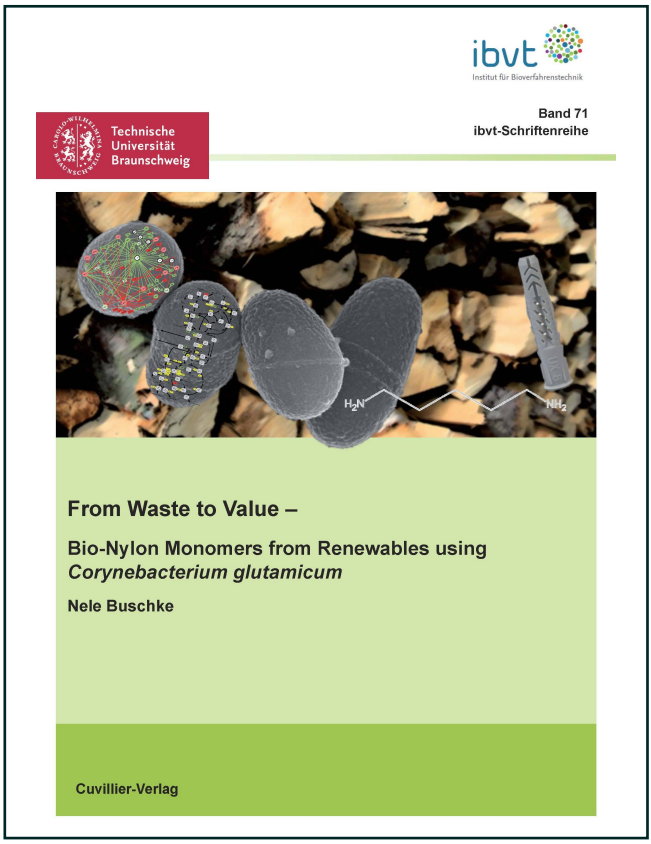
Nele Buschke (Autor)

From Waste to Value

Bio-Nylon Monomers from Renewables using Corynebacterium glutamicum



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1 Introduction

Sustainable supply of chemicals, materials and fuels has become a key issue in industrial production as it protects global climate and prevents shortening of fossil resources (Aristidou and Penttilä, 2000; Luo et al., 2010; Sánchez and Cardona, 2008; Steinbüchel, 2005; Willke and Vorlop, 2004; Wittmann and Lee, 2012). In recent years, the total biotechnological sector has grown to about 8% of the total sales of the chemical industry and is expected to further expand to 15% until 2017 (Festel, 2010). Strategies to achieve sustainability base on a new biosynthetic chemistry that utilizes renewable feedstocks by highly engineered microbial cell factories to convert the bio-based raw materials into value-added products. Particularly, biotechnological platform producers such as *Corynebacterium glutamicum* are most promising in this new era of bio-production (Becker and Wittmann, 2012). They already cover a substantial part of industrial bio-production and provide a broad spectrum of chemicals (e.g. succinic acid, lactic acid) (Okino et al., 2008b; Okino et al., 2008a), polymers (e.g. polyhydroxyalkanoates and polygalacturonic acid) (Liu et al., 2007; Wu et al., 2010) and also polymer building blocks (e.g. diaminopentane, putrescine) (Kind et al., 2010a; Schneider and Wendisch, 2010). Moreover, *C. glutamicum* has shown an efficient production of biofuels as exemplified by ethanol, 1,2-propandiol, isopropanol, 1-butanol, and isobutanol (Inui et al., 2004a; Niimi et al., 2011; Smith et al., 2010). This is complemented by high-value compounds for nutritional and pharmaceutical applications, including flavonoids, vitamins and amino acids (Ault, 2004; Becker et al., 2011; Dickschat et al., 2010). Today, major industrial raw materials comprise especially sugar-based feedstocks from starch, sugar cane, and sugar beet (Wittmann and Becker, 2007). An evaluation of their ecologic and economic classification has been especially attempted for biofuel production. Regarding the primary energy (PE) and the greenhouse gas (GHG) emission, sugar cane and sugar beet show the highest advantages whereas starch and rape seed based biofuel production have only small savings in tons CO_2-equivalents $(ha \cdot a)^{-1}$ and PE in GJ $(ha \cdot a)^{-1}$ (Reinhardt and Helms,

