Part 3: Detoxification of Spent Sulphite Liquor to Remove Growth Inhibitors in Microbial Transformations

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Abstract

Within the framework of this work, the spent sulphite liquor (SSL) was detoxified through different detoxification technologies, which help selectively to remove toxic substances and thereby improve fermentation. To determine the most effective method for SSL detoxification, the growth of *Clostridium saccharoperbutylacetonicum* was observed after the addition of different amounts of detoxified SSL. The comparison of these techniques shows that the detoxification with hydrogen peroxide and peroxidase as a catalyst is the most effective method for the detoxification of the SSL. The second-best methods are detoxifications with activated carbon and lignin, respectively. The effect of ion exchangers is less significant, whereby the detoxification with anion exchangers yields better results than with cation exchangers. The comparison of the alkaline pretreatments shows that treatment with calcium hydroxide is more effective than treatment with ammonium hydroxide and magnesium hydroxide.

Keywords: *Sulphite spent liquor valorisation, fermentation, growth inhibitors, detoxification of hydrolysates*

Introduction

During sulphite pulping large amounts of spent sulphite liquor are produced containing lignosulphonates, extractives hemicelluloses, monosaccharides and various degradation products. The monosaccharides from the SSL are valuable carbohydrate sources for various biotechnological processes [1-5].

In previous investigations, we screened the effect of organic acids, phenols, furan derivatives and alcohols contained in the SSL on several microorganisms. Certain phenol derivatives show pronounced inhibiting effects on those microorganisms during fermentation. In this work, we screened for the best detoxification method using a microtiter plate setup. Our working hypothesis was that those techniques that remove phenolic compounds should be most successful. *Clostridium saccharoperbutylacetonicum* was used for the tests. In earlier works, this strain proved to be more susceptible to inhibition than the other two strains of the investigations *Thermoanaerobacter mathranii* and *Halomonas halophila* [6].

In the literature, various detoxification methods for hydrolysates of lignocellulosic materials are described. Depending on the starting material and the type of hydrolysis, biological, chemical, physical, or combined processes are used (Table 1).

***Table 1:** Detoxification methods in ethanol production*

Detoxification	Process lye	Conditions	Effect	Reference
Peroxidase	Model inhibitors in model medium	0.01 µM of enzyme	p-Coumaric acid, ferulic acid, vanillic acid, and vanillin, the removal efficiency ≈100%	[7]
Laccase	Willow, impregnated with SO_2, steam-treated	1mM laccase, pH 5.3, 30°C, 12h	Removal of monoaromatic phenols, improved fermentation	[8]
Coniochaeta ligniaria	Model inhibitors in model media, corn stover dilute acid hydrolysate	Selected on feedstock phenols and furfural	*Coniochaeta ligniaria* NRRL30616 metabolises furfural, HMF*, aromatic, aliphatic acids and aldehydes	[9,10]
Trichoderma reesei	Willow, impregnated with SO2, steam-treated	Shake flasks, 30°C, 350rpm	*Trichoderma reesei* digests pentoses, produces cellulolytic enzymes and detoxifies the hydrolysate	[11]
Ureibacillus thermosphaericus	Waste, wood hydrolysate	Incubated at 50°C, 24h	Removes furfural, HMF; increases fermentability by *S. cerevisiae*	[12]
Activated carbon	SSL from acid hydrolysis pre-treated *Eucalyptus globulus* wood chips,	a) 2.5g AC/l hydrolysate, 1 day, at RT; b) 2%, pH 7, 1h	a) Improved fermentability; b) low benefit	[13,14]
Anion exchange chromatography	Norway spruce, impregnated with sulfuric acid, steam-treated	pH 10	Removal of phenols, furan derivatives and organic acids	[15,16]
Lignin	Spruce dilute acid hydrolysate	Treated with lignin residue	Removal of 53% phenolic compounds, 68% furan derivatives, improved fermentability	[17]
Alkaline treatment	Dilute acid hydrolysate	pH 9 /80°C-pH 12 /30°C, NaOH for 3h	Improved fermentability	[14,15,18-20]
Reducing agents	Spruce/sugar cane bagasse, thermochemical treatment	dithionite and sulphite 5.0-17.5 mM	Improved fermentability (SHF, SSF)	[21]
Ethyl acetate extraction of wood	Steam exploded (SE) poplar wood	H_2O /ethyl acetate (~1/5) added to the wet SE-poplar wood	Ineffective	[18]
Ethyl acetate extraction of hydrolysate	Steam exploded (SE) aspen wood	SE-hydrolysate four times extracted with ethyl acetate	Increased fermentability	[22]
Trialkylamine extraction	Corn stover prehydrolysate	30% trialkyl amine, 50% n-octanol, 20% kerosene	Removal of 73.3% acetic acid, 45.7% HMF and 100% furfural, improved fermentability	[23]
Supercritical CO_2 extraction	Spruce hydrolysate	CO_2 pressure 200 bar, density 0,84 g/ml, 40°C	Increased fermentability as well as lower concentrations of inhibitors such as phenolics and furan derivatives	[24]
Evaporation	Spruce dilute acid hydrolysate	a) Evaporation 10%; b) evaporation 90%	a) Ineffective; b) improved fermentability	[15]
Heat treatment	Yellow poplar dilute acid + stream treatment	Heat treatment (75°C, 95°C 10 min, 140°C 2,5-3h) + ion exchange	Reduction of acetic acid, phenols	[20]

