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Biotechnological Generation of Value Added Products from Spent Pulping Liquors: Assessing the Potential of Extremophiles

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Abstract

Carbon rich industrial waste streams are interesting raw materials for biorefineries, due to their high abundance and relatively low price. However, they are a challenging habitat for microorganisms, due to extreme pH and temperature levels, as well as high abundance of inhibiting substances. Extreme conditions call for extreme microorganisms.

This contribution aims to show the potential of extremophilic bioprocesses for the generation of valuable products from industrial waste streams on the example of the pulping waste streams spent sulfite liquor (SSL) and Black Liquor (BL). It provides an overview of products, which can be produced biotechnologically by extremophilic organisms and compares their performance to benchmark biotechnological processes. Furthermore it elucidates the factors to be considered for bioprocesses on industrial waste streams, thereby providing a toolset for selecting a bioprocess based on the waste stream. Finally bioprocesses are proposed for SSL and BL, two of which are already investigated in our workgroup.

Keywords: Extremophile bioprocesses; Chemical building blocks; Pulping waste streams; Waste to value

Introduction

Worldwide bio-economy concepts foster the conversion of biomass into a range of food, health, fiber, industrial products, and energy. However, there is a risk that the diversion of farmland or crops for the production of biofuels and bio-based products compromises the food supply-the food versus fuel dilemma. One way to circumvent this dilemma is the use of spent liquors from the pulping industry in terms of a wood biorefinery. The pulping process leads to a solid fraction mainly consisting of cellulose (pulp) and a liquid fraction containing a mixture of the other wood components and their degradation products, which can be further processed. The economical success of the utilization of these liquid fractions largely depends on an efficient separation and conversion of the organic compounds-predominantly the carbohydrates. Integrating biotechnological processes into existing pulp mills is expected to achieve those requirements. Employing extremophilic microorganisms, e.g. *Acidothermophiles* or *Alkalithermophiles*, in the bioprocesses could lead to further process intensifications by saving chemicals, cooling energy and sterilization steps.

In 2004 the US Department of Energy published a list of chemical building blocks, which are likely to become a commercial success [1]. This list was used as guidance for identifying promising target products for bioprocesses. In 2014 the Obama administration identified advanced materials including composites and bio-based materials as one of three technologies critical to U.S. competitiveness [2]. Therefore, biopolymers like polyhydroxyalkanoates or their precursors like lactic acid and succinic acid are included. And last but not least ethanol and butanol are considered, which can be used as chemicals or biofuels (Table 1).

This review article, therefore, summarizes

- a short description of the commercial pulping processes (Sulfite and Kraft) highlighting the parameters relevant for the bioprocessing
- the commercially realized processes yielding products from spent liquor
- target products accessible by bioprocesses, their (potential) markets and state of the art of the bioprocesses
- an overview of potential candidates (excluding GMOs) for future bioprocesses in the pulping industry with special emphasis on extremophiles
- two examples for employing extremophiles from our research

The Commercial Pulping Processes

Lignocellulose (wood) is composed of strands of cellulose molecules embedded in a matrix of hemicellulose and lignin. Hemicelluloses are plant heteropolysaccharides whose chemical nature varies from tissue to tissue and from species to species [3]. It can generally be assumed, that softwood hemicelluloses contain high amounts of mannose, whereas hardwood hemicelluloses contain high amounts of xylose.

In order to obtain chemical pulp the cellulose has to be separated from the other wood components. To date the two major commercial pulping processes are the Kraft process and the Sulfite process. Figure 1 summarizes the pulping conditions of both processes, the "pedigree" of the major organic components present in the spent liquors and a rough

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estimate of the composition of the spent liquors (spent sulfite liquor = SSL and black liquor = BL).

Kraft process

The Kraft process uses an aqueous solution of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) at elevated temperatures (155-175°C), elevated pressure (7-10 bar) and pH values beyond 10 for the digestion of wood. It is the dominant pulping process in the pulp and paper industry. Kraft pulp accounts for two-thirds of the world's virgin pulp production and for over 90% of chemical pulp with a global production of about 130 million tons per year [4]. The compositions of the black liquors derived from hardwood and softwood, respectively, are shown in Table 2.

Target molecule
1,3-propanediol[2]
3-hydroxy propionic acid[1]
aspartic acid[1]
fumaric acid[1]
glucaric acid[1]
itaconic acid[1]
lactic acid[2]
succinic acid[2]
polyhydroxyalkanoates (PHA)[2]
ethanol
butanol
sorbitol[1]
xylitol

Table 1: Promising target products for the bioprocessing of spent pulping liquors [1].

Sulfite process

The Sulfite process uses an aqueous solution of acidic bisulfite at elevated temperatures (130-160°C), elevated pressure (7-10 bar) and pH values ranging from 1-4 for the digestion of wood. The industrially used cations in the sulfite pulping processes are calcium, magnesium, sodium and ammonium. The generated spent liquor is called spent sulfite liquor (SSL) and contains dissolved solids such as lignosulfonates and hemicellulose hydrolysis products, which comprise about 40-50 g l^{-1} of hexoses and pentoses [5]. The composition of the sugar fraction in SSL depends on the type of wood used for pulping. Coniferous "soft" wood yields high proportion of hexose sugars (predominantly mannose and glucose), whereas deciduous "hard" woods yield a high proportion of the pentose sugar xylose. The compositions of the sulfite spent liquors derived from hardwood and softwood, respectively, are shown in Table 2.

State of the Art Spent Liquor Utilization

To date the major use of spent liquors is energy generation and merely a handful of products are obtained: One strategy focuses on reducing the high fossil energy need by thermal utilization of the spent liquors. This has the advantage that the cooking chemicals can be recovered [6]. Pulp mills have used black liquor as an energy source since at least 1930 [7]. Recovery boilers are used to burn the black liquor to recover the chemicals sodium hydroxide and sodium sulfide, which are used to separate lignin from the cellulose fiber. Furthermore, they produce steam used for the generation of energy. This reduced problems with water emissions as well as the use of chemicals due to recovery and reuse. Spent sulfite liquors are treated accordingly. Therefore, paper mills are nearly energy self-sufficient by producing, on average, 66% of their own electricity needs on-site. Hence, the forest

