0273-2289/02/98-100/0765/$12.25

Thermophilic Fermentation of Hydrolysates

The Effect of Inhibitors on Growth of Thermophilic Bacteria

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Abstract

Lignocellulosic biomass has great potential as a cheap feedstock in biological processes to produce biofuels or chemicals; however, dilute acid pretreatment at high temperatures produces undesirable compounds. Toxicity tests were done with inhibitors in standard media, to predict the growth-limiting effects on thermophilic strains. The 22 inhibitors included furfural, levulinic acid, acetic acid, and cinnamaldehyde. Neutralizing reagents and additional treatment steps have been tested.

Index Entries: Softwood hydrolysates; inhibitors; thermophiles.

Introduction

Softwood lignocellulose is an abundant, renewable resource that has been proposed as feedstock for biofuels and plastics production *(1, 2)*. The hemicellulose fraction of lignocellulose is easily hydrolyzed and extracted by a mild acid-treatment yielding mainly D-xylose and varying amounts of D-glucose, D-mannose, D-galactose, L-arabinose and L-rhamnose *(3)*. However, compounds toxic to microorganisms are also produced during acid hydrolysis which must be mitigated or removed *(1,2,4)*.

Although several inhibition studies have already been conducted *(5–9)*, relatively little has been published concerning the inhibition of thermophilic bacteria by toxic compounds. The advantages of the thermophilic process are the fermentation of a wide range of substrates including cellulose and starch, less biomass production with high product yields, rapid fermentation due to high metabolic activity, little risk of contamination by

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undesirable microbes, and the possibility of the continuous recovery of products directly from the bioreactor during fermentation *(10)*.

This paper focuses on the determination of the toxicity of several inhibitors found in hydrolysates that are able to reduce growth of thermophiles.

Materials and Methods

Microorganism

The experiments were carried out with a single thermophilic *Bacillus* strain (IFA 119) that was isolated from a compost heap as described recently *(11)*.

Media

Standard cultivation medium for bacterial growth toxicity tests consists of 9 g/L nutrient broth (consisting of 5.0 g/L of pepton and 3.0 g/L of meat extract; Merck) enriched with 4 g/L of yeast extract (spray dried, autolyzed; Sigma). Inhibitor-test solutions were made by analytical grade chemicals obtained from Sigma and Fluka.

Analytics

Growth of the strain was determined by an optical density reader (SLT Grödig/Salzburg) at 0 and 24 h incubation.

Bacterial Growth Toxicity Tests

Twenty-one inhibitors were tested in microtiter plates in 200 µL scale. The concentration range used for each compound was determined by what was likely to be found in the hemicellulose hydrolysate. For each compound, a stock solution was prepared and diluted to obtain the concentration ranges as described in Table 1. The solutions were filter sterilized (0.22 µm, Cameo 25NS, Osmonics).

For each substrate, a negative and positive control was tested as described in Table 2. For the bacterial suspension, a volume of 20 mL of the inoculum cell suspension was centrifuged to pellet the cells and the supernatant was discarded. The pellet was resuspended in 5 mL of respective medium and the cell concentration adjusted to 1×10^5 cells/mL using a "Thoma Kammer" (0.01 mm depth). Incubation temperature was 60°C, the incubation time was 24 h.

Results

Bacterial Growth Toxicity Tests

The growth of bacteria strains was expressed as a percentage of the positive control with no sugar supplements. The results of the inhibition studies are listed in Table 3.