*5-hydroxymethylfurfural

In biological detoxification techniques, hydrolysates are treated with specific enzymes or whole cells. Enzymatic detoxification is described as one of the most effective methods for removing phenols from lignocellulosic substrates. Mostly laccases and peroxidases are used for this purpose. A distinct advantage of enzyme use is that carbohydrates are not consumed. Cho *et al*., for example, investigated six phenolic model substances that inhibit the production of butanol. Complete removal of these phenols was achieved with a peroxidase from *Coprinus cinereus*. The treatment significantly increased the butanol yields [7]. In the work of Jönsson et al., the effects of a laccase, a phenoloxidase and lignin peroxidase from *Trametes versicolor* were studied on real substrate namely the hydrolysate from willow pretreated with SO_2 and steam. The treatment with laccase and lignin peroxidase resulted in the removal of phenolic compounds and improved ethanol fermentability of the hydrolysate [8].

Some microorganisms can metabolise lignin as well as furan derivatives and acetic acid. Lignocellulosic substrates detoxified with those microorganisms can be hydrolysed more easily into fermentable sugars in further stages of the pretreatment process. This results in reduced demand for chemicals and process heat as well as shorter hydrolysis times. Treatment with the fungus *Coniochaeta ligniaria* NRRL30616, which can metabolize furan derivatives, aromatic and aliphatic acids and aldehydes, resulted in an improvement in the fermentation efficiency of corn stover hydrolysate [9,10]. To improve the efficiency of ethanol production from hydrolysate obtained after steam-pretreatment of willow, the fungus *Trichoderma reesei* is used. This fungus utilizes pentoses, simultaneously removes water-soluble inhibitors and, as an additional benefit produces cellulolytic enzymes [11]. Another example of a microorganism used for detoxification is the thermophilic bacterium, *Ureibacillus thermosphaericus*, which degrades toxic compounds in the hydrolysate of waste house wood. It does not metabolize sugars and improves ethanol production by *Saccharomyces cerevisiae* or the ethanologenic recombinant *Escherichia coli* KO11 [12].

The chemical and physical methods for the detoxification of the hydrolysates lignocellulosic materials are manifold. They can roughly be summarised in the following categories:

a) Interaction with a carrier material, such as adsorption to activated carbon or interaction with an ion-exchange resin
b) Extraction with (organic) solvents
c) Overliming
d) Chemical modification
e) Other

Using **activated carbon**, Parajo *et al*. reduced the concentrations of phenols and acetic acid in acid hydrolysis pre-treated *Eucalyptus globulus* wood [13]. Whereas in the work of Helle *et al*., this method was not the most effective [14]. Tesfaw et al. also saw some effect when using activated carbon alone, but in combination with overliming it was more effective [25]

A low-cost and effective detoxification method is the solid-phase extraction of hydrolysates with **lignin**, which is produced in large quantities as a by-product during pulp production. Its hydrophobic properties make the separation of aromatic and furan derivatives possible [17].