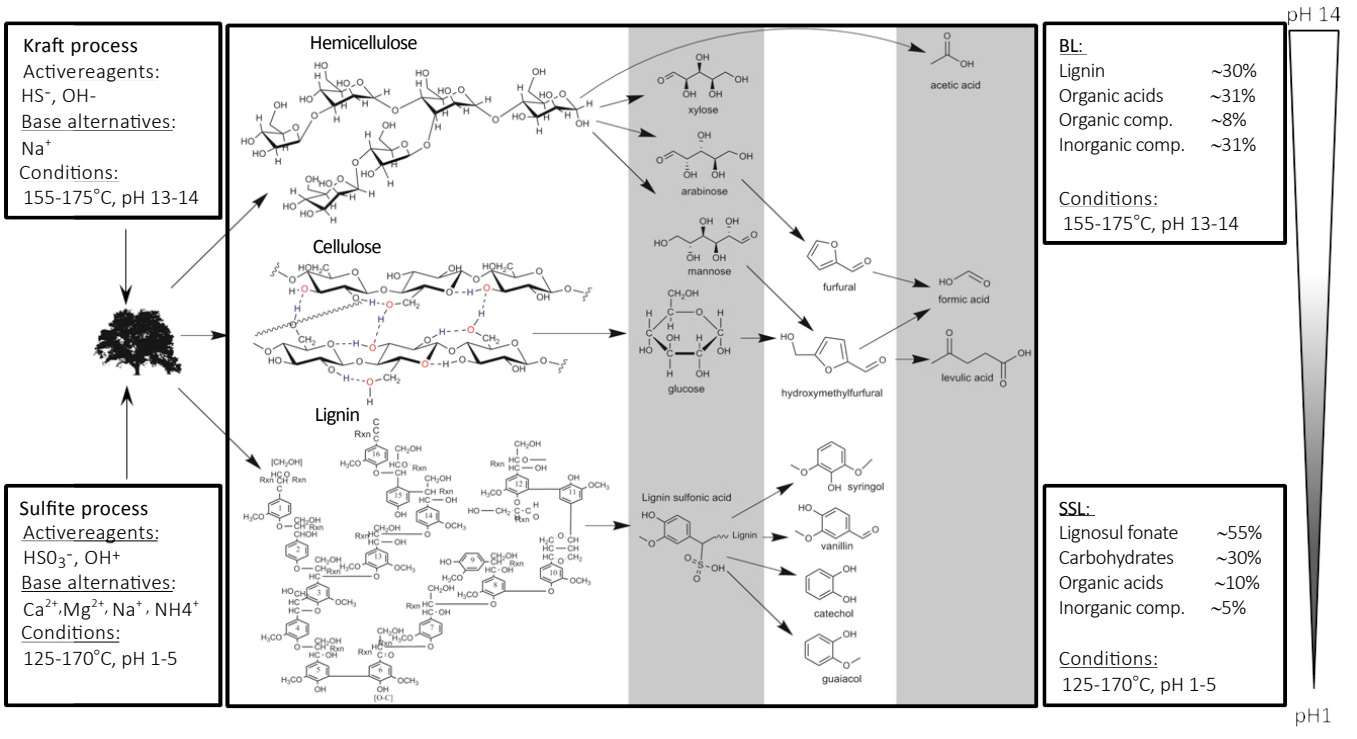

Figure 1: Process scheme of acidic and alkaline wood hydrolysis.

	Kraft process		Sulfite process	
pH	13-14		1-4	
temperature	160-180°C		125-170°C	
	Pine %w/w DM	**Birch %w/w DM**	**Spruce %w/w DM**	**Birch %w/w DM**
Monosaccharides arabinose			1	0
galactose			5	1
glucose			4	1
mannose			12	6
xylose			6	21
Organic acids acetic acid	4	9	4	
aldonic acids			5	
formic acid	6	4		
glucuronic acid				2
glucoisosaccharinic acid	7	3		
glycolic acid	2	2		
lactic acid	3	2		
2-hydroxybutanoic acid	1	5		
3,4-dideoxy pentanoic acid	2	1		
3-deoxypentanoic acid	1	1		
xyloisosaccharinic acid	1	2		
other acids	4			
Others				
lignin	33	27		
lignosulfonate			55	
hemicellulose	8	12		

Table 2: List of components in pulp and paper waste waters [105-107].

products industry is among the leading carbon-neutral industries using renewable energy.

The second strategy is to use the streams as biofuel feedstock for gasification, which has the potential to achieve higher overall energy efficiency than the conventional recovery boiler, while generating an energy-rich syngas from the liquor. The syngas can be burnt in a gas turbine combined cycle to produce electricity, or converted into chemicals or fuels such as methanol or dimethyl ether (DME). There is a 3 MW pilot plant under operation in Piteå, Sweden, where Chemrec runs tests on substrate from Smurfit Kappa for the production of BioDME. Since 2012 this BioDME plant constantly produces high quality DME that is used in trucks [8].

SSL has been used as a substrate for Single-Cell Protein (SCP) (*Candida utilis* or *torula* yeast) or alcohol production [9,10]: Yeasts have been used since the early 20th century for the production of ethanol from Spent Sulfite Liquor (SSL). In 1909 the first sulfite ethanol plant opened in Skutskär, Sweden. Since then many of those plants had to close down because of the low fossil energy prices, the high costs for the substrate pretreatment and the inability of non-genetically modified yeasts to ferment the highly abundant C5-sugar xylose. Today merely a few companies are operating sulfite ethanol mills. Among them are Borregaard (Sarpsborg, Norway), Domsjø (Domsjø, Sweden), Tembec (Temiscaming, Quebec, Canada), Kirov (Kondopoga, Karelia, Russia), Kimberly- Clark (Everett Mill, USA) and Nippon Paper (Gutsu mill, Japan). Yeasts were also used to remove the carbohydrates from SSL in order to improve the quality of the lignosulfonates, which are sold as bulk chemicals. The pulp mill Biocel Paskov, for example, used *Kluyveromyces fragilis,* which was sold as fodder yeast under the brand name Vitex [11].

The chemical production of vanillin from SSL started in the 1930's with the in North America [12] and is nowadays mainly produced by Borregaard. The DuPont wood based process utilizes the SSL to chemically produce xylitol.

Kraft cooking of pine wood yields turpentine as well as tall oil, which is the only noteworthy product from black liquor and which is further processed to bulk chemicals. Additionally, small amounts of lignin are isolated from black liquors for chemical application. The carbohydrate-derived fractions, polysaccharides and aliphatic carboxylic acids, are currently not exploited due to scattered information on their chemical nature or composition, isolation and purification problems, as well as limited markets [13].