2008) (Figure 1). In contrast, woody material and other lignocellulosic biomass for production of the so called “biomass to liquid”-fuels (BtL) have a great availability, a low price, and relative high savings on PE and GHG and are therefore considered the most promising future feedstocks (Balat, 2011) (Figure 1). Today’s biofuel production from lignocellulose is, however, not yet as cost-effective due to the rather complex composition and the correlating pre-treatment costs of US$ 0.08 per liter produced bioethanol (Balat, 2011). With regard to limited land-use and expectable constrain for usage of food biomass, biofuel industry will soon depend on new developments in conversion of lignocellulosic biomass from non-food crops and waste materials such as corn stover, bagasse, or straw.

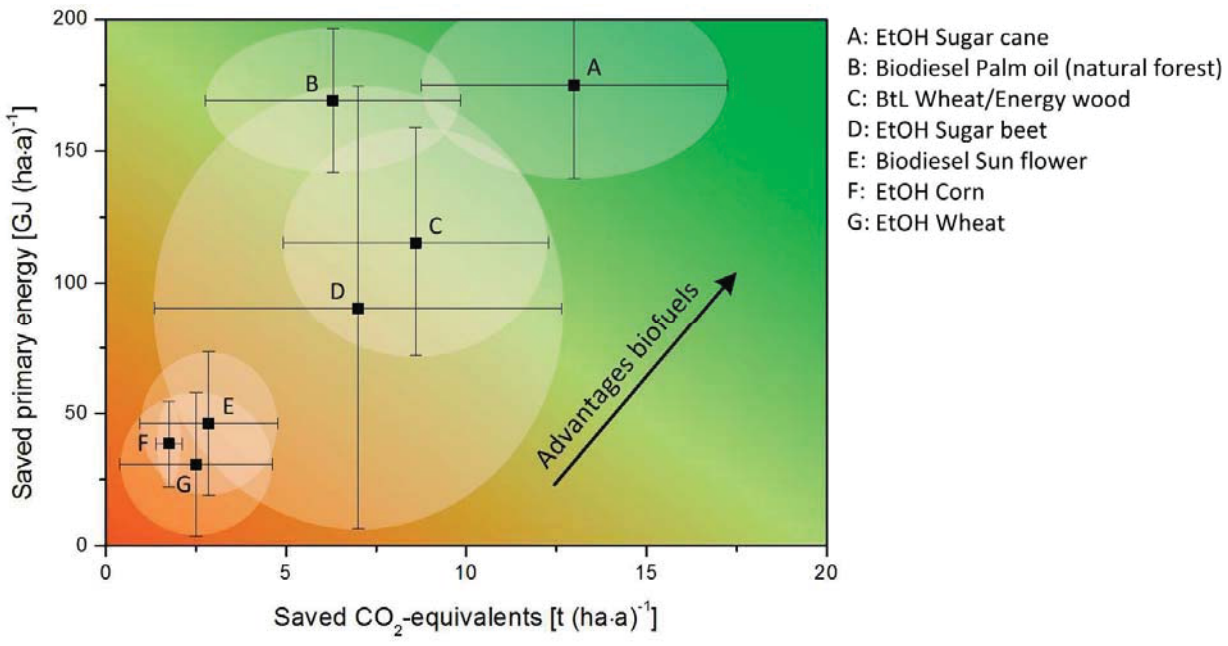

Figure 1: Savings in greenhouse gas emission in tons CO_2-equivalents (ha·a)$^{-1}$ and primary energy input (GJ (ha·a)$^{-1}$) of biofuels compared with fossil fuels. Figure is based on data published earlier (Reinhardt and Helms, 2008).