The use of **anion exchange resins** is an effective method for the removal of phenols, organic acids and furan derivatives. Unfortunately, it causes an undesirable sugar loss of up to 75 %, which can be reduced to 1 % by the addition of sodium sulphate [15-16]. The combination of heat treatment and treatment with ion exchangers is well suited for the separation of acetic acid and phenolic compounds from lignocellulosic hydrolysate [20].

The **extraction** with ethyl acetate was used for the removal of low molecular weight phenolic substances. Cantarella *et al*., extracted sulphite and steam-treated poplar wood with water and ethyl acetate. Despite the removal of a large number of phenols the desired improvement in fermentability was not achieved [18]. In contrast, the fermentability of steam-pretreated aspen wood hemicellulose could be improved by extraction with ethyl acetate. Not only phenolic compounds but also other inhibitors (except for acetic acid) were removed in this process [22]. The difference in those two processes is that Cantarella *et al*. applied the treatment to solid biomass, whereas Wilson *et al*. treated the hydrolysates. Although the latter treatment was very efficient, using ethyl acetate on large scale requires an explosion-proof installation and thus high capital expenditure. Therefore, ethyl acetate was ruled out from this investigation.

Zhu *et al*. used a mix of trialkyl amines, n-octanol and kerosene as extracting agents for the treatment of the corn stover prehydrolysate. This method removed acetic acid, HMF, and furfural, which significantly improved the fermentation [23]. However, those mol-

ecules are not our detoxification targets and the recovery of the solvent mixture does not seem feasible in large-scale production. A promising solvent represents supercritical CO_2, which is perfectly suited to the separation of phenols and furan derivatives. The advantage of the use of supercritical CO_2 is that no sugar loss and pH value changes take place [24].

Overliming or alkaline treatment is also an important detoxification method. Generally, it describes the alkaline treatment of acidic hydrolysates which promotes precipitation of low molecular weight components, for example, phenols or furan derivatives [18]. Most commonly used are $Ca(OH)_2$, NaOH or NH_4OH to achieve a basic pH (about 10), followed by readjustment to a neutral pH usually using H_2SO_4. [14,15,19]. This procedure largely enhances the fermentability although the rationale behind this effect is yet to be understood [20]. $Mg(OH)_2$ was included in the tests since the Mg^{2+}ion is a common counterion in nowadays sulphite processes. This way the introduction of another ion species complicating the recovery process would be avoided.

Also **reducing agents** were used as pretreatment agents. The number of furan derivatives in lignocellulose hydrolysates was reduced with dithionite and sulphite, which improved the fermentability of these hydrolysates. The detoxification can be carried out directly in a fermenter at the same reaction conditions (pH value and temperature) which prevail during the fermentation [21]. Although bisulfite and dithionite are used as bleaching agents in mechanical pulping they are not used in chemical pulping. Moreover, we decided against using potentially hazardous chemicals for detoxification purposes.

Another example of detoxification is evaporation. Evaporation helps to remove volatile components, such as acetic acid, formic acid, and furfural [15]. However, this method is not suited for the removal of the inhibiting phenolic substances identified in earlier studies [6]. Also, membrane separation processes were not included in our study because of the expected loss of significant amounts of the monomeric sugars while trying to remove the inhibitor molecules that are in the same size range.

Among the above-listed processes, Fernandez *et al.* consider adsorption an advantageous technique in terms of cost, environmental impact and detoxification performances [26]. Zhang *et al.* concluded that sequential treatment of overliming and active carbon of prehydrolysates was necessary to vastly improve the fermentation performance of *C. saccharobutylicum* [27]. Tesfaw *et al.* came to similar conclusions [25].

While the use of adsorbents to detoxify lignocellulosic hydrolysates was the object of a wide number of studies, the specific implementation of adsorption to detoxify SSL was only studied by a limited number of authors. Xavier *et al.* developed a two-step adsorption process on ion-exchange resins [28] for subsequent ethanol fermentation with *Pichia stipitis*. SSL was initially treated with a cation-exchange resin to remove Mg^{2+} and other cations from the pulping process. In the second step, organic acids, polyphenols, and lignosulphonates were separated from carbohydrates by employing an anion-exchange resin. This process led to a dilute, almost transparent solution containing mainly neutral monomeric sugars. Takahashi *et al.* studied the adsorptive removal of inhibitors from a model SSL in the production of ethanol with *Saccharomyces cerevisiae*. They compared the effectiveness of activated carbon, precipitated calcium carbonate (PCC) or XAD-4 resin. Activated carbon proved to be the most effective adsorbent by removing 100 % furfural, 48 % acetic acid, and 70 % lignosulfonate from SSL [29].