Generally, the use of microorganisms on black liquor and spent sulfite liquor has limitations. The carbohydrate fraction of SSL contains C6-sugars as well as C5-sugars derived from the hemicelluloses, whereas hardwood SSL contains a significantly higher portion of C5-sugars than softwood SSL. Unfortunately, many microorganisms are only capable of fermenting hexoses, which lowers the overall efficiency and yield of the bioprocess. On the other hand, those microorganisms that can metabolize pentoses usually are less robust than their C6-converting relatives. Black liquor is a challenging substrate since it only contains small amounts of carbohydrates and high amounts of organic acids, which can be utilized by very few strains. Furthermore, both spent liquors contain significant amounts of substances that are known for their inhibitory effects on microorganisms.

Common substances in lignocellulose hydrolysates, known to inhibit microbial growth

Several compounds of the lignocellulosic degradation products common in spent pulping liquors are known to inhibit microbial growth. Their effects on the microorganism have been widely discussed

in the context of hydrolysates from the ethanol production, e.g. [14,15]. A general description of inhibitors found in lignocellulosic hydrolysates was published by Olsson and Hahn-Hägerdal in 1996 [16]. They were divided into four groups: furan derivatives, organic acids, phenols, and inorganic ions. The furan derivatives, furfural and hydroxymethylfurfural (HMF), are degradation products of pentoses and hexoses, respectively. Their inhibition mechanism is not fully clear.

Further degradation of the furan aldehydes leads to the generation of the organic acids formic acid and/or levulinic acid. Another organic acid namely acetic acid is a hydrolysis product derived from the hemicellulose fraction. Undissociated acids may pass through the cell membrane. They can dissociate inside the cell due to the neutral cytosolic pH value. The dissociation of the acids then lowers the pH in the cytosol, which may lead to cell death (Figure 2).

Phenolic derivatives are formed during the degradation of lignin. There is a large variety of structurally closely related phenols present, which renders their identification and quantification a challenging task. Various phenolic compounds have been found to inhibit cell growth [17]. According to the literature the more hydrophobic a phenol is, the more harmful it is to cells [18,19]. The mechanism of toxicity of the phenolic compounds has not been elucidated in most cases. One theory suggests that the phenols interfere with the cell membrane, which will influence its function and change its protein-to-lipid ratio [20].

Inorganic ions may originate from the wood, the pulping chemicals, or the pulping equipment. Another source are the chemicals added for the adjustment of the pH-value before the fermentation. A proposed inhibitory mechanism is osmotic stress [21]. Also, synergistic effects between the inhibiting substances have been proposed [19].

In a nutshell: the inhibitory effects of the furan derivatives, the organic acids, the phenols, and the inorganic ions are not yet fully understood, they are largely dependent on the concentration of the substances and vary with the respective microorganism. For the microbial production of other products than ethanol the available literature is yet rather limited. However, some detailed studies have been performed for *T. mathranii*, *C. beijerinckii* and a *Bacillus* strain.

The effect of wood degradation products such as salts, furfural,

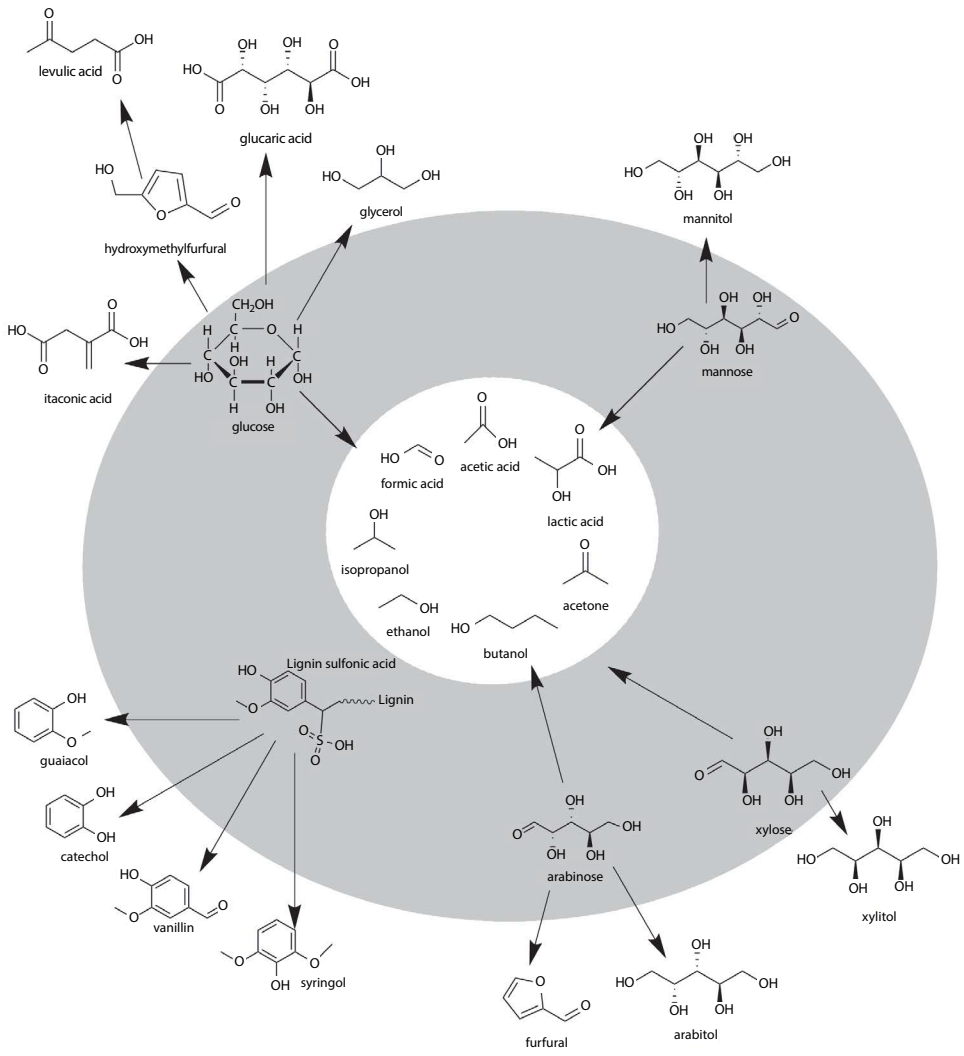

Figure 2: Chemicals (white circle) that can be prepared from precursors in lignocellulose (grey circle).

hydroxymethyl furfural (HMF), syringealdhyde, and acetic, ferulic, ρ-coumaric, and glucuronic acids on cell growth and production of butanol has been investigated for *C. beijerinckii* [22]. While furfural, HMF, and acetic acid (as acetate) did not inhibit fermentation at the tested concentrations, ferulic and ρ-coumaric acids lowered cell growth and ABE production significantly.