Commonly applied biotechnological producers, such as *C. glutamicum*, can only partly degrade and utilize the novel feedstocks. This requires substantial strain engineering for efficient substrate utilization and also strategies to increase tolerance against toxic ingredients typically associated with pre-treated biomass. Clearly, this is far from traditional biotechnology, that mainly utilized easy accessible raw materials, and poses new challenges on the microbial strains used.

2 Objectives

This thesis aims at systems metabolic engineering of *Corynebacterium glutamicum* to extend its substrate spectrum towards lignocellulosic feedstocks including waste material from pulp and paper industry.

In this regard the work focusses on xylose, a major constituent of these non-food raw materials, as substrate. First a basic production strain should be constructed to prove the feasibility of using xylose for production. As host a diaminopentane producing strain was chosen, with the product being an important high-value building block for bio-based polyamides. Subsequently, a systems biology approach should be applied to provide a global insight into the underlying metabolism of glucose and xylose utilizing *C. glutamicum* and to identify genetic targets to increase the production performance from xylose. The potential of the engineered strain should then be determined in industrial relevant fed-batch fermentation. The work should furthermore involve preparation of lignocellulosic feedstocks such as oat spelt xylan and the industrial waste black liquor.

The work was embedded into the collaborative initiative Bioindustry21 funded by the BMBF grant ‘‘Bio-based Polyamides through Fermentation’’ (No. 0315239A) including partners from BIOPRO Baden-Württemberg GmbH, BASF SE, Daimler AG, Fischerwerke GmbH, and Robert Bosch GmbH.

3 Theoretical Background

3.1 Feedstocks for biorefinery applications

The use of typical biotechnological feedstocks as starch and sugars (e.g. glucose or fructose) is discussed critically, due to the competing land-use with agricultural products for human nutrition and their direct use as food products (Aristidou and Penttilä, 2000; Dale, 1987; Luo et al., 2010). Therefore, the feedstock trend is directed towards non-food biomass such as lignocellulose (Aristidou and Penttilä, 2000) green biomass (from grass, alfalfa and clover) (Kromus et al., 2004) and also industrial waste streams (e.g. from pulp mills) (Chaudhary and Qazi, 2011; Whitfield et al., 2012; Xu et al., 2012). These feedstocks are especially attractive for sustainable biorefinery concepts related to high availability (Table 1), low price and eco-friendliness.

Table 1: World-wide availability of biomass feedstocks including crop residues and industrial waste streams that display interesting renewable raw materials for biorefineries.

Material	Availability (Mt/a)	Reference
Corn stover	204.0	(Dale and Kim, 2006)
Barley straw	58.5	(Dale and Kim, 2006)
Oat straw	10.6	(Dale and Kim, 2006)
Rice straw	731.0	(Dale and Kim, 2006)
Wheat straw	354.0	(Dale and Kim, 2006)
Sorghum straw	10.3	(Dale and Kim, 2006)
Bagasse	181.0	(Dale and Kim, 2006)
Molasses	91.0[a]	(FAOSTAT, 2010)
Glycerol	0.9[b]	(Chatzifragkou and Papanikolaou, 2012; Scheffran, 2010)

[a] estimated from world-wide sugar cane and sugar beet production (228 Mt and 1685 Mt (FAOSTAT, 2010)) assuming a molasses production of 4.75 tons/100 tons processed sugar cane and sugar beet containing 45% total sugar.

[b] estimated from world-wide biodiesel production (~$10.2 \cdot 10^9$ L in 2007 (Scheffran, 2010)) assuming a biodiesel density of 0.9 kg L^{-1} and a glycerol waste stream of 10 tons glycerol/100 tons biodiesel (Chatzifragkou and Papanikolaou, 2012) with a concentration of 60-80% (Thompson and He, 2006).

