Enzymatic Lignocellulosic Feedstock Degradation and Lactic Acid Production by *Bacillus coagulans*

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Abstract

For an operator of a lignocellulosic biorefinery plant, simple and fast methods are needed to reproducibly define and optimise processes. For the development of enzymes, methods are required which allow for the assessment of the effectiveness of cellulase as close to the process as possible, and which guarantee quick and easy technology transfers to the industry. Currently, quality parameters and methods to rapidly implement technology transfers into lignocellulosic biorefinery processes are lacking. The first part of this work develops a method that characterizes the effectiveness and efficiency of cellulases using process-related parameters. Results of hydrolyses with the model substrate wheat straw show that, in the investigated case, the particle size distribution has a greater impact on the effectiveness of the hydrolysis than the amount of enzyme used. Using this information, a kinetic parameter was defined, which allows for the description of the effectiveness of cellulase on the substrate lignocellulose. Then, this kinetic approach is used to define an empirical-mathematical model equation. Along with the known Sauter diameter and carbohydrate composition of the substrate, as well as the amount of enzyme used, this allows one to determine the time course of hydrolysis and the expected yield.

For natural scientists and operators of lignocellulosic biorefineries for biotechnological production of chemicals, certainty about the effectiveness of the microorganisms that can be used for production is desired. Which organism already covers a broad spectrum of usable sugars as wild-type, yields the best yield and quality with the highest possible resistance to inhibitors? In the second part of the thesis, fermentation processes of lignocellulose hydrolysates are considered. Isolates of *Bacillus coagulans* serve as model organisms. A high-throughput optical screening method has been developed to study how the microorganisms utilise lignocellulose-based sugars. Starting from this method, a mathematical approach was defined, and an additional quality parameter introduced. This parameter can be used to determine the uppermost level of lignin concentration that still allows for growth of the microorganism. Fermentations with defined media and lignocellulose hydrolysates confirmed the behaviour of the *Bacillus coagulans* isolates in the fermentation of different lignocellulose-based sugars and lignin concentrations. Based on the fermentation data, growth models for process characterisation and prediction were defined. Measurements showed that there was a potential decrease in dissolved lignin in the medium during fermentation. Tests have shown that the isolates of *Bacillus coagulans* used are capable of potentially reducing the concentration of dissolved lignin, vanillin, and ferulic acid in the medium.

Zusammenfassung

Lignozellulose-Bioraffinerien benötigen robuste Methoden zur Prozessführung und Charakterisierung. Für den Betrieb und die Optimierung solcher Anlagen sind einfache, schnelle Methoden notwendig. Methoden für den enzymbasierten Aufschluss der Lignozellulose sollten die Einschätzung der Effektivität von Enzymen wie Zellulasen/Hemizellulasen bereits während der Prozessentwicklung möglichst prozessnah erlauben und einen Technologietransfer in die Industrie einfach und schnell gewährleisten. Derzeit fehlen Qualitätsparameter und Methoden, um den Technologietransfer in Prozesse einer Lignozellulose-Bioraffinerie schnell umzusetzen. Im ersten Teil dieser Arbeit wird eine Methode entwickelt, die die Effektivität und Effizienz von Zellulasen anhand prozessnaher Parameter charakterisiert. Ergebnisse von Hydrolysen u.a. mit dem Modellsubstrat Weizenstroh zeigen, dass im untersuchten Fall die Partikelgrößenverteilung einen stärkeren Einfluss auf die Effektivität der Hydrolyse hat, als die eingesetzte Enzymmenge. Mittels dieser Information wird ein kinetischer Parameter definiert, der es erlaubt, die Effektivität von Zellulasen mit Lignozellulose als Substrat zu beschreiben. Weiterführend wird dieser kinetische Ansatz genutzt, um eine empirisch-mathematische Modellgleichung zu definieren. Diese erlaubt es, bei bekanntem Sauter–Durchmesser und Kohlenhydratzusammensetzung des Substrates, sowie der eingesetzten Enzymmenge, den zeitlichen Verlauf einer Hydrolyse und den zu erwartenden Ertrag zu bestimmen.

Von Betreibern von Lignozellulose-Bioraffinerien zur biotechnologischen Erzeugung von Chemikalien werden sichere Informationen über die Effektivität der zur Produktion einsetzbaren Mikroorganismen benötigt. Welcher Organismus deckt bereits als Wildtyp ein breites Spektrum an verwertbaren Zuckern ab, bringt die beste Ausbeute und Qualität bei möglichst hoher Resistenz gegenüber Inhibitoren? Im zweiten Teil der Arbeit werden Fermentation-Prozesse von Lignozellulose-Hydrolysaten betrachtet. Als Modellorganismen dienen Isolate von *Bacillus coagulans*. Zur Charakterisierung der Mikroorganismen wird eine Methode für ein optisches Hochdurchsatzscreening entwickelt. Mit dieser kann untersucht werden ob die beschriebenen Mikroorganismen Lignozellulose-basierende Zucker verwerten. Ausgehend von dieser Methode wird ein mathematischer Ansatz definiert und ein zusätzlicher Qualitätsparameter eingeführt. Durch diesen Parameter kann bestimmt werden, bis zu welcher Lignin-Konzentration ein Wachstum der Mikroorganismen noch möglich ist. Fermentationen mit definierten Medien und Lignozellulose-Hydrolysaten bestätigten das Verhalten der *B. coagulans* Isolate bzgl. der Fermentation unterschiedlicher Lignozellulose basierender Zucker und Lignin-Konzentrationen. Anhand der Fermentationsdaten werden Wachstumsmodelle zur Prozesscharakterisierung und Prädiktion definiert. Die Messungen zeigen eine Abnahme des gelösten Lignins im Medium während der Fermentation. Die genutzten *Bacillus coagulans* Isolate scheinen in der Lage zu sein, die Mengen an gelöstem Lignin, Vanillin und Ferulasäure im Medium zu verringern. Die Ergebnisse dieser Arbeit zeigen, dass *B. coagulans* ein sicherer Stamm zur effektiven und effizienten Produktion von Milchsäure ist.

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List of Abbreviations

General

AL	Alkaline lignin
Ara	Arabinose
AM	Artificial medium
AW	Aspen wood
AWH	Aspen wood hydrolysate
BM	Biomass
CBU	Cellobiase units
CB	Cellobiose
etc.	*et cetera*
e.g.	*exempli gratia*
FT	Fermentation time
FA	Ferulic acid
FPA	Filter paper assay
FHF	Filter paper hydrolysis factor
FPU	Filter paper unit
Glc	Glucose
g_p	Gram protein
g_s	Gram substrate
i.e.	*id est*
LA	Lactic acid
LAB	Lactic acid bacteria
DSMZ	Leibniz Institute's German Collection of Microorganisms and Cell Cultures
MO	Microorganism
MTM	Monod type model
CT2	Novozymes enzyme Cellic CTec2
HT2	Novozymes enzyme Cellic HTec2
PS	Particle size
PW	Pine wood
PWH	Pine wood hydrolysate
pWS	Pulverised wheat straw
RS	Rice straw
RMS	Root mean square
SMD	Sauter mean diameter
VAN	Vanillin
w/v	Weight dry mass per volume
WS	Wheat straw
WSA	Wheat straw assay
WSH	Wheat straw hydrolysis
WSU	Wheat straw units
Xyl	Xylose

Symbols

Chapter 4.1.