A similar study was conducted for *T. mathranii*: nine phenols and 2-furoic acid were each tested in concentrations of 10-100 times the concentration found in the hydrolysate for their effect on fermentation by *T. mathranii.* The study showed that the concentration of phenol aldehydes (4-hydroxybenzaldehyde, vanillin, syringaldehyde) higher than 10 mM severely inhibited fermentation, while phenol ketones (4-hydroxyacetophenone, acetovanillone, acetosyringone, 4-hydroxybenzoic acid, vanillic acid, syringic acid) inhibited fermentation to a lesser extent [23].

Another study performed with a *Bacillus* strain (IFA 119) showed high inhibition by cinnamaldehyde, p-hydroxybenzaldehyde, and syringealdehyde, as well as for high levels of HMF and furfural (low concentrations had a stimulatory effect). Catechol and hydrochinone were the most toxic lignin derived components in this study; however, also iron (3+) and chromium (3+) were very toxic for the used strain.

These three studies show that for the investigated species phenols are very problematic. There are various strategies to overcome the inhibitory effects of phenolic components.

Several physicochemical methods have been proposed for the detoxification of the hydrolysates prior to the actual fermentation [24]. Proposed pretreatment steps include alkaline treatment [14], overliming, adsorption into activated charcoal and treatment with anion- and cation-exchange resins at different pH [25], or a nonionic polymeric adsorbent resin [26]. Salts can be successfully removed by electrodialysis [27]. Furthermore, total separation of lignosulfonates (LS) by an integrated process of ultrafiltration coupled to ion exchange or a membrane process can efficiently separate high molecular weight LS from the low-medium molecular weight LS and sugars [28].

A modified fermentation process may overcome the inhibition phenomena, for example, during a fed-batch process the inhibiting substances are added in small amounts below the inhibiting concentration. Finally the microorganisms themselves could be altered either by adaptive evolution [29,30] or genetic engineering [31,32].

Methods and Criteria for Microbial Utilization

Sufficient amounts of carbohydrates need to be present and the organisms requirements for nitrogen, phosphor and sulfur supply as well as trace elements and vitamins need to be fulfilled for a substrate to be utilized by microorganisms. For this purpose the favored spent liquor has to be characterized. From a technological point of view there are factors that can be easily adjusted, like temperature and pH, factors that can be changed with a considerable amount of effort, like amount of inhibitors and factors that cannot be changed at all, like the available substrates. Based on this evaluation a set of microorganisms, which can thrive on the chosen substrate can be identified.

Next, a portfolio of desired products is investigated, leading to a set of microorganisms capable of producing them. These two resulting sets of microorganisms (the one with the organisms able to thrive on the available substrate and the one with microorganisms producing desirable products) are compared and the intersecting set is identified.

The spent liquors present a substrate full of inhibiting substances, they leave the digesters with a high temperature and an extreme pH-value and they contain a mixture of carbohydrates and their degradation products (Table 2). Therefore, organisms, which are able to withstand and thrive in these conditions, have to be identified. The main criteria for identifying strains suitable for biotechnological processes on pulping waste were determined according to their favored growing pH value, high temperature and utilization of at least cellulose, glucose, mannose, arabinose, galactose or xylose, but ideally also hemicelluloses. The most promising microorganisms for black liquor seem to be thermoalkaliphiles. However, the abilities to degrade oligosaccharides and organic acids will also be taken into account [33]. The same decision criteria apply for fermentations on SSL. However, due to the low pH value of SSL thermoacidophile strains are favored.

Microorganisms Generating Target Products

Some of these products have been generated biotechnologically over a long period. In addition to ethanol, and butanol (1861, Louis Pasteur) 1,3-propanediol is probably among the oldest known fermentation products. It was reliably identified by August Freund in 1881, in a glycerol- fermenting mixed culture obviously containing *Clostridium pasteurianum* as an active organism [34]. Another well investigated process is the biotechnical production of succinic acid, which has been produced since the 1930's [35]. The following section lists target products accessible by bioprocesses, describes their (potential) markets and state of the art of the bioprocesses.

Where applicable, organisms, which are already used in a respective bioprocess, will be introduced as a benchmark for comparison to identified potential extremophile candidates. This benchmark organism can be an extremophile, but also a mesophile strain to show what an extremophile bioprocess has to compete with.

3-Hydroxypropionic Acid and 1,3- propanediol

The C3-building block 3-Hydroxypropionic acid (3-HP) is a valuable platform chemical, which can be produced biologically from glucose or glycerol [36]. By coupling the fermentation with chemical processing, this intermediate may then be used for the production of commercially valuable chemicals. Such chemicals include 1,3-propanediol, acrylic acid, malonic acid, and acryl amide, which are used in large quantities for adhesives, polymers, plastic packaging, fibers, and resins.

1,3-Propanediol (1,3-PDO) is an important chemical product, which can be used for synthesis reactions, in particular, as a monomer for polycondensations to produce polyesters, polyethers and polyurethanes [37]. It has been proposed by DuPont as a potential monomer for use in fibers for carpet for its improved dyeability and elasticity. So far only mesophilic organisms, including *Clostridium ssp.* [38,39] and *Lactobacillus ssp.*[40] species and genetically modified *E. coli* [41] have been reported to produce 1,3-PDO. *Desulfovibrio* sp., *D. carbinolicus* and *D. fructosovorans* can produce 3-HP when growing on glycerol [42,43]. However no extremophiles producing 3-HP have been found. A benchmark organism for the production of 3-HP and 1, 3-PDO can be seen in Table 3.