Material	Availability (Mt/a)	Reference
Starch	48.5	
Corn	39.2	
Wheat	2.6	(Grüll et al., 2006)
Potatoe	4.2	
Tapioca & rice	2.5	
Sugar	140.0	(Thoen and Busch, 2006)
Cellulose	320.0	(Thoen and Busch, 2006)

3.1.1 Structure and properties of lignocellulose

Lignocellulose is the most abundant polymer on earth. This global availability and also the unsuitability for human nutrition have brought this raw material into the focus for biorefinery applications (Luo et al., 2010). Lignocellulose makes up the scaffold of cell walls of woody plants and consists of cellulose, hemicellulose and lignin (Figure 2).

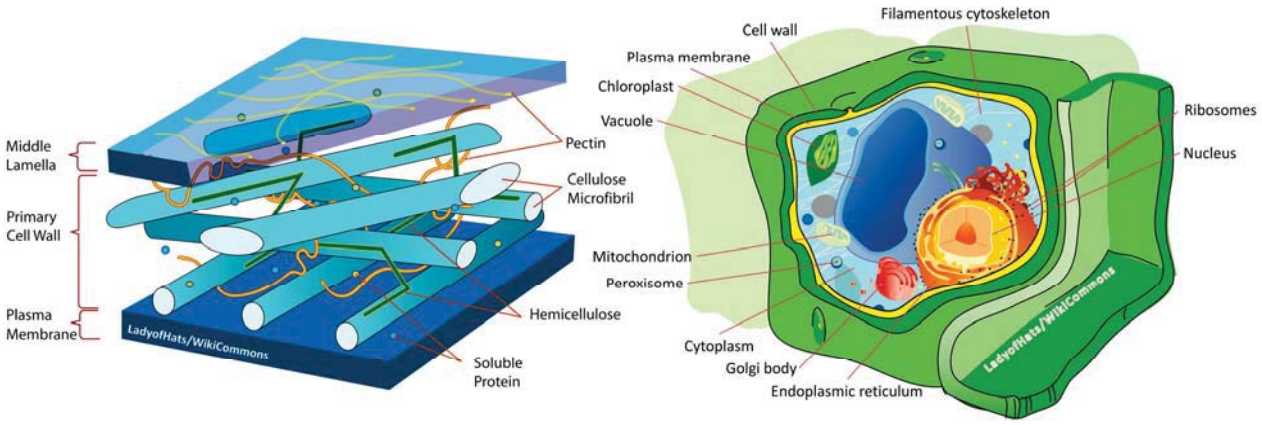

Figure 2: Plant cell composition and cell wall structure in detail. Photo reference: LadyoftheHats (WikiCommons).

The composition of the lignocellulosic material depends on the resource (Table 2), namely cellulose 40-50%, hemicellulose 20-30% and lignin 10-25% (Dale, 1987). Besides the listed compounds also ash and protein are lignocellulosic constituents. In general, hardwood contains a higher amount of hemicellulose then softwood and a lower lignin concentration.

Table 2: Composition of lignocellulosic feedstocks in percentage of the entire dry weight.

Feedstock	Cellulose [%]	Hemicellulose [%]	Lignin [%]	Reference
Corn stover	36.5	28.1	10.4	(Ackerson et al., 1991)
Rice straw	36.2	24.5	11.9	(Fan et al., 1987)
Wheat straw	39.9	28.2	16.7	(Fan et al., 1987)
Oat straw	39.4	27.1	17.5	(Fan et al., 1987)
Switch grass	31.0	20.4	17.6	(Wiselogel et al., 1996)
Popular	43.0	31.0	23.0	(Lengyel and Morvay, 1973)
Beech	38.0	35.0	25.0	(Lengyel and Morvay, 1973)
Oak	35.0	32.0	26.0	(Lengyel and Morvay, 1973)
Spruce	43.0	36.0	29.0	(Lengyel and Morvay, 1973)
Newspaper	61.0	16.0	12.0	(Ackerson et al., 1991)
Processed municipal waste	47.0	25.0	12.0	(Ackerson et al., 1991)