Symbol	Explanation	Unit
α	offset for basal concentration of sugars	[-]
a	model fitting parameter	[-]
af	adjustment factor	[-]
af_{Glc_CB}	adjustment factor for glucose from cellobiose	[-]
$af_{Glc\_cellulose}$	adjustment factor for glucose from cellulose	[-]
af_{Xyl}	adjustment factor for xylose from xylan	[-]
b	model fitting parameter	[-]
$cf_{cellulose}$	conversion factor describes the amount of cellulose bound in lignocellulose	$[g_{cellulose}/g_{substrate}]$
$cf_{hemicellulose}$	conversion factor describes the amount of hemicellulose bound in lignocellulose	$[g_{hemicell.}/g_{substrate}]$
cf_{PS}	particle size-based conversion factor describes the accessibility for hydrolyses	$[g_{Glc}/g_{substrate}]$
hf	hydrolysis factor	[-]
hf_{CBA}	hydrolysis factor for the standard cellobiose-assay	[g]
hf_{FPA}	hydrolysis factor for the standard filter-paper-assay	[-]
hf_{XA}	hydrolysis factor for the xylan-assay	[g]
Ne	Newton number of the stirrer setting	[-]
Re	Reynolds number	[-]
sf	scale factor	[-]
$S_{cellulose}$	Substrate amount of cellulose bound in the cellulose fraction	[g]
E_p	enzyme protein amount	[g]
m_{FP}	mass of the filter paper used for the Filter-Paper-Assay	[mg]
$m_{Cellulose}$	mass of α-cellulose used in the α-CU test	[mg]
m_{Glc}	mass of glucose	[mg]
m_{Glc_100mg}	mass of glucose based on conversion of x mg α-cellulose	[mg]
m_{Glc_100mg}	mass of glucose based on conversion of 100 mg α-cellulose	[mg]
m_{Glc_133mg}	mass of glucose based on conversion of 133.33 mg α-cellulose	[mg]
m_{Glc_200mg}	mass of glucose based on conversion of 200 mg α-cellulose	[mg]
m_{Glc_300mg}	mass of glucose based on conversion of 300 mg α-cellulose	[mg]
$m_{Glc\_7.5mM}$	mass of glucose based on conversion of 7.5 mM cellobiose	[mg]
m_{Glc_15mM}	mass of glucose based on conversion of 15 mM cellobiose	[mg]
m_{Glc_30mM}	mass of glucose based on conversion of 30 mM cellobiose	[mg]
$m_{Xyl\_1\%}$	mass of glucose based on conversion of 1% w/v xylan	[mg]
$m_{Xyl\_2\%}$	mass of glucose based on conversion of 2% w/v xylan	[mg]
$m_{Xyl\_3\%}$	mass of glucose based on conversion of 3% w/v xylan	[mg]
$m_{hemicellulose}$	mass of hemicellulose	[mg]
m_{Xyl}	mass of xylose	[mg]
$m_{Enzyme}(m_{Glc})$	mass of enzyme protein which releases x mg glucose	[mg]
$m_{Enzyme}(m_{Xyl})$	mass of enzyme protein which releases x mg xylose	[mg]
$V_{Enzyme}(m_{Glc})$	enzyme concentration to release x mg glucose	[mL]
V_{FP}	Volume used for the Filter-Paper-Assay	[mL]
V_{RT}	Volume used in the reaction tube	[mL]
M_{Glc}	molar mass of glucose	[mg/µmol]
M_{xyl}	molar mass of xylose	[mg/µmol]
r_{c_i}	conversion rates factor of the component i	[µmol/min]
r_{c_FPA}	conversion rates factor for 50mg filter-paper of the standard FPA	[µmol/min]
r_{c_100mg}	conversion rates factor for 100 mg α -cellulose substrate	[µmol/min]
r_{c_133mg}	conversion rates factor for 133.33 mg α -cellulose substrate	[µmol/min]
r_{c_200mg}	conversion rates factor for 200 mg α -cellulose substrate	[µmol/min]
r_{c_300mg}	conversion rates factor for 300 mg α -cellulose substrate	[µmol/min]
$r_{c\_7.5mM}$	conversion rates factor for 7.5 mM cellobiose substrate	[µmol/min]
r_{c_15mM}	conversion rates factor for 15 mM cellobiose substrate	[µmol/min]
$r_{c\_30\ mM}$	conversion rates factor for 30 mM cellobiose substrate	[µmol/min]
$r_{c\_1\%}$	conversion rates factor for 1% w/v xylan substrate	[µmol/min]
$r_{c\_2\%}$	conversion rates factor for 2% w/v xylan substrate	[µmol/min]
$r_{c\_3\%}$	conversion rates factor for 3% w/v xylan substrate	[µmol/min]
WSU	Wheat straw units (glucose)	$[g_{Glc}/(min*g_p)]$
WSU_{Xyl}	Wheat straw units (xylose)	$[g_{Xyl}/(min*g_p)]$
FPU	Filter-paper-units	$[\mu mol_{Glc}/(min*mg_p)]$
αCU	α-cellulose-units	$[\mu mol_{Glc}/(min*mg_p)]$
αCU_i	α-cellulose-units of the substrate component i	$[\mu mol_{Glc}/(min*mg_p)]$
αCU	α-cellulose-units for 100 mg α-cellulose substrate	$[\mu mol_{Glc}/(min*mg_p)]$

αCU	α-cellulose-units for 133.33 mg α-cellulose substrate	[$\mu mol_{Glc}/(min*mg_p)$]
αCU	α-cellulose-units for 200 mg α-cellulose substrate	[$\mu mol_{Glc}/(min*mg_p)$]
αCU	α-cellulose-units for 300 mg α-cellulose substrate	[$\mu mol_{Glc}/(min*mg_p)$]
CBU	Cellobiose-units	[$\mu mol_{Glc}/(min*mg_p)$]
CBU_i	Cellobiose-units of the substrate component i	[$\mu mol_{Glc}/(min*mg_p)$]
$CBU_{7.5mM}$	Cellobiose-units for 7.5 mM cellobiose substrate	[$\mu mol_{Glc}/(min*mg_p)$]
CBU_{15mM}	Cellobiose-units for 15 mM cellobiose substrate	[$\mu mol_{Glc}/(min*mg_p)$]
CBU_{30mM}	Cellobiose-units for 30 mM cellobiose substrate	[$\mu mol_{Glc}/(min*mg_p)$]
XU	Xylose-units of the substrate component i	[$\mu mol_{Xyl}/(min*mg_p)$]
XU_i	Xylose-units of the substrate component i	[$\mu mol_{Xyl}/(min*mg_p)$]
$XU_{1\%}$	Xylose-units of the substrate component of 1% w/v xylan	[$\mu mol_{Xyl}/(min*mg_p)$]
$XU_{2\%}$	Xylose-units of the substrate component of 2% w/v xylan	[$\mu mol_{Xyl}/(min*mg_p)$]
$XU_{3\%}$	Xylose-units of the substrate component of 3% w/v xylan	[$\mu mol_{Xyl}/(min*mg_p)$]
ν	kinetic viscosity	[m^2/s]
R	amplitude of the planar shaker	[m]
t	run time of the process	[h]
t_R	reaction time	[min]
μ_p	activity in the context of product release per hour	[1/h]
N	shaking frequency/ rotation speed	[1/s]

Chapter 4.2.