Aspartic acid

Major amounts of L-aspartic acid are used for the production of the sweetener aspartame. Furthermore it is used as educt for the stereo selective synthesis of many chiral organo-chemical molecules, such as polyene macrolide class antibiotics [44]. N-substituted polyaspartic acid esters are used as reactive components in modern lacquer systems

Product	Strain	$Y_{p/s}$ $Cmol_i$ $(Cmol_s)^{-1}$	C-Source	References	Notes
1,3 - PDO	*Clostridium diolis*	0.71	glycerol	[108]	wt/BM
3-HP	*Lactobacillus reuteri*	0.914	glycerol	[40,109]	wt/BM
L-aspartic acid	*E. coli* EAPc-7		ammonium fumarate	[46]	GMO/BM pH 8.9
Fumaric acid	*Rhizopus sp*		glucose	[50,51]	wt/BM
	Alkalibacterium subtropicum		glucose	[110]	alkali-halophile
	Alkalibacterium gilvum		glucose	[111]	alkali-halophile
Glucaric acid	*E. coli*		glucose	[112]	GMO/BM
Itaconic acid	*A. terreus*	0.94	glucose	[113]	ME/BM
Lactic acid	*Lactobacillus pentosus*	0.435	hydrolyzed wheat straw	[57]	ME/BM
	Bacillus sp. 36D1		sugar cane hemicellulose hydrolysate	[114]	acidophile, thermotolerant
	Bacillus sp. XZL4	0.98	corn stover hydrolysate	[115]	thermophile
	Marinilactibacillus psychrotolerans	0.99	glucose	[116]	halophile
	Alkalibacterium subtropicum		glucose	[110]	alkali-halophile
	Alkalibacterium gilvum		glucose	[111]	alkali-halophile
Succinic acid	*Actinobacillus succhinigenes*	0.91	glucose	[117,118]	wt/BM
	Clostridium thermosuccinogenes	0.43	inulin, fructose	[119,120]	thermo-phile
PHA	*Ralstonia Eutropha*	- 0.76 g g^{-1}	palm oil, soybean oil	[121-124]	BM
	Haloferax mediderranei	0.33 g g^{-1} 0.9 mol mol^{-1}	starch glycerol whey	[97,125-127]	halophile
	Halomonas boliviensis	0.1 g g^{-1}	hydrolyzed wheat bran	[99,128]	halophile
	Halomonas elongata		glucose, mannose, xylose SSL	[99,100,129,130]	halophile
BM GMO ME wt	– benchmark organism – genetically modified organisms – metabolic engineering – wild type organism, mesophile				

Table 3: Summary of microorganisms producing organic acids and interesting bulk chemicals.

[45]. L-aspartic acid is also used as part of infusion solutions, for parenteral nutrition and for salification. To date it is only produced by mutant strains utilizing specific substrates like ammonium fumarate instead of simple sugars [46,47].

Fumaric acid

Fumaric acid is used as a food additive, in beverages and baking powders mainly regulating acidity. It substitutes tartaric acid and occasionally replaces citric acid. Furthermore it is used in the manufacture of polyester resins and polyhydric alcohols and as a mordant for dyes. Currently, fumaric acid is produced chemically from maleic anhydride, which in turn is produced from butane [48]. With rising petroleum prices, maleic anhydride as a petroleum derivative has increased in price as well, renewing the interest in fumaric acid production by fermentation as was operational during the 1940s [49]. Although the chemical process yields 112% *w*/*w* fumaric acid from maleic anhydride and the fermentation process yields only 85% *w*/*w* from glucose, the latter raw material is three times cheaper [48]. Additionally, the fermentation fixes CO_2, a fact that can be crucial in terms of CO_2-neutrality of a process. Production of fumaric acid by *Rhizopus* species and the involved metabolic pathways have been reviewed in literature [48]. So far mainly *Rhizopus sp.* [50,51] is known to be industrially interesting fumaric acid producers, thereby being the benchmark organism. Several extremophiles have been reported to produce at least trace amounts of fumarate [52], however the biotechnological feasibility and yield has not been reported so far.

Glucaric acid

Glucaric acid is a starting material for polymers like poly-(hexamethylene glucaramide). No wild type microorganism is known to produce glucaric acid, however, several genetically engineered microorganisms were designed to produce glucaric acid.

Itaconic acid

Itaconic acid (IA) is an unsaturated dicarboxylic acid. It can easily substitute acrylic or methacrylic acid in polymers. It is used at 1-5% as a co-monomer in resins, in the manufacture of synthetic fibers, in coatings, in adhesives, and also in thickeners and binders [53]. The favored production process is the fermentation of carbohydrates by fungi (mainly *Aspergillus sp.)*, with a current market volume of about 15,000 t a^{-1} [53]. At present, the production rates using *A. terreus* do not exceed 1 g l^{-1} h^{-1}, and product concentrations of about 80 g l^{-1} [53], The achieved titers for itaconic acid are still low. Furthermore, purified substrates are required for optimal productivity, making the overall process expensive. Due to the high price of about 4 USD kg^{-1}, the use of itaconic acid is restricted [53]. A productivity of 2.5 g l^{-1}

h^{-1} was proposed for the process to be economically competitive [1]. Fermentations are run below pH 3.5 and the strain can, therefore, be considered an extremophile [54].

Lactic acid

Lactic acid is commonly used in food, pharmaceuticals and cosmetics. In the last years the interest in its use for the production of poly-lactic acid (PLA), a biobased polymer with properties of a thermoplast, has grown. An ideal lactic acid-producing strain converts the substrate fast and complete, with high yields of preferred stereospecific lactic acid under low pH value and high temperature conditions. Preferred raw materials are common, cheap carbon sources like sucrose, lactose, starch, or sulfite waste liquor, which are fermented by numerous species of the genus *Lactobacillus* [55]. *L. amylophylus* and *L. amylovirus* are able to ferment starch, *L. lactis* can ferment glucose, sucrose and galactose and *L. pentosus* have been used to ferment sulfite waste liquor [56]. Using acid hydrolyzed wheat straw, a yield of 0.435 Cmol $(Cmol)^{-1}$ lactic acid could be observed in *L. pentosus* [57], thereby posing as benchmark organism. However, several extremophile, mainly halophilic lactic acid bacteria have been identified. A summary of these bacteria is listed in Table 3.

Succinic acid

Nowadays, the succinic acid market is about 20000-30000 ton per year worldwide and is manufactured on industrial scale by catalytic hydrogenation of petrochemically derived maleic acid or maleic anhydride [58,59]. The four existing succinic acid markets are the detergent/surfactant market, the ion chelator market, food market (e.g. acidulants, flavors or antimicrobials) and the pharmaceutical market [60]. These markets have high added value and do not require very cheap feedstock [61]. However, succinic acid derived commodity chemicals like butanediol (BDO), tetrahydrofuran (THF) and gamma-butyrolactone (GBL) are mostly low cost bulk chemicals [60]. In order to compete with products derived from petrochemicals, the fermentation cost needs to be at or below $0.25 per pound. A minimum productivity of 2.5 g l^{-1} h^{-1} needs to be achieved in order to make the bioconversion process economically competitive [1]. Several microorganisms have been proposed for biotechnological succinic acid production, some are shown in Table 3.

Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are biobased polyesters that can be produced directly via single step bioprocesses fermenting carbon sources from renewable feedstocks. The PHA serves as a microbial energy reserve material that is stored as granules within the cytoplasm of the cell and created, once the nutrients are reduced to create an imbalance, which puts the microorganisms under stress. Under ideal conditions, typically, from 80% to 90% of the cell weight can comprise PHA. Technologically PHA has highly attractive qualities for thermo-processing applications as bioplastics and their mechanic characteristics are similar to polypropylene [62].

At the moment there are several companies that provide a large scale PHA based product. Metabolix Inc.,which produces PHA latex that is derived from renewable raw materials and can be used to replace PE as moisture-resistant barrier coatings for paper and corrugated cardboard applications, is planning to establish an intermediate-scale manufacturing capacity of 2.5 to 5.0 kilo tons per year in late 2015 (Quarter Report Sept. 2014). Other companies are TianAn, producing about 2000 t. p a. of PHB/V or Meredian. Typical PHA producing strains can be found in Table 3.

Butanol

Similarly to ethanol, butanol is a solvent regularly produced by microorganisms during the ABE fermentation, named after the main products acetone, butanol and ethanol. It has various uses, including the use as a solvent in dyes, the use as a fuel and various applications in the polymer industry.

As a biofuel, bio-butanol shows even greater potential than bioethanol in the transportation industry as it contains 25% more energy than bioethanol (per volume). Microbial production of butanol is well documented and a variety of strains are available. Most commonly the used substrate is a monosaccharide, but some microorganisms are also able to utilize lactate [63]. Selected strains are listed in Table 4.

Ethanol

Alcoholic beverages produced by microbes are probably the oldest biotechnological processes used by mankind. Since the invention of the Otto engine in the 1860's ethanol has been successfully used as a fuel. However it is also an important industrial ingredient and has widespread use as a base chemical for other organic compounds, including ethyl esters, diethyl ether, acetic acid, ethyl amines, and, to a lesser extent, butadiene. Furthermore it is an excellent water soluble solvent and has a variety of medical uses. The annual worldwide production was about 21,812 Million U.S. gallons in 2012 [64]. Prices for ethanol vary widely, ranging from 2.3 $/gal for bulk vendors at the stock market to high prices for ultra-pure analytical ethanol.

The production of ethanol by microorganisms has been excessively reviewed before [65-69]. Therefore, only a small variety of the most prominent extremophilic, especially thermoacidophilic candidates is mentioned in Table 4.

Sorbitol

Sorbitol is of increasing industrial interest as a sweetener, humectant, texturizer and softener [70]. Further applications include pharmaceutical products, sorbose, ascorbic acid, propylene glycol [71], synthetic plasticizers [72] and alkyd resins [73], among others. According to a market report sorbitol demand was 1,699.7 kilo tons in 2011 and is expected to reach 2,148.9 kilo tons in 2018 [74]. Liquid sorbitol presently dominates the product market and accounted for 83.3 % of the overall demand in 2011 [74]. At present, it is produced chemically [75]. The mesophile bacterium *Zymomonas mobilis* is able to produce sorbitol and gluconic acid from fructose and glucose [76]. Furthermore sorbitol production has been described in *Candida boidinii* [77] and *Candida famata* [78], however no extremophile microorganism could be identified.

Xylitol

Xylitol is a sugar alcohol with an increasing global market potential. It has beneficial health properties and is used as a nutritive sweetener and food additive [79]. Its major use is for the prevention of dental caries, as xylitol prevents the growth of microorganisms responsible for tooth decay. Increasing commercial demand and scientific interest in xylitol has led to a demand of more than 125,000 tons per year for this product, with a value that is relatively high (4.5-5.5 US$ kg^{-1} for bulk purchase by pharma / chewing gum companies 20 US$ kg^{-1} in supermarkets) makes it an attractive proposition for commercialization [80]. Several microorganisms have been reported to produce considerable amounts of xylitol. An exhaustive screening, investigating xylitol production was performed on 128 yeasts, resulting in several promising candidates. Among them are several strains of *S.*

Product	Strain	$Y_{p/s}$ $Cmol_l (Cmol_s)^{-1}$	C-Source	References	Notes
Butanol	*Clostridium acetobutylicum*	0.55	glucose	[31]	wt/BM
		0.23	SO_2-ethaol-water hydrolyzed spruce	[131]	wt/BM
	Clostridium thermosaccharolyticum		starch, glucose oak saw dust hydrolysate (1% H_2SO_4)	[132] [133]	thermo-phile
	Hypothermus butylicus		starch	[52]	thermo-phile
	Pyrodictum abyssi		carbohydrates	[134,135]	thermo-phile
Ethanol	*Caldicellulosiruptor sp.*		lignocellulose	[136]	thermo-phile
	Caloramata boliviensis	0.52-0.6	sugarcane bargasse hydrolysate	[137,138]	thermo-phile
	Clostridium thermocellum	0.35	cellulose	[139,140]	thermo-phile
		0.32	filter paper	[141]	thermo-phile
		0.30	pretreated wood	[142]	thermo-phile
	Clostridium thermosaccharolyticum	0.39	oak saw dust hydrolysate (1% H_2SO_4)	[133]	thermo-phile
	Bacillus stearothermophilus	0.36	sucrose	[143]	thermo-phile
	Geobacillus stearothermophilus		glucose, xylose, arabinose, starch	[144,145]	thermo-phile
	Geobacillus thermoglucosidasius		glucose, xylose, cellulose, starch	[144,146]	thermo-phile
		0.5-0.55	glucose	[147]	ME
	Thermoanaerobacter AK5	0.42	wheat straw hydrolysate	[148]	wt isolate
	Thermoanaerobacter brockii	0.29	glucose	[149,150]	thermo-phile
	Thermoanaerobacter ethanolicus	0.63	glucose	[151,152]	in test tubes
		0.55	xylose	[153]	batch
		0.27	birch wood hydrolysate	[154]	batch
		0.29	beet molasses	[155]	in test tubes
	Thermoanaerobacter mathranii	0.49/0.59	glucose	[136,156,157]	wt/GMO
		0.55/0.56	xylose	[157-159]	wt/GMO
		0.6/0.64	mannitol	[157]	wt/GMO
		0.19	wet oxidized wheat straw	[160]	in test tubes
		0.38	wet oxidized wheat straw	[23]	in test tubes
	Thermoanaerobacter pseudoethanolicus (C. thermohydro-sulfuricum)	0.65	glucose	[161-164]	in test tubes thermo-phile
	Thermoanaerobacter saccharolyticum	0.61	xylose	[165]	ME
Sorbitol	*Zymomonas mobilis ZM31*	0.162	fructose, sucrose	[76]	BM
Xylitol	*Candida guilliermondii*	0.83	xylose	[166]	BM
	Debaryomyces hansenii	0.507	hydrolyzed brewery waste	[25]	osmo-tolerant
	Gluconobacter oxydans	0.98	D-arabitol +D-glucose + EtOH	[167]	BM

Table 4: Microorganisms producing alcohols.

rouxii, *S. acidifaciens*, *P. farinosa, Hansenula anomala, H. suaveolens, Endomycopsis chodatii, C. melibiosii and Cryptococcus neoformans* [81]. Furthermore the osmotolerant yeast *D. hansenii* is capable of xylitol production [82].