Materials and Methods

Detoxification

For all applied detoxification methods, the SSL solution was sterile-filtered before the fermentation.

Enzymatic detoxification

345.000 u/l horseradish peroxidase was added to 10 ml SSL. 1.3 µl H_2O_2 (8 mmol) was diluted with 98.7 µl deionised water. The H_2O_2 solution was added dropwise to the SSL. The mixture was stirred for 1 h at 100 rpm at room temperature.

Detoxification with lignin

Lignin (Indulin AT) was washed with deionised water until the wash liquor was colourless and had a neutral pH. 2, 5 and 10 wt. % Indulin AT were added to 10 ml SSL. The mixtures were stirred for 1 h at 100 rpm at room temperature. Thereafter, the lignin was removed by filtration.

Detoxification with activated carbon

2, 5 and 10 wt. % activated carbon was added to 10 ml SSL. The mixtures were stirred for 1 h at 100 rpm at room temperature. Thereafter, the activated carbon was removed by filtration.

Detoxification with cation exchange

2 g cation exchange (Lewatit, 1368 Ca/320, Lanxess) was washed with deionised water and added to 10 ml SSL. The mixture was stirred for 1 h at 100 rpm at room temperature. Thereafter, the cation exchange resin was filtered off.

Detoxification with anion exchange

2 g anion exchange (AG 1-X8, 20-50 mesh, chloride form, Bio-Rad) was washed with deionised water and added to 10 ml SSL. The mixture was stirred for 1 h at 100 rpm at room temperature. Thereafter, the anion exchange resin was filtered off.

Detoxification with $Ca(OH)_2$

$Ca(OH)_2$ was added to 10 ml SSL and the pH was adjusted to 11. The mixture was stirred for 3 h at 100 rpm at 60 °C. Thereafter, the precipitate was filtered off.

Detoxification with $Mg(OH)_2$

$Mg(OH)_2$ was added to 10 ml SSL and the pH was adjusted to 8.5. The mixture was stirred for 3 h at 100 rpm at 60 °C. Thereafter, the precipitate was filtered off.

Detoxification with NH_4OH

NH_4OH was added to 10 ml SSL and the pH was adjusted to 8.5. The mixture was stirred for 3 h at 100 rpm at 60 °C. Thereafter, the precipitate was filtered off.

Growth experiments with *Clostridium saccharoperbutylacetonicum* (DSMZ 14923)

Medium preparation

For the cultivation of *C. saccharoperbutylacetonicum* (DSMZ 14923) was used a medium, which contains 0.3 g/l magnesium sulfate heptahydrate, 2 g/l yeast extract, 6 g/l peptone from casein, 3 g/l ammonium acetate, 1.5 g/l potassium dihydrogen phosphate, 1.2 g/l dipotassium hydrogen phosphate, 0.01 mg/l Iron(II) sulfate heptahydrate, 0.5 g/l L-cysteine. The pH value of the solution was adjusted to 7. The medium was autoclaved for 10 min at 120 °C. Glucose (20 g/l) was used as a carbon source. The concentrated sterile glucose solution was added to the medium before the inoculation of microorganisms.

Cultivation

The work was carried out in a glovebox under forming gas atmosphere. 1 ml cryo stock (-80 °C) of cells in glycerol was thawed at room temperature and then added to 9 ml medium. The cells were incubated with agitation for 24 h at 30 °C. To preserve vital cells culture was re-inoculated in a medium once again and incubated for 18 h. These cells were used for the microtiter plate experiments.

Microtiter plate experiments

The effect of various detoxification methods on the growth of *Clostridium saccharoperbutylacetonicum* was tested in a microtiter plate under forming gas atmosphere. For the determination of detoxification effects on the growth of *C. saccharoperbutylacetonicum*, the ΔOD (optical density) of microorganisms in a medium with the sugar mixture was compared with the ΔOD of microorganisms in the non-detoxified as well as detoxified SSL. The sugar mixture in the medium corresponds to the composition of the SSL (1.4 mg/l arabinose, 2.9 mg/l galactose, 6.8mg/l glucose, 20.1 mg/l mannose and 8.0 mg/l xylose). The pH of the non-detoxified, as well as detoxified SSL, was set to 7. A dilution range of the SSL from 1:2 to 1:30 (Figure 1) was prepared. For the preparation of the dilutions, the medium with a sugar content corresponding to the SSL sugar concentration was used. 50 µl medium with sugar mixture or SSL were pipetted into each well. Thereafter, 30 µl cultures were added to the solutions. The microtiter plate was sealed with a transparent oxygen-impermeable adhesive film. The cells were incubated for 10 h at 30 °C. The OD measurements were carried out at regular intervals in triplicate.