Symbol	Explanation	Unit
λ	Lag time	[h]
μ_m	Maximum specific growth rate, mixed substrate	[1/h]
γ	Parameter for growth evaluation	[$1/h^2$]
δ	Parameter for growth evaluation	[$1/h^2$]
N	cell concentration	[cfu/mL]
N_0	initial cell concentration	[cfu/mL]
N_{min}	minimum cell concentration	[cfu/mL]
N_{max}	asymptotic maximum cell concentration	[cfu/mL]
y	natural logarithm of the quotient of the cell concentration (N) and minimal cell concentration (N_{min})	[-]
y_0	natural logarithm of the quotient of the initial cell concentration (N_0) and minimal cell concentration (N_{min})	[-]
y_{max}	natural logarithm of the quotient of the asymptotic cell concentration (N_{max}) and the minimum cell concentration (N_{min})	[-]
Δy	difference of logarithmic cell concentrations y_{max} & y_0	[-]

Chapter 4.3.

Symbol	Explanation	Unit
$\mu_{max,sim,mix}$	model fitting parameter	[-]
k_1	model fitting parameter	[-]
k_2	model fitting parameter	[-]
k_3	model fitting parameter	[-]
y	natural logarithm of the quotient of the cell concentration and minimal cell concentration	[-]
y_0	natural logarithm of the normed minimum cell concentration	[-]
y_{max}	natural logarithm of the normed maximum asymptotic cell concentration	[-]
b	corresponds to natural logarithm of the normalized cell concentration (Schnute growth model)	[-]
q_0	Physiological state of the inoculum	[-]
$q_{0,Glc}$	Physiological state of the inoculum toward glucose	[-]
$q_{0,Xyl}$	Physiological state of the inoculum toward xylose	[-]
$q_{0,Ara}$	Physiological state of the inoculum toward arabinose	[-]
m	constant fitting parameter (Baranyi growth model)	[-]
h_o	constant fitting parameter (Baranyi growth model)	[-]
ω	constant fitting parameter (Richard & Stannard)	[-]
ξ	constant fitting parameter (Richard & Stannard)	[-]
$\mu_{BM,max}$	Maximum specific growth rate, mixed substrate	[1/h]
$\mu_{Glc,\ max}$	Maximum specific uptake rate, glucose	[1/h]
$\mu_{Xyl,\ max}$	Maximum specific uptake rate, xylose	[1/h]

$\mu_{Ara,max}$	Maximum specific uptake rate, arabinose	[1/h]
a	fitting parameter (Schnute growth model)	[1/h]
ν	fitting parameter (Baranyi growth model)	[1/h]
υ	constant fitting parameter (Baranyi growth model)	[1/h]
β	Parameter for growth evaluation	[1/h²]
γ	Parameter for growth evaluation	[1/h²]
δ	Parameter for growth evaluation	[1/h²]
$Y^{BM/Glc}$	Biomass to glucose yield coefficient	[g/g]
$Y^{BM/Xyl}$	Biomass to xylose yield coefficient	[g/g]
$Y^{BM/Ara}$	Biomass to arabinose yield coefficient	[g/g]
$Y^{LA/BM}$	Lactate to substrate yield coefficient	[g/g]
$Y^{LA/Sub}$	Lactate to biomass yield coefficient	[g/g]
$Y^{\Delta BM/\Delta Glc}$	produced biomass to consumed glucose yield coefficient	[g/g]
$Y^{\Delta BM/\Delta Xyl}$	produced biomass to consumed xylose yield coefficient	[g/g]
$Y^{\Delta BM/\Delta ara}$	produced biomass to consumed arabinose yield coefficient	[g/g]
$Y^{LA/\Delta BM}$	produced lactate to produced biomass yield coefficient	[g/g]
$Y^{LA/\Delta Sub}$	produced lactate to depleted substrate yield coefficient	[g/g]
C_{BM}	Concentration of biomass	[g/L]
C_{Glc}	initial substrate concentration, glucose	[g/L]
C_{Xyl}	initial substrate concentration, xylose	[g/L]
C_{Ara}	initial substrate concentration, arabinose	[g/L]
C_{Sub}	total initial substrate concentration	[g/L]
C_{LA}	total product concentration, lactate	[g/L]
$C_{\Delta Glc}$	Concentration of consumed glucose	[g/L]
$C_{\Delta Xyl}$	Concentration of consumed xylose	[g/L]
$C_{\Delta Ara}$	Concentration of consumed arabinose	[g/L]
$C_{\Delta Sub}$	Concentration of consumed substrate	[g/L]
K_{Glc}	Saturation constant, glucose	[g/L]
K_{xyl}	Saturation constant, xylose	[g/L]
K_{Ara}	Saturation constant, arabinose	[g/L]
K_D	Biomass reduction constant	[g/L]
k_D	Maximum specific biomass reduction rate	[g/(L*h)]
dC_{BM}/dt	Biomass formation rate	[g/(L*h)]
dC_{Glc}/dt	Substrate depletion rate, glucose	[g/(L*h)]
dC_{Xyl}/dt	Substrate depletion rate, xylose	[g/(L*h)]
dC_{Ara}/dt	Substrate depletion rate, arabinose	[g/(L*h)]
dC_{LA}/dt	Product formation rate, lactate	[g/(L*h)]
P	Productivity	[g/(L*h)]
FT	Fermentation Time	[h]
R^2	Correlation coefficient	[-]
σ	Standard deviation	[1]

Chapter 4.4.

Symbol	Explanation	Unit
$\mu_{max,sim,mix}$	model fitting parameter	[-]
k_1	model fitting parameter	[-]
k_2	model fitting parameter	[-]
k_3	model fitting parameter	[-]
$q_{0,Glc}$	Physiological state of the inoculum toward glucose	[-]
$q_{0,Xyl}$	Physiological state of the inoculum toward xylose	[-]
$q_{0,CB}$	Physiological state of the inoculum toward cellobiose	[-]
$\mu_{BM,max}$	Maximum specific growth rate, mixed substrate	[1/h]
$\mu_{Glc,\ max}$	Maximum specific uptake rate, glucose	[1/h]
$\mu_{Xyl,\ max}$	Maximum specific uptake rate, xylose	[1/h]
$\mu_{CB,max}$	Maximum specific uptake rate, cellobiose	[1/h]
$Y^{BM/Glc}$	Biomass to glucose yield coefficient	[g/g]
$Y^{BM/Xyl}$	Biomass to xylose yield coefficient	[g/g]
$Y^{BM/CB}$	Biomass to arabinose yield coefficient	[g/g]
$Y^{LA/Sub}$	Lactate to substrate yield coefficient	[g/g]
$Y^{LA/BM}$	Lactate to biomass yield coefficient	[g/g]