Cellulose, the main component in lignocellulose, is made up of glucose building blocks and tends to self-associated in microfibrils which are responsible for the insolubility and the complicate hydrolysis of cellulose (Hayashi et al., 2005). Cellulose is a linear polymer that consists of thousands of glucose units which are linked by 1,4-β glycosidic bonds (Figure 3). The macroscopic microfibrils are formed by hydrogen bonds between the glucose oxygen atoms O_3-O_5 and O_6-O_2. Cellulose can be used as substrate by various fungi, but most bacteria and yeasts lack endoglucanase activity for degradation (Lynd et al., 2002).

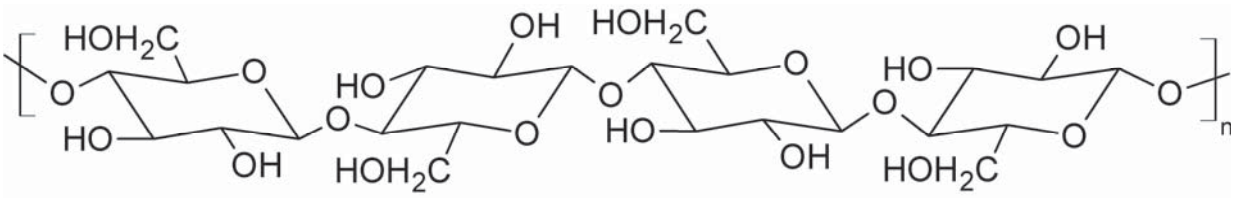

Figure 3: Cellulose structure consisting of β-1,4-linked glucose units with the formal composition $(C_6H_{10}O_5)n$.

The heteropolysaccharidic structure of hemicellulose consists of a β-1,4-linked D-xylopyranosyl main chain with a variable number of neutral and uronic monosaccharide subunits, as well as short oligosaccharide side chains (Figure 4). In soft wood plants, the xylan contains mainly arabino-4-O-methyl glucuronoxylan, and is further characterized by a α-arabinofuranoside unit every eight xylose residues (Subramaniyan and Prema, 2002).