Table 1
Concentration of the Inhibitor Test Solutions[a]

Concentration-steps tested (g/L)	1	2	3	4	5	6
Compound	Concentration (g/L)					
Furfural	0.1	0.2	0.4	0.6	0.8	1.0
5-Hydroxymethylfurfural	0.5	1	1.5	2	2.5	3
Levulinic acid	0.5	1	1.5	2	2.5	3
Formic acid	0.5	1	1.5	2	2.5	3
Acetaldehyde	1	2	3	4	5	6
Acetic acid	1	2	4	6	8	10
Ethanol	1	2	4	6	8	10
Lactic acid	5	10	15	20	30	40
Catechol	0.01	0.05	0.1			
Cinnamaldehyde	0.1	0.2	0.4	0.6	0.8	1.0
Hydrochinone	0.01	0.05	0.1			
p-Hydroxybenzaldehyde	0.1	0.2	0.4	0.6	0.8	1.0
p-Hydroxybenzoic acid	0.01	0.05	0.1			
Homovanillic acid	0.01	0.05	0.1			
Syringealdehyde	0.1	0.2	0.4	0.6	0.8	1.0
Vanillin	0.01	0.05	0.1	0.15		
Vanillic acid	0.01	0.05	0.1			
Copper (+2)	0.01	0.02	0.03	0.04	0.05	0.06
Chromium (+3)	0.01	0.02	0.03	0.04	0.05	0.06
Iron (+3)	0.1	0.2	0.3	0.4	0.5	0.6
Nickel (+2)	0.01	0.02	0.03	0.04	0.05	0.06

[a]Each substance was tested in several concentration steps with two replicates. The levels used for each compound were determined by what was likely to be found in the hemicellulose hydrolysate.

Table 2
Composition of Toxicity Test-Media in Microtiter Scale[a]

Sample	Composition
Positive control	150 µL nutrient broth/yeast extract 50 µL cell suspension
Negative control	200 µL nutrient broth/yeast extract
Test wells	100 µL inhibitors in various concentrations 50 µL nutrient broth/yeast extract 50 µL cell suspension

[a]A positive and a negative control was incubated together with the test wells, where inhibitors were added to the media in various concentrations.

Sugar-Degradation Products

Levulinic acid was the most toxic compound in the group of sugar-degradation compounds and caused 100% growth inhibition of the IFA 119 strain at 0.5 g/L, while the total inhibition level of furfural was attained with 0.8 g/L. Furfural did not inhibit growth more than 25% at concentrations up to 0.6 g/L.

Table 3
Inhibition Values of Each Compound in Each Concentration Tested[a]

Concentration-steps tested (g/L)	1	2	3	4	5	6
Compound	Growth inhibition (%)					
Furfural	8.4	20	24.5	19.9	96	78
5-Hydroxymethylfurfural	−134	83	91	73	93	70
Levulinic acid	102	87	98	84	103	95
Formic acid	13	17	37	50	47	63
Acetaldehyde	−102	100	100	99	100	99
Acetic acid	−15	48	73	92	94	91
Ethanol	−0.61	−4.1	−3.5	−1.6	1.7	−7.5
Lactic acid	−15	48	73	92	94	91
Catechol	−2.7	88	90	84		
Cinnamaldehyde	17	27	45	97	101	99
Hydrochinone	99	95	94	94		
p-Hydroxybenzaldehyde	8.1	54	93	92	94	90
p-hydroxybenzoic acid	9.2	6.9	17	1.4		
Homovanillic acid	15	14	8	38		
Syringealdehyde	−9.7	28	88	90	89	88
Vanillin	−31	−34	−18	−19		
Vanillic acid	−32	−30	−19	2.9		
Copper (+2)	−3.9	6.1	−14.1	−25	−33	−32
Chromium (+3)	20	101	101	100	101	100
Iron (+3)	12	48	53	51	57	46
Nickel (+2)	27	21	17	25	28	29

[a]The growth of the bacteria strain was expressed as a percentage of the positive control with no sugar supplements. The values are obtained from microtiter platetests after 24 h incubation (60°C) with two replicates. Negative values indicate enhancement of growth.