$Y^{\Delta BM/\Delta Glc}$	produced biomass to consumed glucose yield coefficient	[g/g]
$Y^{\Delta BM/\Delta Xyl}$	produced biomass to consumed xylose yield coefficient	[g/g]
$Y^{\Delta BM/\Delta CB}$	produced biomass to consumed cellobiose yield coefficient	[g/g]
$Y^{LA/\Delta BM}$	produced lactate to produced biomass yield coefficient	[g/g]
$Y^{LA/\Delta Sub}$	produced lactate to consumed substrate yield coefficient	[g/g]
$Y^{BM/Alk}$	Biomass to alkaline yield coefficient	[g/g]
C_{BM}	Concentration of biomass	[g/L]
C_{Glc}	initial substrate concentration, glucose	[g/L]
C_{Xyl}	initial substrate concentration, xylose	[g/L]
C_{Ara}	initial substrate concentration, arabinose	[g/L]
C_{Sub}	total initial substrate concentration	[g/L]
C_{LA}	total product concentration, lactate	[g/L]
$C_{\Delta Glc}$	Concentration of consumed glucose	[g/L]
$C_{\Delta Xyl}$	Concentration of consumed xylose	[g/L]
$C_{\Delta Ara}$	Concentration of consumed arabinose	[g/L]
$C_{\Delta Sub}$	Concentration of consumed substrate	[g/L]
K_{Glc}	Saturation constant, glucose	[g/L]
K_{xyl}	Saturation constant, xylose	[g/L]
K_{CB}	Saturation constant, cellobiose	[g/L]
dC_{BM}/dt	Biomass formation rate	[g/(L*h)]
dC_{Glc}/dt	Substrate depletion rate, glucose	[g/(L*h)]
dC_{Xyl}/dt	Substrate depletion rate, xylose	[g/(L*h)]
dC_{CB}/dt	Substrate depletion rate, cellobiose	[g/(L*h)]
dC_{LA}/dt	Product formation rate, lactate	[g/(L*h)]
P	Productivity	[g/(L*h)]
V_R	Bioreactor working volume	[L]
F_{ALk}	addition rate of NaOH base	[L/h]
c_{OH^-}	NaOH base concentration	[mol/L]
FT	Fermentation Time	[h]
R^2	Correlation coefficient	[-]
σ	Standard deviation	[1]

List of publications

My participation in the publications extended from the creation, planning, execution and evaluation of the experiments to contributing to the content and writing of the publications. I defined the described mathematical equations of the proposed models of enzymatic hydrolyses.

Robert Glaser and Joachim Venus, 2014. Screening of *Bacillus coagulans* strains in lignin supplemented minimal medium with high throughput turbidity measurements
Biotechnology Reports 4 (2014) 60–65. http://dx.doi.org/10.1016/j.btre.2014.08.001

Robert Glaser, 2015. Enzyme-based lignocellulose hydrolyzation – Sauter mean diameter of raw materials as a basis for cellulase performance characterization and yield prediction. Journal of Biotechnology 214; 9–16. http://dx.doi.org/10.1016/j.jbiotec.2015.08.024

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Robert Glaser and Joachim Venus, 2017. Model-based characterisation of growth performance and L-lactic acid production with high optical purity by thermophilic *Bacillus coagulans in* a lignin-supplemented mixed substrate medium. New Biotechnology 37; 180–193. http://dx.doi.org/10.1016/j.nbt.2016.12.006

Robert Glaser and Joachim Venus, 2017. A brief data set on the model-based evaluation of the growth performance of *Bacillus coagulans* and L-lactic acid production in a lignin-supplemented medium. Data in Brief 11; 236–244. http://dx.doi.org/10.1016/j.dib.2017.02.010

Robert Glaser and Joachim Venus, 2018. Co-fermentation of the main sugar types from a beechwood organosolv hydrolysate by several strains of *Bacillus coagulans* results in effective lactic acid production. Biotechnol. Rep. 18; e00245
https://doi.org/10.1016/j.btre.2018.e00245

1. Introduction

The adverse effects of climate change will pose many social and economic challenges for current and future generations of humanity, who will have to deal with issues such as scarcity of natural resources, waste and energy problems, and a growing world population. It has become an urgent requirement to develop sustainable, practical, and efficient strategies that ensure that our natural resources will provide sufficient foodstuffs and renewable commodities to sustain the world's population (BMBF, 2011, 2015; European Commission, 2012). Therefore, that humanity must radically change its approach and views of production, consumption, processing, storage, recycling, and of disposal of goods and biological resources (United Nations Framework Convention on Climate Change, UNFCCC). Therefore, it is crucial to broaden and increase the production of renewable resources and them into food, feed, bio-based products and bioenergy, to achieve global demands for sustainable fuel and food supplies. Therefore, the bioconversion of renewable lignocellulosic biomass to biofuels and value-added products is significantly gaining in importance. (Oertel, 2007; BMBF, 2011, 2015; European Commission, 2012; Siebert et al., 2016).

The different areas of the bioeconomy should offer the opportunity to achieve economic growth while also protecting nature and the environment. Biomass is considered to be sustainable and renewable. Therefore, it is currently considered as an inexpensive feedstock. Furthermore, it is seen as a potential alternative to a wide variety of fossil-based products within different industry sectors (e.g. energy, heat, power, fuels, materials, chemicals). Biomass can substitute fuels to produce heat and power. Biological products such as enzymes can be used to develop biotechnological processes that substitute chemical processes. Therefore, the use of abundant lignocellulosic biomass available from the agricultural and forestry industry provides the foundation for the structuring and development of the bioeconomy and for the concept of biorefinery. The use of lignocellulosic biomass may be advantageous from both the energy and environmental point of views.

Lignocellulose is a major structural component of woody and non-woody plants. Feedstocks such as hardwood (e.g. aspen), softwood (e.g. pine), or straws (e.g. wheat, rice straw) or grasses are widely discussed for lignocellulose biorefineries. The primary step in the development and performance of biorefinery processes should be the availability of a supply chain of lignocellulosic biomass (Oertel, 2007; BMBF, 2011, 2015; European Commission, 2012; Siebert et al., 2016). Other research on the use of fast-growing energy crops and woods (e.g. short rotation poplar) addresses these specific supply chains.

The chemical structure of lignocellulose contains carbohydrate polymers (cellulose, hemicellulose) and phenolic polymeric structures (lignin). These structures, in turn, hinder the

degradation of lignocellulose by enzymes or microorganisms (MOs) and therefore make the efficient processing of biomass more difficult. Based on its polymeric structure, lignocellulose is highly recalcitrant towards environmental breakdown, such as enzymatic breakdown. The effective utilisation of all its components (cellulose, hemicellulose, lignin) would play a significant role in the economic viability of the biorefinery and of its value-added products. Biomass conversion of these polymers into fermentable sugars (hexoses and pentoses) is crucial for fermentation and further downstream processes and can only be achieved using intensive pre-treatment processes. The success and yield gained from pre-treatment processes–such as milling or diluted acidic or alkaline pre-treatment, use of saccharolytic enzymes-cellulases and hemicellulases mixtures–depends on the choice of specific feedstock (hardwood, softwood, and straw). Therefore, pre-treatment processes must be optimised accordingly.