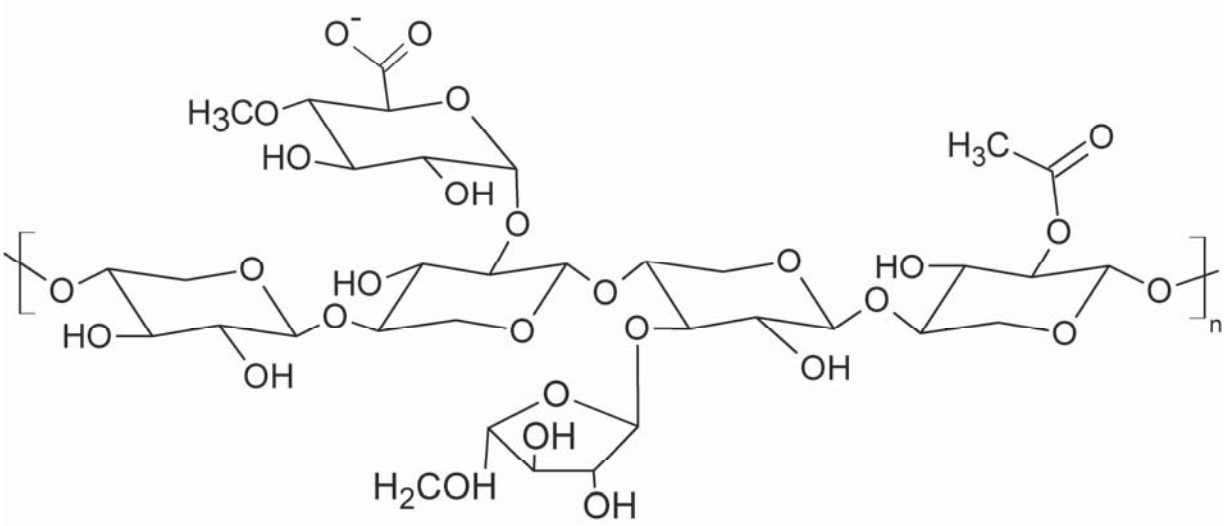

Figure 4: Arabino-4-O-methyl-glucuronoxylan the most typical hemicellulose structure in soft wood plants (Subramaniyan and Prema, 2002).

In contrast, the most abundant hemicellulose in hardwood is O-acetyl-(4-O-methylglucuron) xylan. This polymer is characterized by acetic acid groups that are esterified by the hydroxyl groups of the sugar chain (Subramaniyan and Prema, 2002).

The utilization of hemicellulose and its main component xylose, in particular in biotechnological fermentation processes, is in the center of extensive research efforts and has been so far over decades (Adham et al., 2001; Bettiga et al., 2009; Burchhardt and Ingram, 1992; Katahira et al., 2004; Sun et al., 2012). Applicable lignocelluloses can be obtained from the wood industry, from agriculture and also from sugar-processing industrial branches that produce high amounts of bagasse (Table 1) as solid remains of the sugar refineries (Dale and Kim, 2006) or from pulp mills that produce a waste stream (black liquor) with a high hemicellulose content. The utilization of

lignocellulose in biotechnological production typically requires extensive pre-treatment to alter the macroscopic and microscopic structure and eventually to facilitate a rapid hydrolysis of the carbohydrate fraction (Mosier et al., 2005).

3.1.2 Lignocellulose processing – Extraction and saccharification

Particle size, insolubility and macroscopic composition are all characteristics that contribute to the recalcitrance of lignocellulose and impose challenges to its use in biorefineries. An effective processing is indispensable for further biotechnological application. The processing steps can be separated into pre-treatment that includes polysaccharide extraction and separation, and subsequent hydrolysis to obtain fermentable monosaccharides (Chandra et al., 2007; FitzPatrick et al., 2010; Mosier et al., 2005). Pre-treatment procedures can be classified in physical and chemical methods. Comminution, steam explosion and hydrothermolysis are physical methods that enable a reduction of the particle size and therefore an easier handling in the following steps (Bobleter, 1994; Millett et al., 1979; Saddler et al., 1993). Chemical methods include treatment with bases or acids (NaOH, NH_3, H_2SO_4, HCl) and especially support the separation of lignin and hemicellulose. Solvents as alkaline H_2O_2, ozone, glycerol, dioxane, phenols or ethylene glycol are used to dissolve the cellulose (Mosier et al., 2005). An effective pre-treatment is very important as it affects the preservation of monosaccharides, formation of degradation products that inhibit bacterial growth, and the energy demand (Lynd et al., 1996; Mosier et al., 2005; Palmqvist and Hahn-Hägerdal, 2000a). The separated polysaccharides cellulose and hemicellulose can be hydrolyzed by enzymatic or acidic treatment or can be directly used by degrading organism as several fungi. Enzymatic hydrolysis, using enzymes from lignocellulose degrading microorganisms, is a more gentle method at moderate temperatures < 50°C and pH values > 5, whereas acidic hydrolysis requires more extreme conditions at temperature > 100°C and pH values < 2. The heterologous expression of xylanases and cellulose has been reported for several microorganisms. Different strategies are conceivable which include secretion and surface display of the enzymes (Burchhardt

and Ingram, 1992; Hyeon et al., 2011; Katahira et al., 2004; Kondo et al., 2010; Tsuchidate et al., 2011). By these techniques a simultaneous saccharification and fermentation is possible reducing production time and operating cost.