A test-solution of 0.5 g/L 5-hydroxymethylfurfural stimulated 100% growth of the IFA 119 strain before the bacteria were inhibited. The inhibition curve of 5-hydroxymethylfurfural showed a value of 82.72% at a concentration of 1 g/L and stayed in that range when testing more concentrated solutions (Fig. 1). Growth inhibition by formic acid was proportional to the amount of acid in the medium. The maximum inhibition was attained at the highest concentration tested (3 g/L) and it was more than 60% for the IFA 119 strain.

Lignin Derived Compounds

Cinnamaldehyde, p-hydroxybenzaldehyde, and syringealdehyde caused more than 90% inhibition of the growth of the IFA 119 strain at 0.6 g/L. While cinnamaldehyde showed an inhibition at a concentration of 44.90% at 0.4 g/L, this value was doubled at 0.6 g/L. The same effect was seen with solutions of p-hydroxybenzaldehyde, where inhibition values of 54.15 and 93.24% were determined when testing the microor-

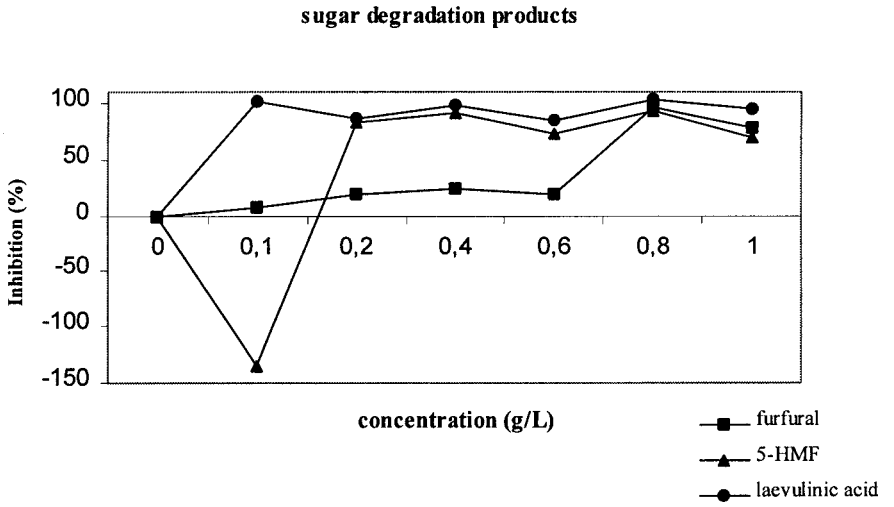

Fig. 1. Dose-response curves of sugar degradation products: while levulinic acid shows high inhibition values, 5-hydroxymethylfurfural promotes growth in low doses.

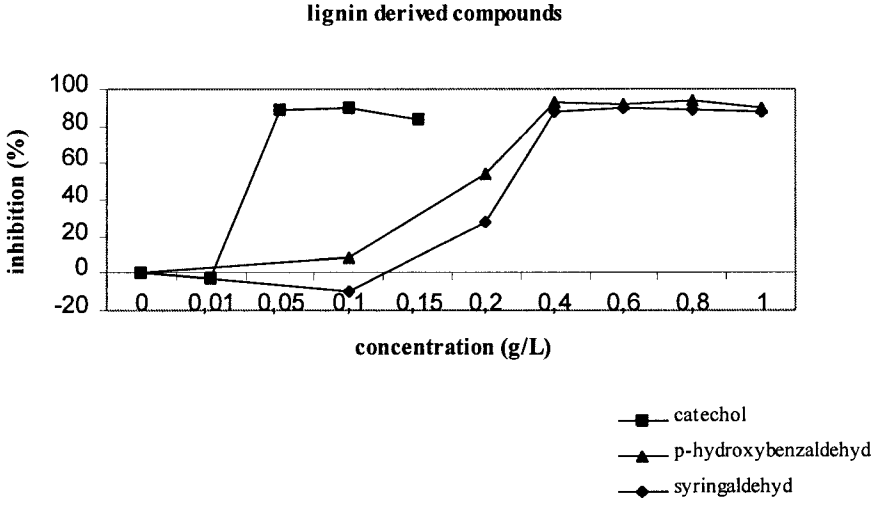

Fig. 2. Dose-response curves of lignin-derived compounds: hyperbolic curves of p-hydroxybenzaldehyde and syringealdehyde; catechol is very toxic to IFA 119.

ganism in solutions of 0.2 and 0.4 g/L. A sharp increase showed the dose-response curve of syringealdehyde between the growth inhibition values at concentrations of 0.2 and 0.4 g/L.