Pre-treatment processes have been identified as the most challenging steps of the conversion process–technically and economically–owing to their required conditions. Within the context of the production of chemicals from biomass, pre-treatment processes include steps by which cellulosic biomass is made amenable using hydrolytic enzymes. Fungi, bacteria, and plants can synthesize cellulases, hemicellulases, and ligniases. Information on MOs producing cellulases is given by Menon and Roa (2012).

Cellulases and hemicellulases must show a high level of activity in an ideal enzyme mixture aimed at complete hydrolysis for lignocellulosic raw material. In addition, the enzymes have to withstand product inhibition while operating well at a mildly acidic pH and at mostly thermophilic temperature, withstanding process stresses such as shear force, all while being highly cost effective. Today's research efforts are centred on the development and optimisation of enzyme systems for the enhancement of the cellulase activity through enzyme mixture composition and recovery strategies. They are also centred on the reaction conditions, through approaches of protein engineering, over expression techniques and through the development of optimal enzyme mixtures (Maki et al., 2009). The yield and turnover rates of enzyme-based hydrolysis are affected mainly by the substrate type and concentration. Current enzymatic processes on lignocellulose hydrolyses are of limited effectiveness due to a lack of key quality parameters. Realistic quality parameters and methods should be based on physically relevant industrial substrates. Finding good quality parameters would help solve the question of which practice is best-suited to match the methods and processes of feedstock characterisation, pre-treatment and enzyme characterisation and therefore achieve standardized and predictable results.

Hydrolysed lignocellulose-based sugars can be fermented using processes such as separate hydrolysis and fermentation, simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation, and consolidated biomass processing. However, the employed microbial strains must be able to utilise the wide range of lignocellulosic sugars while being inert to inhibitors–ideally without genetic engineering. To obtain such strains, screening methods are necessary to get process information on the strains of interest.

This work is aimed at two aspects of lignocellulosic bioconversion into biochemicals: the use of enzymatic systems for lignocellulose hydrolysation and the co-fermentation of lignocellulosic sugars by *Bacillus coagulans* into L-(+)-lactic acid. To achieve the highest informational outcome, a process of separate hydrolysis and fermentation is used in this study. Such an approach can be beneficial for a later process integration using another process type, such as simultaneous saccharification and fermentation. The separate hydrolysis and fermentation, as a conventional two-phase process, considers the lignocellulose hydrolysis using enzymes to form lignocellulose-derived sugars as a first step before the sugars are fermented into lactic acid (LA) by various *Bacillus coagulans* isolates. If using this approach, each process step can be run with optimum conditions such as pH, temperature, substrate concentration and substrate type. Possible points of action are needed to identify ways to improve cellulase enzyme characterization. In addition, quality control parameters for substrate characterization and pre-treatment optimization need to be defined. A new set of definitions for enzyme units is needed for effective and efficient hydrolysis processes in larger-scale applications. The process can be used to get kinetic information (e.g. enzyme turnover rates) on hydrolysis processes for various lignocellulose substrates. This should enable and improve conversion processes in industrial (non-food) applications, by supplying fermentable sugars from lignocellulosic non-starchy plants, such as softwoods and hardwoods, grass, and straw. Lignocellulose hydrolysates must be prepared for fermentations. For these fermentations, the potential of MOs needs to be characterised for the utilisation of different lignocellulosic sugars (hexoses, pentoses, oligosaccharides), and the performance needs to be analysed. Using the hydrolysate directly after hydrolysis without detoxification, which increases complexity and costs, enables the examination of the co-fermentation of lignocellulosic sugars and of resistance of the bacteria towards inhibitors. Of interest is the tolerance against inhibitory compounds (soluble lignin), which must be examined. Methods for screening and characterisation of MOs, such as mathematical modelling approaches for process simulation and prediction, need to be explored.

2. Scientific Background

2.1. Brief introduction to bioeconomy

The substitution of bio-based products for fossil-based products to achieve sustainable supply is a worthy goal. To understand the potential impact of substituting bio-based products for fossil-based products, there must be serious consideration of the effects on biomass demands (e.g. biomass for chemicals and fuels versus biomass for food and feed in the face of finite landmass for agriculture). Scenarios need to be discussed: calculations, analyses, and estimations of current fossil-based and bio-based capacities in light of the projected demands for biomass feedstocks have to be made, and specific achievable goals over a period of time have to be defined. For an intensive management of biomass, official biomass monitoring must be implemented (i.e. forestry management), substitution targets must be set (stepwise increase of biodiesel instead of diesel), and clear definitions of products and feedstocks are needed. Pre-treatment and conversion technologies must be introduced to mitigate climate change in the coming decades and major social barriers must be discussed and ultimately overcome (Siebert et al., 2016), such as

i) The pressure on drinking water supplies;
ii) Social and demographic progress and its impact on the industrial sectors;
iii) Global and regional climate change, which may lead to ecological changes;
iv) Alterations in lifestyles and dietary habits of the human population;
v) Demand for the global supply of safe and healthy food;
vi) The crucial need to curb the continuously increasing demands for fuel; and
vii) The control and prevention of diseases

Therefore, to integrate the bioeconomy into daily life and industry, active work is required, such as increasing education and communication, research and innovation, and a steady process optimisation. Furthermore, researchers, engineers, and the public can work together and help to improve bio-based technologies; this could be done by seeing waste not as a problem but as an opportunity for new products. Biorefineries, a technical concept that can provide opportunities for new products, can be a central focus in solving some of these future demands.

2.2. Biorefineries

Biorefineries are defined as an effective, efficient, and sustainable utilisation of biomass resources to ensure the supply of energy, chemicals, and materials, ultimately mitigating climate change. Many different definitions for the term ‘biorefinery’ are currently used (see also Oertel 2006; BMBF 2011; 2015; VDI 2016). A selection of examples, according to Diep et al. (2012), is listed below:

• A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and value-added chemicals from biomass. The biorefinery concept is analogous to today's petroleum refinery, which produces multiple fuels and products from petroleum (NREL, 2007; Demirbas 2010).

• Biorefining is the transfer of the efficiency and logic of fossil-based chemistry and the substantial converting industries, as well as the production of energy, to the biomass industry (Kamm et al., 2006).

• Biorefinery is the concept of converting plant-based biomass to chemicals, energy, and materials that may run out in our civilisation, replacing the needs for petroleum, coal, natural gas and other non-renewable energy and chemical sources (Liu S, et al., 2011).

• Biorefineries are integrated bio-based industries, using a variety of technologies to produce chemicals, biofuels, food and feed ingredients, biomaterials (including fibres) and power from raw biomass materials (EU Biorefinery Euroview, 2007).

• Bio-refining is the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, and materials) and bioenergy (biofuels, power and/or heat).
The framework of IEA Bioenergy Task 42 used this definition for the BIOPOL Status Report Biorefinery 2007.

Following these approaches, the biorefinery is to be understood as an industrial facility, or a cluster of facilities. This cluster has to use diverse concepts and technologies to enable the maximum sustainable transformation of biomass feedstocks into building blocks such as biofuels, energy, chemicals and materials, preferably of added value (Cherubini et al., 2009, 2010; Diep et al., 2012; Jungmeier et al., 2013; Fernando et al., 2006).