Results and discussion

Spent sulphite liquor from a dissolving pulp mill was subjected to the following detoxification treatments:

a) Enzymatic: horseradish peroxidase (HRP)/ H_2O_2 because HRP is significantly cheaper than laccase
b) Adsorption on 2%, 5% and 10% (w/v) Indulin AT
c) Adsorption on 2%, 5% and 10% (w/v) activated carbon (AC)
d) Cation and anion exchange, respectively
e) Alkaline treatment with $Ca(OH)_2$, $Mg(OH)_2$ and NH_4OH; $Mg(OH)_2$ is particularly interesting for SSLs derived from Mg-sulphite pulping because it does not introduce new chemicals into the SSL.

The detoxified SSLs were then tested in a microtitre plate setup with *C. saccharoperbutylacetonicum* as the reference microorganism. The growth of microorganisms was investigated after the addition of different amounts of SSLs, which were detoxified by the

above-mentioned techniques. Growth was monitored by OD measurements and expressed as % growth compared to the inhibitor-free control (=100%). Because of the experimental setup, the highest amount of SSL possible is 63%. The remaining volume is taken up by the inoculum.

Figure 1 summarises the results of all types of treatments. Changing the concentration of the SSL is not very effective. Lowering the concentration to 1/30 only triples the growth of the microorganisms. It has practical disadvantages as well. Dilution means additional water use in the pulp mill, which is nowadays undesired. All pulp mills try to significantly reduce the amount of process water they use. In addition, a huge energetic effort is required to evaporate the added water for further use of the sugar-free SSL. Last but not least an external carbohydrate source is required to make up for lowering the monosaccharide concentration as well. The figure also shows that the treatment with peroxidase/H_2O_2 is the most effective, followed by activated carbon. Lignin seems to have somewhat better effectiveness than ion exchange resin and may also be the more economical solution of the two. Overliming has a very limited positive effect on bacterial growth.

