrid** and **Kiira** thanks for your help with some microbiology techniques. **Michel** (Eppink) thank you for your support in enzyme purification. I am grateful to FBR for allowing me to use some of their equipment. I would like to give special tanks to **Carmen** for useful discussions on my work and to **Guus**, **Jan**, **Frits** and **Marc** for their kind help with the use of some equipment.

I would like to acknowledge **Susan** (and her magical troubleshooting hands) for the help on the analysis part. Next to the technical support, you and **Annemarie** were there for me in

the happy and in the difficult moments of my PhD. Thank you both so much, I will never forget this.

**Gerda**, you helped me with so much paperwork in such a professional way and you always made the coffee breaks cheerful and fun. Thanks for everything.

Along the four years of my PhD I shared my office with nice people. **Chen,** I enjoyed the discussions on Chinese and Dutch culture or practicing some English pronunciation. Thanks for being our guide & host in China (Jurjen and I enjoyed the trip a lot). **Pascal**, **Ruben**, **Luana**, **Gerben** and **Jin** it was a pleasure sharing the office with you.

I enjoyed the time at BCT and the PhD study trips to Brazil and China along with: **Daniel**, **Yu**, **Neus**, **Elvira**, **Farnoosh**, **Nurul**, **Piet** (van de Zaal), **Sanne**, **Nathan**, **Tim**, **Gwen**, **Ischa**, **Yessie**, **Rani**, **Tomas**, **Roxani**, and **Piet** (Buwalda). It was a pleasure to meet BCT members (**Karel**, **Rachel**, **Gerard**, **Ton**) with research interests quite different than chemistry...it certainly broadened my scientific horizons.

Further, I would like to acknowledge the ORC department where I work as teaching assistant since my PhD contract ended. Special thanks go to **Erik** for giving me the chance to work at ORC. To my office mates at ORC **Fred**, **Alexander, Sevil** and **Pepijn -** with whom I shared the last stretch of my PhD, thanks for all the great fun and your encouragements. **Carel**, **Hendra**, **Barend**, **Kris** and **Milou** thank you for encouragements and good advices.

I would like to acknowledge some people that gave me a good start for my PhD: **prof. dr. Francisc Peter**, **prof. dr. Mircea Dărăbanțiu** and my former **colleagues from Actavis**. Vă mulțumesc pentru tot ceea ce m-ați învățat.

Dear **Gosia**, thanks for all the fun we had during our PhDs and thanks for your 'Rocky style' pep talks. **Anamaria**, mii de mulțumiri pentru dicuțiile pe teme ştiințifice care m-au ajutat să înțeleg mai bine cum să lucrez cu enzimele şi multumesc pentru încurajări. **Anca**, îți mulțumesc pentru încurajările acordate de-a lungul doctoratului. Dear **Evie**, since you started your Master thesis at BCT we had a good connection, I am so glad we became friends. Thanks for the good fun, the insightful discussions and the encouragements. **Evie** and **Fred**, thanks for all the support as paranymphs.

Ik wil graag de **familie van Jurjen** bedanken voor de aanmoedigingen voor het afronden van mijn doctoraat. **Mike**, many thanks for the beautiful design of the cover and invitation.

Aproape de final doresc să îi mulțumesc familiei mele, **mami**, **tati**, **Mirela**, **Vlăduț**, **bunica Margareta**, **bunica Florica** şi **Adina**. Vă mulțumesc din suflet pentru suportul şi încurajările acordate de-a lungul doctoratului şi nu numai. Fără voi nu aş fi reuşit.

Mijn lieve **Jurjen**, het laatste plaats is voor jou omdat jij bent de persoon voor wie heb ik het meest dankbaarheid. Daar zijn niet genoeg dankwoorden voor alle hulp en geduld jij had terwijl mijn promotie.

# About the author

## Curriculum vitae

Andrada But was born on 19th of January 1986 in Satu Mare, Romania.

In 2009 she obtained her bachelor's degree in Biochemical Engineering from Babeş-Bolyai University in Cluj-Napoca, Romania. Her bachelor thesis, the synthesis of hetero functionalised bicyclooctanes, resulted in a publication in Letters in Organic Chemistry in 2010.

Further, from 2009 to 2011 she continued her studies with a master in Food Technology at Politehnica University of Timişoara, Romania. For her master thesis she was granted a six months Erasmus scholarship at Wageningen University (WUR) where she discovered her interest in green and biobased chemistry. Her master thesis was the screening work for the production of biobased nitriles using the enzyme vanadium chloroperoxidase which resulted in a publication in *ChemSusChem*, in 2012.

After graduating her master studies, she worked as a chemical analyst for half a year at Actavis - a pharmaceutical company in Bucharest, Romania.

With a passion for sustainability, biobased chemistry and enzymes, in 2012 she started the research for her PhD at Biobased Chemistry & Technology group, Wageningen University.

Currently, she works as teaching assistant at Laboratory of Organic Chemistry, Wageningen University where next to teaching she is involved in education innovation projects.

## Publication list

**A. But,** E. van der Wijst, J. Le Nôtre, R. Wever, J. P. M.Sanders, J. H. Bitter, E. L. Scott,
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# Overview of completed training activities

## Conferences

- EMBO Conference "Catalytic mechanisms by biological systems: combining computational and experimental approaches", Groningen, 2012
- International Symposium on Green Chemistry Renewable carbon and Eco-Efficient Processes, La Rochelle, France, 2013
- Biotrans Conference, Vienna, Austria, 2015
- The Netherlands' Catalysis and Chemistry Conference (NCCC), Noordwijkerhout, 2015, 2016
- Oxizymes conference, Wageningen, 2016

## Discipline specific courses

- Enzymology, Wageningen, 2014
- Advance course of biocatalysis, Delft, 2014
- Biorefinery training school, Budapest, Hungary 2014
- Master class applied biocatalysis, Wageningen, 2014
- Advance downstream processing course, Delft, 2015
- Sustainable food & biobased production, Wageningen, 2015

## General courses

- Scientific publishing, 2012
- Reviewing a scientific paper, 2013
- Information literacy & EndNote, 2013
- Effective behaviour in professional surroundings, 2013
- Interpersonal communication for PhD students, 2013
- Techniques for writing and presenting a scientific paper, 2014
- Writing grant proposal, 2016

## Other activities

- Writing of research proposal, 2012
- Participant of PhD study trip – Brazil, 2013
- Organisation of PhD study trip – China, 2015
- Weekly group meetings BCT, Wageningen, 2012-2016
- VLAG PhD council activity, Wageningen, 2014-2016

The research described in this thesis was financially supported by Wageningen University.

Cover design by Mike Broad and Andrada But

Printed by ProefschriftMaken || DigiForce on FSC-certified paper