To substitute fossil products, the industry has to replace existing processes with new technologies to meet numerous considerations, such as reducing the environmental footprint of their products (Morais and Bogel-Lukasik, 2013). Technologies that consume less energy and generate less waste need to be developed, or the existing technologies need to be improved. To achieve this goal, there is a need for investment as well as technology adoption and implementation. Ideally, industries should be based on clean processes and closed product life-cycles. Closed loop life-

cycle management integrates all aspects of a product, taking it from conception through the product life-cycle to the disposal of the product and the re-using of its components–starting with non-polluting materials and taking a later reduction of solvent requirements into account.

Biological waste, such as communal green waste and industrial meat waste, is estimated to be up to 138 million tonnes per year in the European Union. Nearly 40% of this waste is disposed of in landfills, which represents a drastic environmental problem and cost (Morais and Bogel-Lukasik, 2013). To reduce this amount by 50% in the European Union by 2030 (European Commission: HORIZON2020, 2013), major changes are needed. One way to reduce waste is by creating high-value products from it, therefore maximising its economic value and replacing raw fossil materials that would have been otherwise needed for the production. To enhance the range of bio-based products, the usable range of biorefineries must be increased, both the products and substrates. Different feedstocks should be processed in biorefineries, including forestry residues, bio-waste, and industrial by-products (crops, organic residues, agro-residues, forest residues, wood, aquatic biomass; Bozell and Pertersen, 2010; Lugue and Clark 2013).

The development of different types of biorefineries will lead to a greater variety of usable feedstocks, technologies, and products. This can increase the sustainable use of biomass while maximising biomass conversion efficiency and minimising raw material requirements. It can also increase the production of bio-based products in suitable markets (at acceptable volumes and prices) and of bioenergy (taking socioeconomic aspects into account, such as economic action in the context of other social, political, demographic, and ecological processes).

2.2.1. Biorefinery platforms

Biomass is the basis that can be converted to a variety of products. Today, two primary biorefinery platforms can be discussed, which are the sugar and thermochemical platforms. Both the sugar platform and the thermochemical platform are used to produce chemicals and fuels (Figure 2.2-1).

The term ‘sugar platform’ comes from the breakdown of biomass into water-soluble sugars that occurs in these platforms. After the breakdown of biomass, sugars can be further processed, for example, by fermentation, to chemicals like LA. Naturally, residues from these biomass breakdown processes–mainly lignin–can be used for power generation or may be used as a basis for products such as composite materials (e.g. Arboform ®).

Through the thermochemical platform, biomass is converted into synthetic gas by gasification or into bio-oils by pyrolysis and hydrothermal conversion. Bio-oils can be further upgraded to liquid fuels such as methanol, gasoline, diesel fuel, and other chemicals. This kind of biorefinery is not addressed in this work.

Table 2.2-1 provides an overview of possible biorefinery products, according to the IEA’s “Bioenergy – task 42 Biorefinery” is given.

Table 2.2-1: Typical biorefinery products

Syngas platform	**Fermentation products from C6/C5 sugar**	**Glucose chemical derivatives**	**Products from the plant-based oil platform**
Methanol	Succinic acid	Sorbitol	Propylene glycol
Ethanol	Itaconic acid	Levulinic acid	Epichlorohydrin
Dimethylether (DME)	Adipic acid	Glucaric acid	1,3-Propanediol
Fischer-Tropsch diesel	3-Hydroxypropionic acid	Hydroxymethyl-furfural	3-Hydroxypropion aldehyde
	Isoprene	2,5-Furan dicarboxylic acid	Acryl acid
	Glutamic acid	p-Xylene	Propylene
	Aspartic acid		

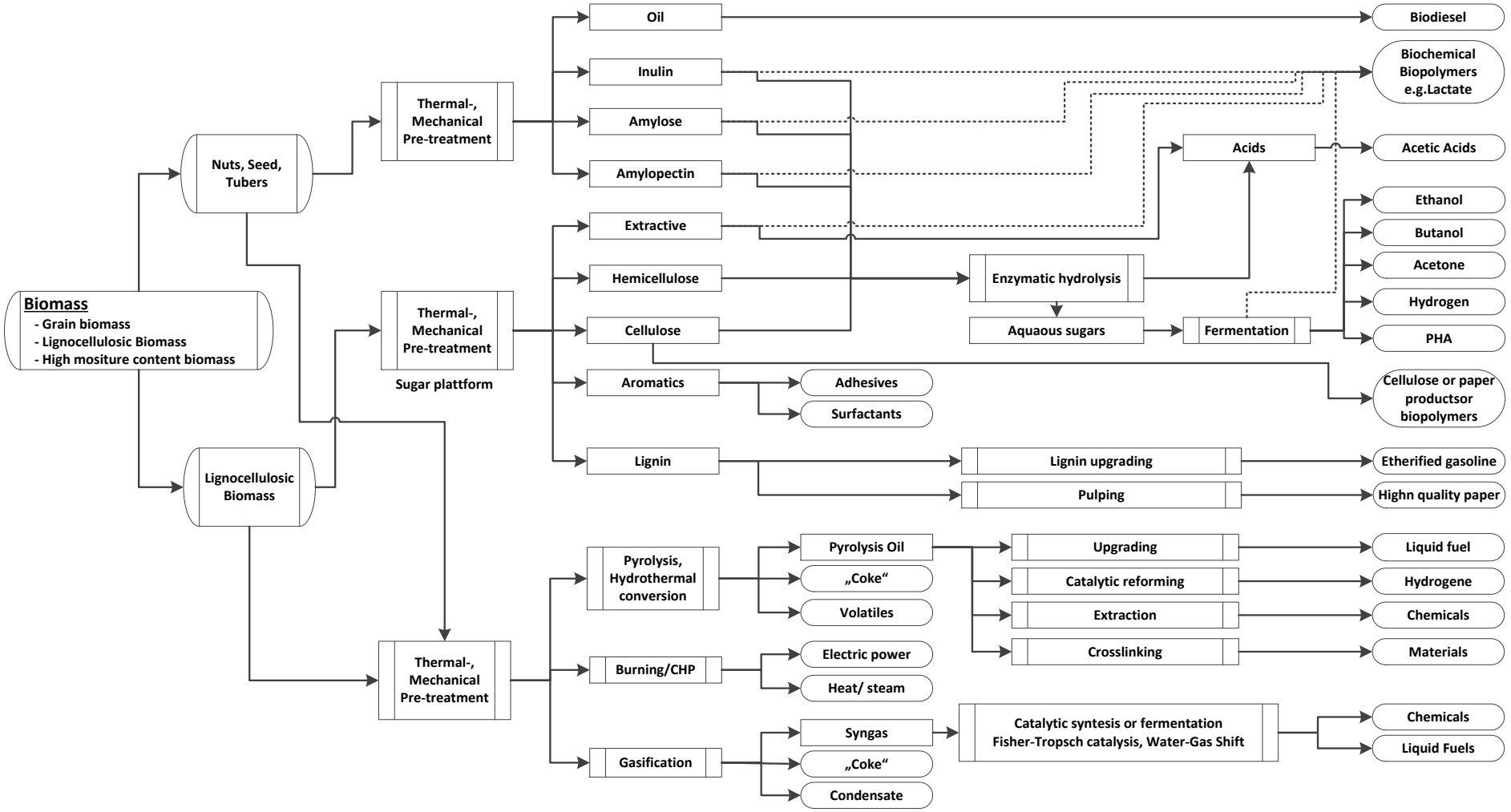


Figure 2.2-1: Biorefinery concepts (based on Xiu et al., 2011, see also BMBF, 2015)