Catechol and hydrochinone were the most toxic compounds in this class. Although they were tested in very minute concentrations, they both show an inhibition of more than 85% at a level of 0.05 g/L. Typical dose-response curves of p-hydroxybenzaldehyde and syringealdehyde, and the high inhibition of catechol are shown in Fig. 2.

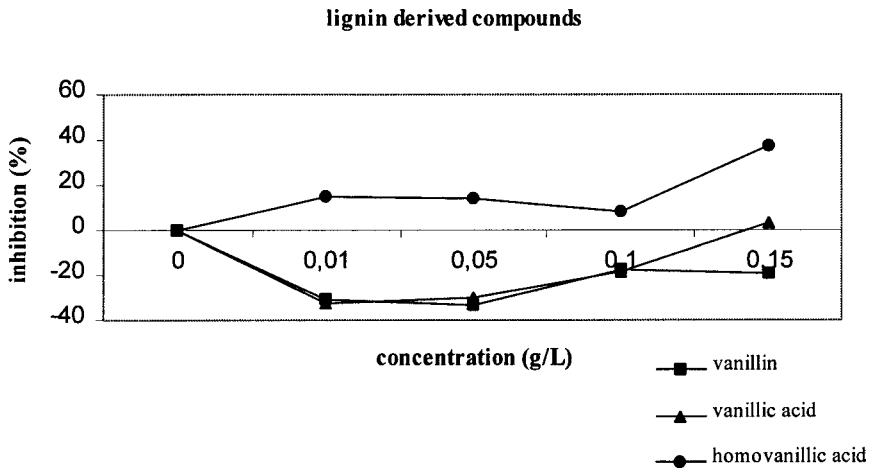

Fig. 3. Dose-response curves of lignin-derived compounds: growth promotion of vanillin and vanillic acid.

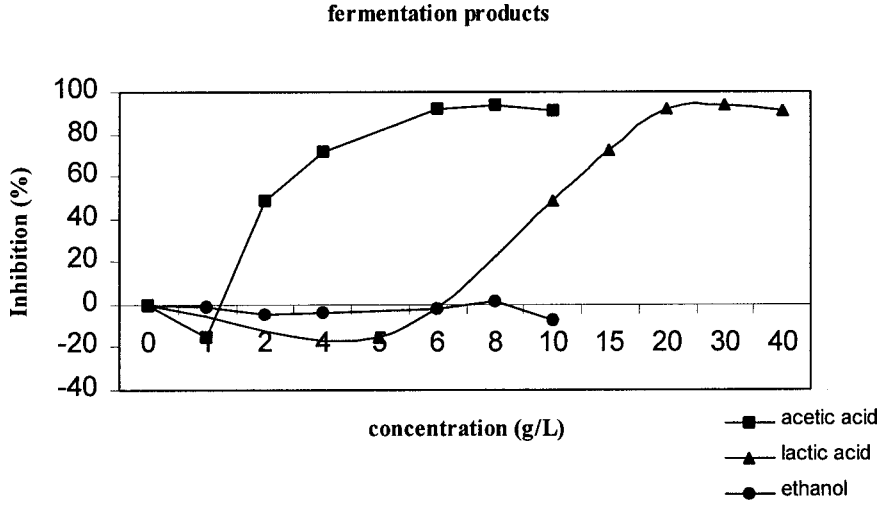

Fig. 4: Dose-response curves of fermentation products: acetic acid shows high inhibition, while ethanol does not have strong effects on growth of IFA 119.