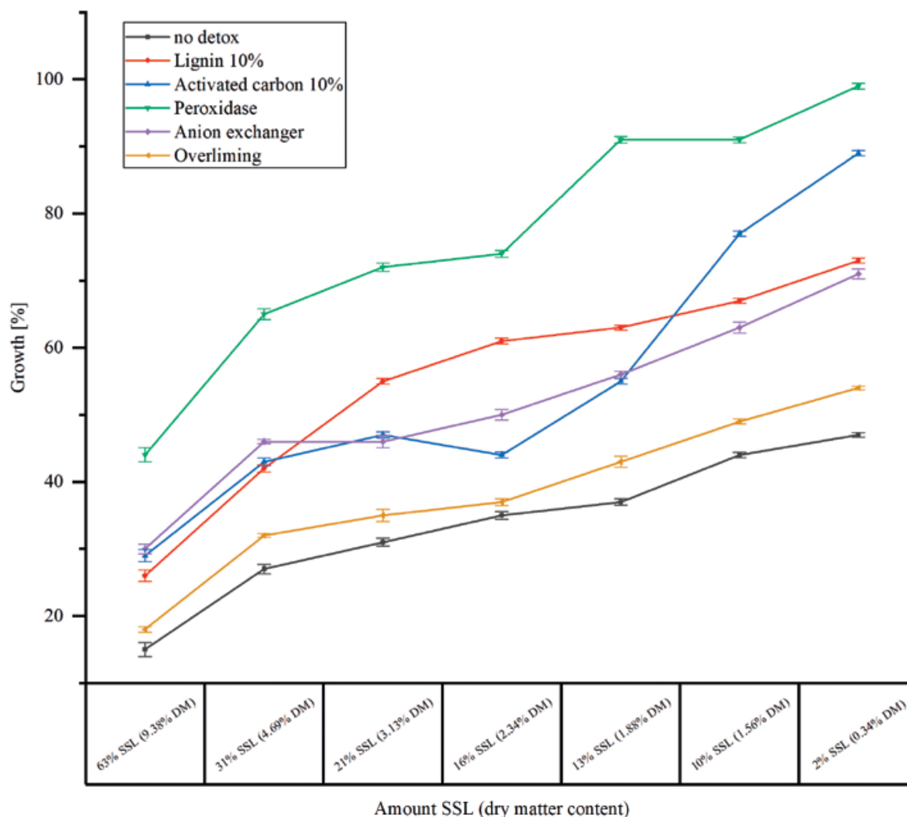

***Figure 1:** Summary of the effects of detoxification on microbial growth with lignin, activated carbon, horseradish peroxidase/H_2O_2, ion exchange resin and overliming in combination with different amounts of SSL.*

Figure 2 summarises the results of all three bases used for the alkaline treatment. Overliming with $Ca(OH)_2$ results in slightly higher microbial growth than treatment with NH_4OH. $Mg(OH)_2$ had no effect at all, which is a little disappointing. From a practical point of view in a magnesium sulphite process only an alkaline treatment with $Mg(OH)_2$ could be easily implemented.

Figure 3 depicts the detoxification effectiveness of ion exchange resins in detail. The anion exchanger performed a little better than the cation exchanger. The detoxification with lignin (Figure 4) is somewhere in the range of the anion exchange, even slightly better. Data on the influence of the amount of lignin applied are inconclusive.

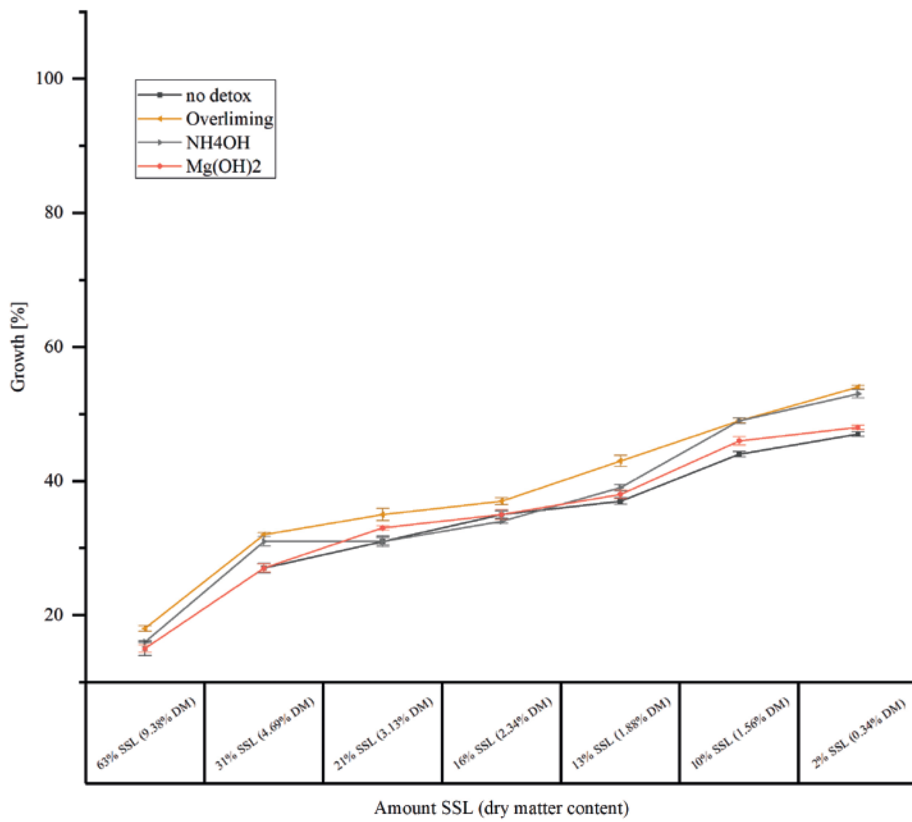

Figure 2: *Alkaline treatment with $Ca(OH)_2$ (overliming), NH_4OH and $Mg(OH)_2$.*