Biotechnological Production of High Value Products from Spent Liquors

As shown in previous chapters, various bioprocesses used for the generation of value added products have been identified. In this chapter we evaluate which microorganisms could ferment pulping spent liquors.

Microorganisms Potentially Capable of Producing Desirable Products from Black Liquor (BL)

The goal of this chapter is to suggest microorganisms capable of producing desirable products that are promising candidates to utilize sugars and organic acids in BL. The main monosaccharide units in the hemicellulose fraction of black liquor include the pentoses as arabinose, and xylose, with only some glucose and mannose, depending on the used wood. The total amount of hemicellulose is about 8% (w/w) of dry matter, as shown in Table 2, whereas 31% of the dry matter comprise of organic acids. Therefore, processes utilizing pentoses and organic acid substrates are of interest. Since xylose is the predominant C5-sugar in hardwood xylitol production from hardwood BL is an interesting option. Butanol and ethanol can be fermented from various monosaccharides. However, the utilization of organic acids is limited. Some microorganisms are able to ferment organic acids to gaseous products; some bacteria isolated from the human colon are able to ferment organic acids to butyrate when they are deprived of sugars [63]. In an example for production of organic acids from Black Liquor (BL) citric acid was produced by *C. tropicalis* at concentrations of up to 15% BL in medium. However, higher concentrations of BL inhibited growth and production [83-85]. Generally a low pH value is preferable for the

production of organic acids, making BL a suboptimal option [86]. Furthermore several halophilic microorganisms have been reported to utilize organic acids [41,70,87]. A group of halophilic microorganisms, belonging to genus *Marinilactibacillus*, is able to produce lactic acid at alkaline conditions [88].

As shown in Table 5 some processes show potential of generating high value compounds from BL, however, most of these processes are favored at neutral pH values. Only the generation of glycerol by the osmotolerant yeast *H. anomala* was reported to be independent of pH in a range from 5.4-8.6 [89]. Fermentation of xylose yielding xylitol is feasible at pH 8, also minimizing the necessary change in pH of substrate BL. Although these organisms are known to be halotolerant, the high levels of salts and inhibitors in the BL make dilution or pretreatment necessary. An interesting option would be the alkalihalophile lactic acid bacteria. Another interesting option would be halophilic bacteria and *Archaea*. Not only could they be able to withstand the high salt load in BL, but they have also been reported to utilize a wide range of organic acids (acetate [90,91], lactate [92], formate [93,94]) and even some phenols [95]. Furthermore they produce a wide spectrum of high value products, among others the compostable bioplastic polyhydroxybutyrate (PHB) [96,97] or the osmolytes ectoine [98,99] and betaine [98]. Although so far no bioprocesses with *Halophiles* utilizing black liquor have been published, several experiments on wood hydrolysate and common inhibiting substances performed in our group could show that they are able to utilize SSL at commonly reported concentrations of 5-15% [w/w] dry matter [85]. The results of these experiments were published recently [100].

Microorganisms Producing Desirable Products from SSL

The goal of this chapter is to suggest microorganisms capable of producing desirable products that are promising candidates to adapt to growth on SSL. The main monosaccharide units in the polysaccharide fraction of spent sulfite liquor include mannose, glucose and xylose, with only some arabinose and galactose, depending on the wood used. As described before, butanol and ethanol, as well as organic acids can be fermented from various monosaccharides. In addition, xylitol production is an interesting use for process streams with high amounts of xylose. The relatively high abundance of glucose would also enable the production of sorbitol and itaconic acid. Furthermore, suitable strains should be able to thrive and produce at elevated temperatures and low pH values. Fermentation at low pH values can increase ethanol production, as for example ATP from ethanol production is needed for the plasma membrane ATPase in yeast, which pumps protons out of the cell to neutralize the inflow of weak acids [86]. Experiments on *C. saccharoperbutylacetonicum* have shown that presence of acetic acid shifts ABE production from butanol to acetone, whereas addition of butyric acid favors butanol production [101]. Furthermore it has been shown, that the maximal butanol production on a glucose limited culture of *C. acetobutylicum* DSM 1731 was achieved at a pH value around pH 4.3 and that a pH value below 4.7 was necessary to show any butanol production [83].

As shown in Table 6 several microorganisms are promising candidates for the production of high value products from spent sulfite liquor (SSL). Especially among the ethanol producing microorganisms several extremophiles can be found. The possibility of process-integrated product purification, when producing ethanol at elevated temperatures, is a very interesting option and explains why these microorganisms are of high scientific interest. Most of the candidates are mesophiles, tolerant to pH values as low as pH 4. Among the yeasts several osmotolerant species are found. This is very important, due to the high salt load of SSL. The osmotolerant yeast *D. hansenii* is an ideal candidate for xylitol production at various pH values. It can produce xylitol and ethanol at pH 2-8, with an optimum for glycerol production at pH 4.5-5.5 and an optimum for ethanol production at pH 6.5-8 [102]. For SSL with pH values lower than 4 a shift in pH will be necessary for most biotechnological processes. Although this can easily be implemented, it can lead to elevated salt levels, which can in return inhibit microbial growth. Hence, in the majority of the mentioned cases pretreatment cannot be avoided.

Work from our Group

Putting our concept to the test we investigated two types of extremophiles -*Thermoacidophiles* and *Halophiles* – for the production of ethanol and polyhydroxyalkanoates, respectively. Their pH-range, temperature range and substrate spectrum (glucose, mannose, galactose, xylose and arabinose) were investigated. They could ferment all sugars at 65°C and a pH of 5 in the area of the *Thermophiles* and at high salt concentrations at 30°C and neutral pH in the case of the *Halophiles* and were able to produce the targeted products on dilutions of industrial SSL, containing 10-12 g of hydrolyzed sugar (2-6% referring to solid content of the SSL) The detailed results for the thermophilic ethanol production are in preparation, whereas the results for halophilic PHB production were recently published [100].