3.1.3 Black liquor – a high potential waste from pulp and paper industry

Black liquor is a major waste product of the pulp and paper making industry (approximately 7 tons per ton of pulp) (Biermann, 1993). It contains inorganic pulping salts and organic compounds and is traditionally burned to recover energy and low value cooking chemicals (Figure 5) (Kang et al., 2012). In this regard, material based utilization of this huge waste stream towards value added chemicals would be highly beneficial. Black liquor is rich in hemicellulose suggesting its potential use by metabolically engineered microorganisms that are capable to degrade such materials. However, black liquor, as starting material, is very basic (pH 13) and contains toxins and huge loads of salts, which complicates its integration into bio-production routes.

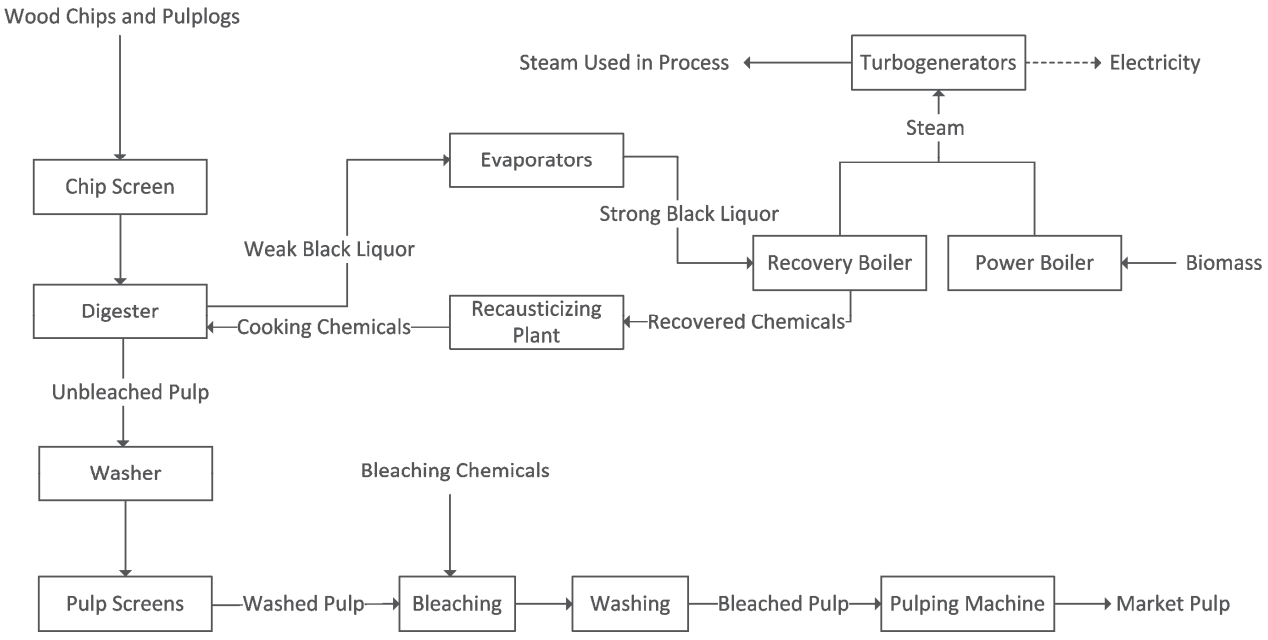

Figure 5: Pulp manufacturing process as realized in Mercer International pulp mills (including Papierfabrik Rosenthal GmbH). The figure represents a schematic illustration of the process shown in the Mercer President's Report 2011 (Mercer, 2011).