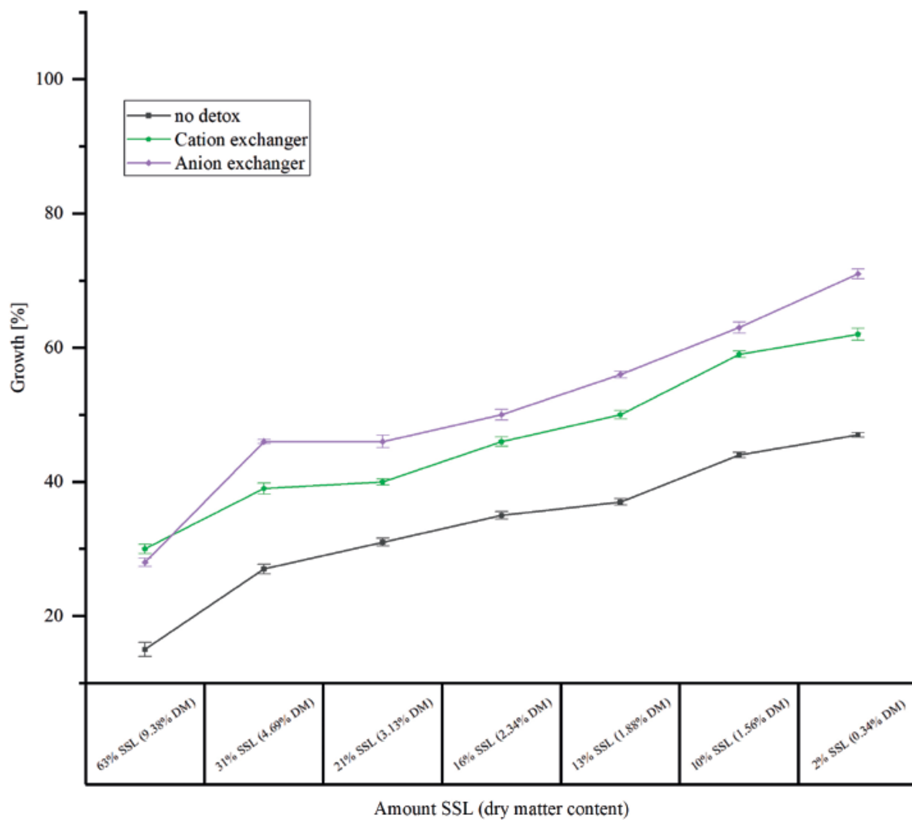

Figure 3: *Treatment of SSL with anion and cation exchangers.*

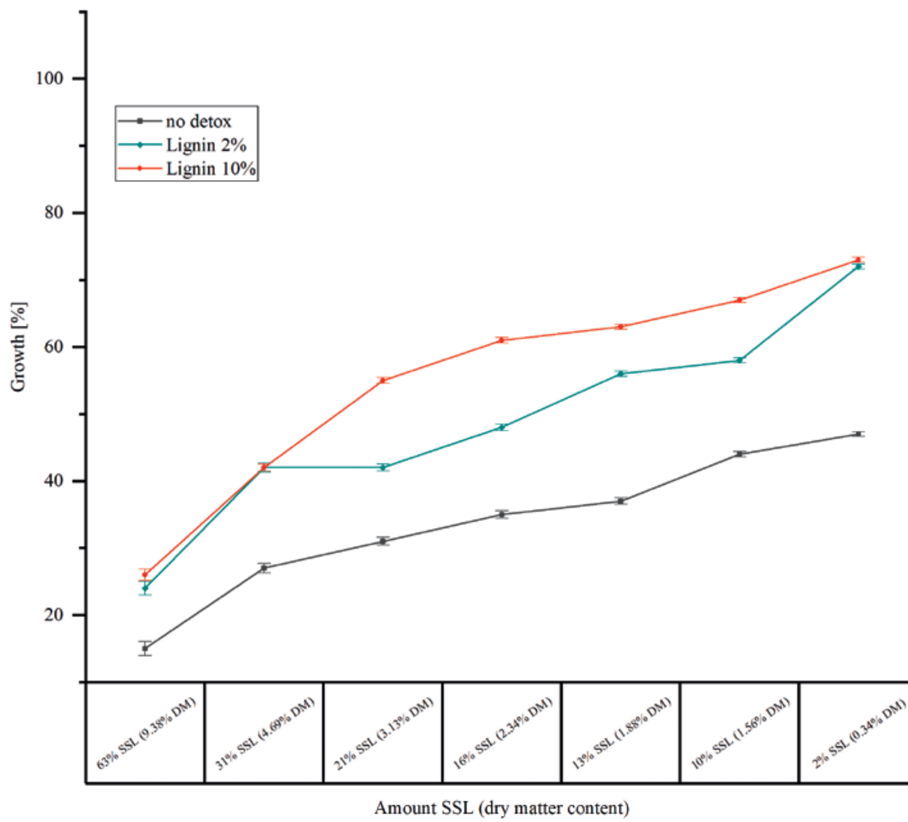

Figure 4: *Treatment of SSL with different amounts of (solid) lignin.*

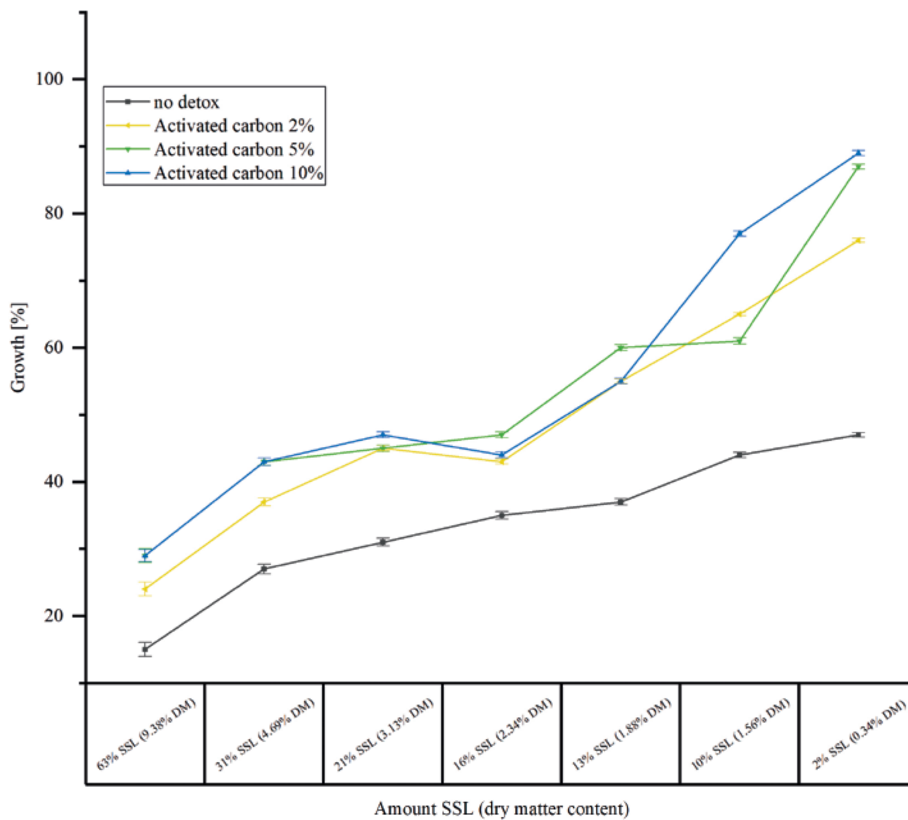

Figure 5: *Treatment of SSL with different amounts of activated carbon.*

The treatment with 10% activated carbon shows the second-best detoxification result. The steep rise at the end of the 5% and 10% curves in Figure 5 allow us to speculate that there is room for improvement in the ratio of activated carbon to dry matter content.

The results are partly in agreement with the trends in the literature and partly not, which is not surprising. Lignocellulose sources, digestion processes and the respective microorganisms vary widely in the literature and, thus, limit comparability.

Conclusions

The most effective detoxification method for *C. saccharoperbutylacetonicum* fermentations of SSL is employing horseradish peroxidase/H_2O_2. The second-best method is the adsorption onto activated carbon closely followed by adsorption onto lignin. Ion-exchange resins show some effect. Alkaline treatment has a very limited effect in the case of $Ca(OH)_2$ and no effect at all for $Mg(OH)_2$ as a base. The results corroborate our working hypothesis that techniques that remove phenolic compounds are the most effective

The next steps are to elaborate the economically and technologically most rewarding detoxification method or to grow microorganisms better adapted to the SSL.

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