IFA 119 strains growth was stimulated by low concentrations of syringealdehyde, vanillin and vanillic acid. This condition was observed in most cases at concentrations below 0.1 g/L. While a slight inhibition was observed at a concentration of 0.15 g/L vanillic acid, vanillin seemed to promote growth all over the concentration range tested (Fig. 3).

Compounds Released During Autohydrolysis Of Hemicellulose

The inhibition curves of the fermentation products are shown in Fig. 4. Acetic acid at 1 g/L stimulated the growth of the IFA 119 strain by 15%,

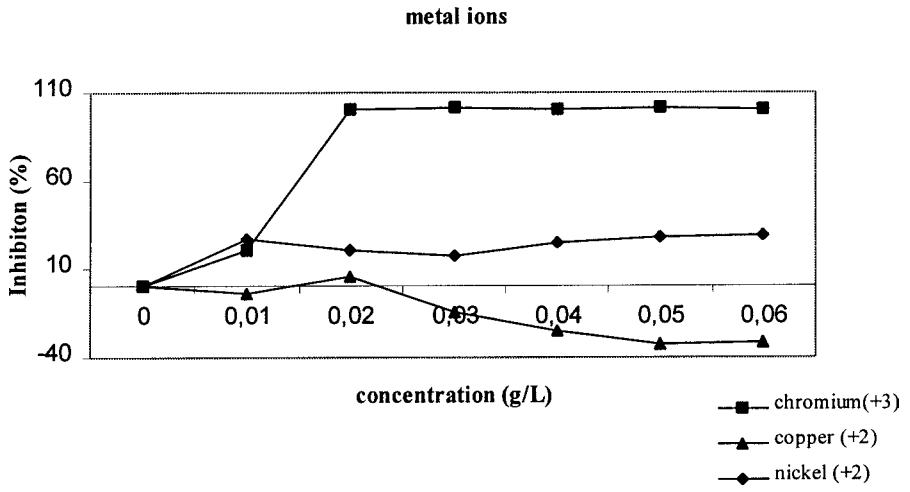

Fig. 5: Dose-response curves of metal ions: while copper (+2) seems to be a growth promoter, chromium (+3) is very toxic to IFA 119.

while the addition of concentrations of 2 g/L resulted in growth inhibition of 50%. No growth was detected when adding concentrations greater than 6 g/L. Growth of the IFA 119 strain was inhibited 100% at a concentration of 2 g/L acetaldehyde. The inhibition effect of lactic acid was doubled between levels of 10 and 20 g/L. Ethanol seemed to be a growth promoter up to 10 g/L.

Metal Ions Released From Soil, Wood, and Hydrolysis Equipment

While iron (+3) and chromium (+3) ions had very toxic effects on the growth of the IFA 119 strain, the maximum inhibition attained for nickel (+2) ions was 30%. Copper (+2) seemed to be a growth promoter.

An inhibition of 100 % at a concentration of 0.02 g/L of a Chromium (+3) solution was observed (Fig. 5), iron (+3) test solutions at levels higher than 0.2 g/L inhibited the microorganisms more than 50%.

Discussion

Growth of Bacteria on Media Supplemented with Varying Concentrations of Inhibitors

The medium used for culturing the IFA 119 strain was a complex media, nutrient broth with yeast extract. When the medium was supplemented with sugars of the same quantity and composition as in the softwood hydrolysate, low growth of the bacteria strains was observed. The growth of the IFA 119 strain in media with sugars was ~30 % of the growth of the same strain in media without sugars. The monomeric sugars were converted to acids by the process organism. The medium that was used for the positive controls did not contain any sugars, which meant that the media were used for growth without formation of products.