3.1.4 Inhibitors from industrial raw materials

Renewable feedstocks, such as lignocellulosic materials and industrial waste streams, often contain toxic compounds or potential growth inhibitors released during the pre-treatment. These are small aromatics such as furfural, hydroxymethylfurfural, and phenols, but also acetic acid. Thermal acidic treatment supports the degradation of pentose sugars into furfural and of hexoses into hydroxymethylfurfural via maillard reactions. Hydrolytic or oxidative cleavage of lignin facilitates the formation of various phenols such as 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, and syringaldehyde. Acids are often found after alkaline carbohydrate degradation or as further degradation products from furfural (glycolic acid, lactic acid, formic acid, levulininc acid) (Liu and Blaschek, 2010). Acetic acid is present in lignocellulosic hydrolysates as acetylation residues of hemicellulose and lignin. During the hydrolysis, the acetic acid is released and may act as inhibitor in subsequent fermentation. Depending on the biomass and the pre-treatment conditions (temperature, time, pressure, pH, redox conditions and catalysts) the degradation products may vary, but can accumulate up to approximately 10 g L^{-1} (Klinke et al., 2004). *C. glutamicum* shows a certain basic tolerance against lignocellulosic inhibitors (Rumbold et al., 2009). Still, a detoxification of the feedstocks is generally required as growth is already 50% inhibited at furfural and hydroxymethylfurfural concentrations of 0.5-2 g L^{-1} and at acetate concentrations of 10 g L^{-1} (Rumbold et al., 2009). Different strategies are possible to either achieve extracellular (*in vitro*) detoxification by chemical or enzymatic treatment or *in situ* detoxification by directed evolutionary or rational strain engineering (Larsson et al., 2001b; Sanda et al., 2011). As example, directed evolutionary engineering has led to promising *S. cerevisiae* strains with a higher tolerance towards the inhibitors, identified by repeated adaptation and screening (Martín et al., 2007). The obtained mutants were able to detoxify inhibitors and to maintain rather high growth and ethanol production rates compared to the non-adapted strains. As shown, they tolerated hydroxymethylfurfural concentrations of 4.5 g L^{-1} whereas the parent strain hardly grew

at 3.8 g L^{-1}. Using diluted hydrolysates with a furfuraldehyde concentration of 2.2 g L^{-1}, the evolved strain even exhibited a twofold increased production rate (Martín et al., 2007). The increased tolerance is mediated by aldehyde reductases, which convert furfural and hydroxymethylfurfural into the less toxic products furanmethanol and furandimethanol, respectively. Several NADPH-dependent multiple aldehyde reductases were found responsible and show a higher expression under inhibitory cell stress (Liu and Blaschek, 2010). *In situ* detoxification by rational engineering has been reported e.g. for overexpression of laccase (Larsson et al., 2001a). Laccase positive strains were able to grow on medium with coniferyl aldehydes and on biomass hydrolysates with 1.4 g L^{-1} furfural, 2.3 g L^{-1} 5- hydroxymethylfurfural and 2.9 g L^{-1} phenolic compounds whereas the laccase negative parent strain was completely inhibited (Larsson et al., 2001a). To address the growth inhibition by weak acids including formate and acetate, transaldolase and formate dehydrogenase activities were enhanced in *S. cerevisiae*. As response the volumetric ethanol productivity was 5-fold higher in the presence of 30 mM acetate and 20 mM formate (Sanda et al., 2011). Analogous detoxification strategies are conceivable for other platform organisms.

3.2 *Corynebacterium glutamicum* as industrial working horse

3.2.1 History – Becoming a platform organism

Corynebacterium glutamicum is a gram-positive rod-shaped soil bacterium. It belongs to the group of *Corynebacteriaceae* including other bacteria as *Mycobacteria, Nocardia and Rhodococci*. The members of this family are connected by the same characteristic cell wall structure (Eggeling and Sahm, 2001; Goodfellow et al., 1976). *Corynebacterium glutamicum* was discovered in the 1950s during a comprehensive screening program that aimed for a natural glutamate secreting bacterium. The ability of this bacterium to produce other amino acids under certain conditions was discovered shortly after (Kinoshita, 1959).