Conclusion and Outlook

Research on the sustainable production of bio- fuels, bio- chemicals and bio- materials is always accompanied by the discussion of fuel versus food, and hence also by the search for ethically justifiable raw materials. Carbon rich waste streams have, therefore, been an interesting and cheap option for decades. However, decades of research have shown, that industrial waste streams are always challenging substrates for bioprocesses due to their extreme pH and temperature levels, as well as high abundance of substances inhibiting microbial growth. Extreme conditions call for extreme microorganisms. This contribution aims to show the potential of extremophilic bioprocesses for the generation of valuable products from industrial waste streams on the example of the pulping waste streams spent sulfite liquor (SSL) and black liquor (BL). One part of this contribution contains an overview of products, which can already be biotechnologically produced by extremophilic organisms. Additionally it shows how well they perform in comparison to benchmark biotechnological processes, often performed by their mesophile cousins, thereby presenting the general potential of extremophilic generation of various bio-chemicals.

Furthermore, the contribution elucidates the factors to be considered for bioprocesses on industrial waste streams. While some factors are a go or no-go criterion, like the general abundance of suitable building blocks for the desired bioprocess, others affect the economy of the process and hence the price of the resulting product. Changes in the medium composition, which often occur when using a natural resource like lignocellulose, can lead to changes in productivity. For some bioprocesses certain media components will have to be added, for others pH and temperature have to be controlled and for some even elaborate pretreatment steps for the removal of inhibiting substances have to be performed. All these factors have a significant impact on the economic feasibility of a bioprocess.

Proposed bioprocesses are summed up in the results shown in Tables 5 and 6. Thermophilic microorganisms have a high potential to

Product	Strain	$Y_{p/s}$ $Cmol_i$ $(Cmol_s)^{-1}$	Fermentation conditions	C-Source	References
Butanol	*Clostridium thermosaccharo-lyticum*		58-69°C, pH neutral	starch, glucose	[132,168]
Lactic acid	*Alkalibacterium subtropicum*		NaCl 0-17% w/v, pH 7.5-9.5	various carbohydrates, organic acids	[110]
	Marinilactibacillus psychrotolerans	0.99	NaCl 0-20.5% w/v, pH 6-10	glucose	[116]
Xylitol	*Debaryomyces hansenii*	0.57	24°C, pH 8	xylose	[102]
PHB	*Haloferax mediderranei*	0.33 g g^{-1} 0.9 mol mol^{-1}	37°C, pH 7-8	starch glycerol	[97,125,126]
	Halomonas boliviensis	0.1 g g^{-1}	NaCl 0-25% w/v, pH 6-11,	hydrolyzed wheat bran	[99,128]
	Halomonas elongata		NaCl 0-20% w/v, pH 5-10;	glucose, mannose, xylose, …	[99,129]

Table 5: Microorganisms suitable to produce desirable products from BL.

Product	Strain	$Y_{p/s}$ $Cmol_i$ $(Cmol_s)^{-1}$	Fermentation conditions	C-Source	References
Ethanol	*Clostridium thermocellum*	0.347	60°C, pH 5-7	cellulose	[164]
		0.35		pretreated wood	[142]
	Clostridium thermosaccharolyticum	0.26	58-69°C, pH 4.5	oak saw dust hydrolysate (1% H_2SO_4)	[133]
	Thermoanaerobacter saccharolyticum	0.616	50°C, pH 5,2-5,4	xylose	[165]
	Thermoanaerobacter mathranii	0.49 0.55	70°C, pH 4.7-8.8	glucose xylose	[156,157]
		0.38		wet oxidized wheat straw	[23]
Butanol	*Clostridium saccharoper-butylacetonicum*	0.51	30°C, pH 5.5	glucose	[101]
	Clostridium thermosaccharolyticum		58-69°C, pH 4.5	starch, glucose oak saw dust hydrolysate (1% H_2SO_4)	[132] [133]
	Clostridium acetobutylicum	0.55	37°C, pH 4.3-5	glucose	[31,83]
		0.23		SO_2-ethaol-water hydrolyzed spruce	[131]
Xylitol	*Debaryomyces hansenii*	0.507-0.81	24°C, pH 5.5	hydrolyzed brewery waste	[25,102]
Itaconic acid	*A. terreus*	0.26	pH 2-3.5-	glucose	[54]
	U. maydis	0.196	30°C, pH 4,5-6	glucose	[169]
Lactic acid	*Bacillus sp. XZL4*	0.98	50°C, 5-6	corn stover hydrolysate	[115]
Succinic acid	*Clostridium thermosuccinogenes*	0.43	60°C, pH neutral	inulin	[119,120]

Table 6: Microorganisms suitable to produce desirable products from SSL.

utilize SSL for the production of ethanol, whereas slightly acidophile microorganisms could produce butanol, itaconic acid and xylitol from SSL at acidic pH values. Itaconic acid is mainly produced by *A. terreus* industrially. This process takes place at low pH and could utilize the glucose in SSL. However IA production is sensitive to inhibitors in lignocellulose hydrolysate [103]. Therefore, a pretreatment with combined anion and cation exchangers is proposed [104]. A productivity of 2.5 g l^{-1} h^{-1} is desired, for the process to be economically competitive [1]. Furthermore *T. saccharolyticum* and *C. acetobutylicum* are able to ferment sugars from SSL to ethanol and butanol respectively. *T. saccharolyticum* is capable of fermenting C5 as well as C6 sugars, which makes this microorganism an attractive opportunity.

The utilization of BL is favored for microorganisms being able to ferment organic acids. Most of these microorganisms transform organic acids to gas. However, there is an additional potential in the utilization of organic acids by mesophile and alkalihalophile microorganisms, leading to innovative products that were not the scope of this publication. *Halomonas elongata* is promising for the production of xylitol due to its pH-range up to pH 10 and its ability to ferment hemicelluloses.

While a decision which factors are a no-go criterion, and what steps could potentially be performed is a decision that has to be taken for each bioprocess, this publication aims to give the reader an overview on what could be potentially possible and what would be the steps that have to be considered for each individual microorganism, therefore, providing a toolset for the decision making process.

From our own experiments (published and in preparation)we could see that the halophilic production of PHAs and the thermophilic production of EtOH show some promise, but the inhibition phenomena have to be overcome and media have to be designed in way that guarantees maximum compatibility with the pulping processes they are intended for.

However, as several of the proposed strains have been successfully

cultivated on various lignocellulosic hydrolysates it could be shown, that extremophiles have some potential for bioprocesses on extreme substrates.

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