The action of some of the inhibitors depicted a clear pattern of the inhibition. The lignin derived compounds' dose-response curves were in most cases hyperbolic. The response could be a result of the mode of action of the compounds on bacteria cells. The compounds are known to damage the morphology of the cells by reacting with macromolecules of the cells *(9)*. The bacteria could metabolize some of the inhibitors at very low concentrations. This condition was observed at very low concentrations of the lignin-derived compounds, in most cases at concentrations below 0.1 g/L. At high concentrations the lignin derived compounds were very toxic. Lignin-derived compounds' toxicity has been found to be linked to the degree of hydrophobicity and the nature and number of the substituent groups on the parent phenyl compound *(5)*. The less substituted and more hydrophobic compounds were more toxic than the heavily substituted compounds. The same result was obtained in this study because catechol and hydroquinone were the most toxic compounds. Vanillin, vanillic acid, and homo vanillic acid were less toxic because they are less hydrophobic than the other lignin compounds, although the concentration ranges tested were at very low levels, i.e., from 0.01 to 0.15 g/L. These compounds were evaluated at this level since they are mostly present in the wood hydrolysates at very minute levels.

A concentration of less than 0.5 g/L of 5-hydroxymethylfurfural even stimulated the growth of the IFA 119 strain. This effect on growth has also been reported previously *(6,9)*. Furfural and 5-hydroxymethylfurfural were reported to increase the lag phase since they were metabolized in preference to the sugars in the media *(7)*. Growth was said to resume when the compounds had been metabolized to their corresponding alcohols. The corresponding alcohols were assimilated and used in the formation of biomass. At high concentrations, 5-hydroxymethylfurfural was toxic to the bacteria strains. Furfural was toxic over a sharp threshold to the IFA 119. The toxicity of furfural is related to its mode of action. Furfural inhibits growth by limiting the amount of energy available for growth. As a rule of thumb it was found that fermentations took place in the media with less than 0.5 g/L *(12)*.

Levulinic acid was toxic to the IFA 119 strain at 0.5 g/L causing 100% inhibition. The toxicity of levulinic acid is related to its structure as it is hydrophilic in nature. Levulinic acid can easily pass through the cell membranes of bacteria and inhibit metabolic reactions. The IFA 119 strain were also sensitive to sugar-degradation compounds compared to yeasts. Yeasts are inhibited by 0.8 g/L of 5-hydroxymethylfurfural, 9 g/L levulinic acid, and 5 g/L formic acid *(12)*.

The inhibition caused by acetic acid is a function of the undissociated form in the media *(8)*. When the undissociated form of the acid enters the cells of the microorganisms, they dissociate, causing a rise in the pH of the cells. The pumping of the hydrogen ions out of the cell by ATPase makes the energy unavailable for growth *(6)*. In this study the IFA 119 strain was able to grow at concentration less than 1 g/L acetic acid without being inhibited.

Acetaldehyde has been described to have the same effects as acetic acid but more potent than acetic acid, since it is volatile. The potency of acetaldehyde was also demonstrated in this study as 4 g/L caused 100% inhibition to the IFA 119.

Metal ions are cofactors in some enzyme complexes and this function could be responsible for the stimulating effect of the copper ions and nickel ions on the growth of the bacteria. The strongly inhibiting effect of chromium ions could be a result of the ions inhibiting the enzyme involved in the metabolic pathways of bacteria. The metal ion could inhibit the enzymes of the metabolic pathways *(13)*.

The single model compound testing system gave an indication of the resistance of the bacteria strains toward the toxic compounds. Results from the toxicity test can be used to accurately predict the effects of the inhibitors in the hydrolysate on growth of the process organisms. As the inhibitor action was found to be cumulative by other researchers *(6,9)*, a combination of the two or three compounds can be used to predict more accurately the effects on growth.

The bacterial strain could be adapted to increase the resistance to the presence of inhibitors and reduce their effects on the fermentation process and growth. The effect of the inhibitors on the fermentation process can be investigated to see if there is a relationship with growth inhibition.

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