2.2.2. Biorefinery concepts of the sugar platform

Two dominant categories, the so-called ‘generations’, are used to distinguish between the feedstocks that are used by biorefineries (Naik et al., 2010; BMBF 2015; VDI). The first-generation biorefinery uses crops containing high amounts of sugar or starch, such as sugar cane, sugar beet, or sweet sorghum. The sugars of those feedstocks can easily be extracted for subsequent fermentation processes by MOs, such as *B. coagulans*, thereby producing LA. Feedstocks that have a high amount of freely accessible sugars are preferred to feedstocks that contain a high amount of starch. However, starch-rich crops, such as wheat, cassava, and corn, can easily be enzymatically hydrolysed before the fermentation process. Another approach of a first-generation biorefinery is using vegetable oil from dedicated oil crops such as palm, soybean, rapeseed, and sunflower seeds to produce biodiesel. The first-generation biorefineries are limited only by the agricultural capacity of the country. One of the drawbacks of the first-generation biorefineries is that they decrease the amount of available agricultural land to use for food and feed production. This issue is generally labelled as the “food vs. fuel” one. The use of vegetable oil from cooking waste or of animal fat seems to be an effective recycling method to lessen the need for land use. However, before it can be used in tightly controlled industrial environments as feedstocks, waste requires a pre-treatment and refinement (e.g. filtration and decantation). Despite the efficiency and success of first-generation feedstock biorefineries, it is important to consider changes in land-use patterns alongside second- and third-generation feedstock biorefineries. For the first-generation biorefinery, more land will be used for fuel and chemical production instead of for food and feed growing. As a result, more land is deforested to be used for their production. In contrast to the first generation biorefinery, which uses basic food components, the second-generation and especially third-generation biorefineries utilise biomass consisting of residual non-food parts of crops, grasses, wood, or algae, by following the approach of utilising industrial or municipal waste. Non-food, inedible parts of crops, such as grasses and woods, consist of lignocellulosic components.

The conversion of lignocellulosic raw material, such as wheat straw or woods, is more difficult than the direct use of feedstocks by the first-generation biorefinery. A mesh of cellulose and hemicellulose in connection with lignin is formed within such feedstocks that leads to inert and protected sugar components. Highly energy-intensive processes must be used to initially break down this mesh of conserved structures.

In general, the descriptions of first- and second-generation biorefineries can be divided into seven different biorefinery concepts (Diep et al., 2012; Naik et al., 2010; BMBF 2015; VDI), which may be described as follows:

I. Conventional Biorefineries
II. Green Biorefineries
III. Whole Crop Biorefineries
IV. Lignocellulosic Feedstock Biorefineries
V. Two Platform Concept Biorefineries
VI. Thermo Chemical Biorefineries
VII. Marine Biorefineries

In the following chapter, the lignocellulosic feedstock biorefinery (LCF-Biorefinery) will be discussed.

2.2.3. Aspects of the lignocellulosic feedstock biorefinery

Lignocellulosic raw materials are usually forestry residues, (e.g. wood chips as residues of tree-cutting or plank-cutting), agricultural residues (e.g. wheat straw, barley straw, corn stover; dedicated energy crops, such as switchgrass, short-rotation poplar; wood chips from residues of the pruning of vines and fruit-bearing trees), industrial residues (e.g. paper, cardboard), and communal residues (e.g. paper and carton, leaves and grass from gardening and organic municipal residues).

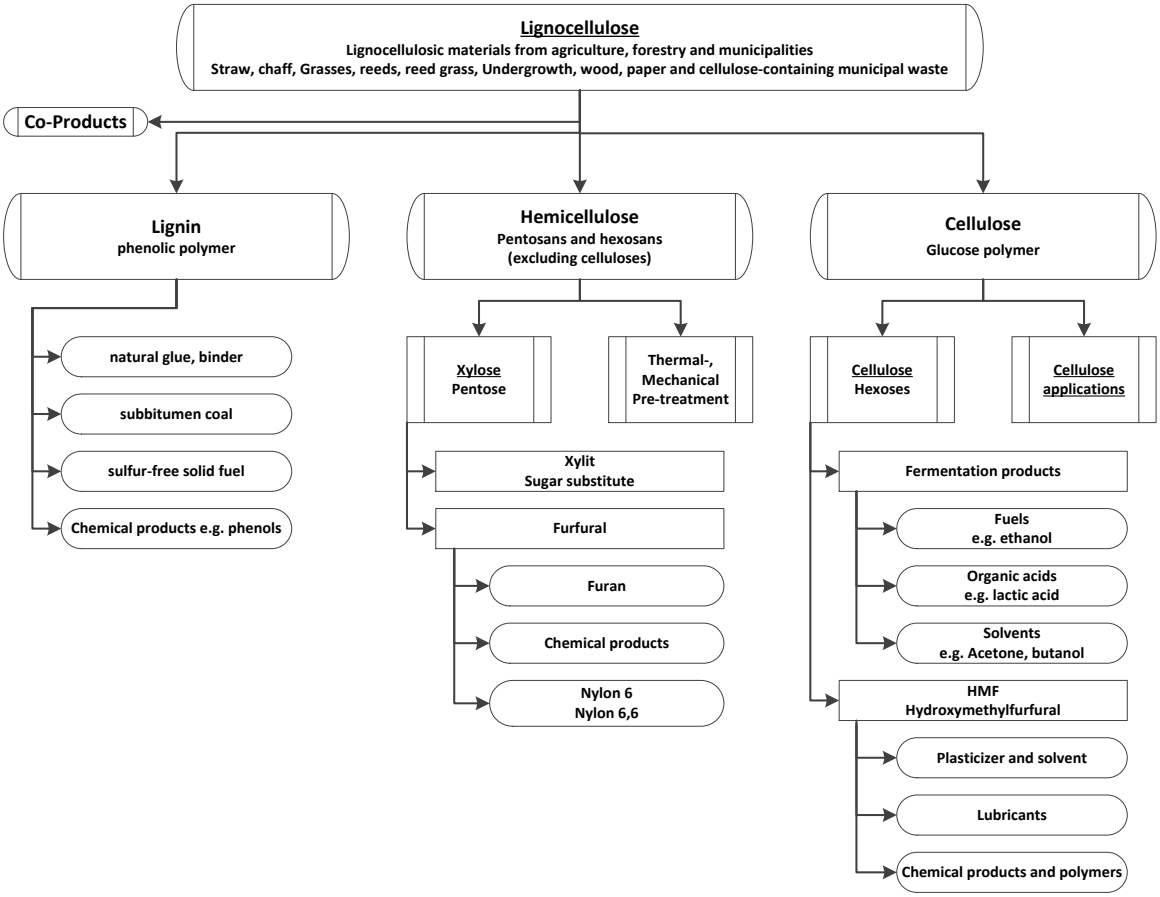


Figure 2.2-2: Lignocellulose feedstock biorefinery concept
(see also Oertel 2006; BMBF 2015; VDI 2016)

2.3. Lignocellulosic biomass

The effective utilisation of lignocellulose–with the component cellulose (35%–50%), hemicellulose (20%–35%), and lignin (12%–20%)–plays a significant role in improving the economic viability of bio-based products and processes. Lignocellulose contains minor components of other polymeric structures: starch, pectin, proteins, minerals, and ash, among others (Gomez et al., 2008; Knauf and Moniruzzaman, 2004; Lynd et al., 2008). The monomeric sugars that are valuable for fermentation–such as glucose, xylose, or arabinose–are only available after a pre-treatment step. This stage typically involves physical, physicochemical, or biological-based methods (Chang and Webb, 2017). Detailed references are provided in Chapter 2.4.

To design a suitable pre-treatment to make the desired components available, it is necessary to understand the complexity of the lignocellulose structure. A representative diagrammatic framework of lignocellulosic biomass is shown in Figure 2.3-1. The general composition of plant cell walls shows they can be subdivided into a primary cell wall (PCW) and a secondary cell wall (SCW). The amount of cellulose, hemicellulose, and lignin varies largely between walls. The secondary wall contains the major portion of cellulose. The middle lamella, which binds to adjacent cells, is mainly comprised of lignin (Pandey, 2009).

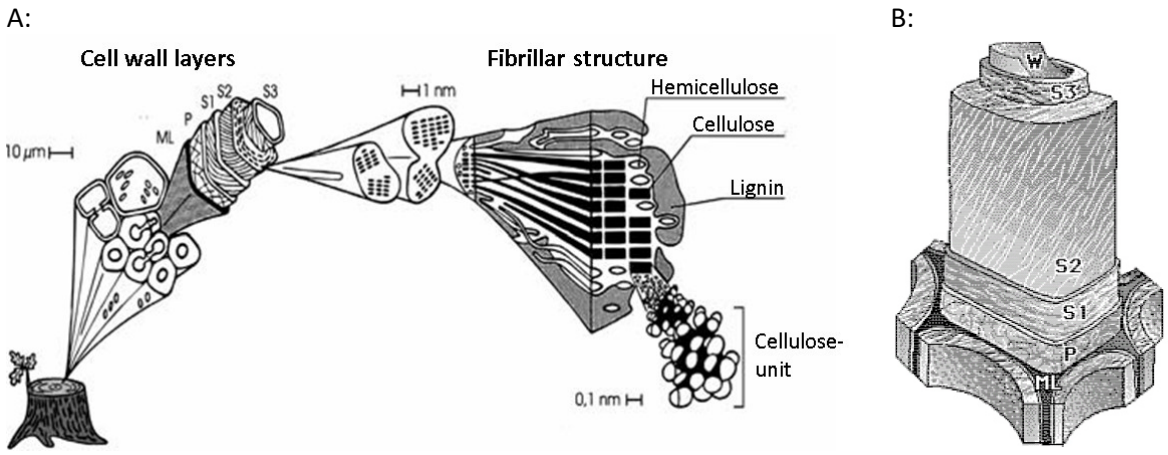
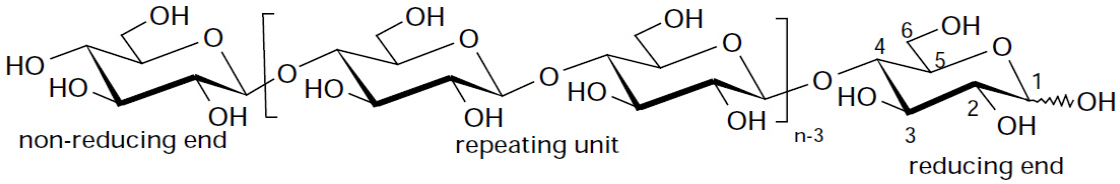


Figure 2.3-1: Lignocellulose structure
A: Schematic illustration of the individual cell's wall layers and their chemical compositions (illustrated by Per Hoffmann) B: Cell wall model with the fibrillar structure of the cell wall's layers.
ML = Middle lamella; P = Primary wall; S = Secondary wall (S1; S2; S3); W = Warty layer.
Adopted from: Fibers in Wood Matrix (Herbert Sixta, Handbook of Pulp, 1, 41, 2006, 3-527-30999-3)

2.3.1. Cellulose

Cellulose is described as a strictly linear syndiotactic polymer of glucose which has a regular alternation of opposite configurations at successive regularly spaced positions along the chain (Menon and Rao, 2012). These glucose molecules are linked together by β-(1-4)-glycosidic bonds linking D-glucopyranose units (anhydroglucose units). Cellobiose is created through a linkage of β-(1-4)-glycosidic bonds and builds the polymeric cellulose chains. Cellulose chains are stabilised by strong hydrogen van der Walls bonds and combine to form microfibrils. The secondary structure of cellulose is shown in Figure 2.3-2. The supramolecular structure leads to the formation of an extremely complex and complicated material in comparison to, for example, starch. Hydrogen bonds can be formed within the same cellulose chain (intramolecular hydrogen bonds) or between the different cellulose chains (intermolecular hydrogen bonds). While the intramolecular bonding is responsible for chain stiffness and conformation, the intermolecular bond is responsible for structures, such as the crystalline domains of fibrils (Menon and Rao, 2012).

A:



B:

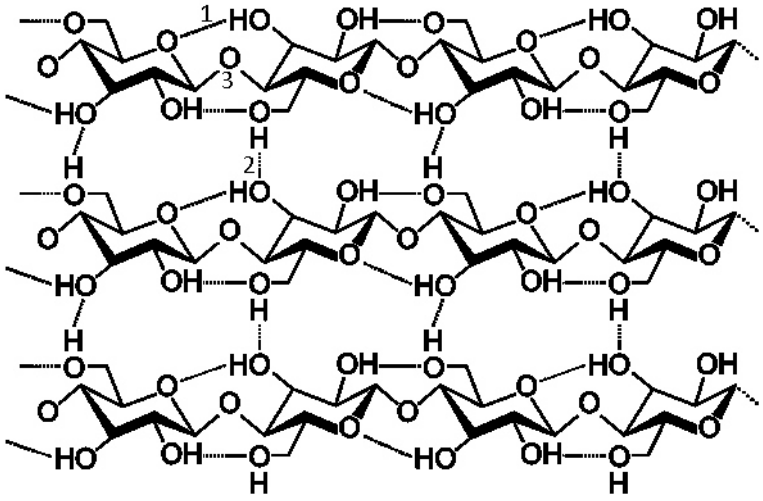


Figure 2.3-2: Secondary structure of cellulose. A: Cellulose strain[a]; B: Cellulose mesh[b];
1: intramolecular hydrogen bond, 2: intermolecular hydrogen bond, 3: β-(1-4)-linkage
[a]: Adapted from: Fibers in Wood Matrix (Herbert Sixta, Handbook of Pulp, 1, 41, 2006, 3-527-30999-3)
[b]: Adapted from: https://en.wikipedia.org/wiki/Cellulose#/media/File:Cellulose_strand.svg (March 2017)

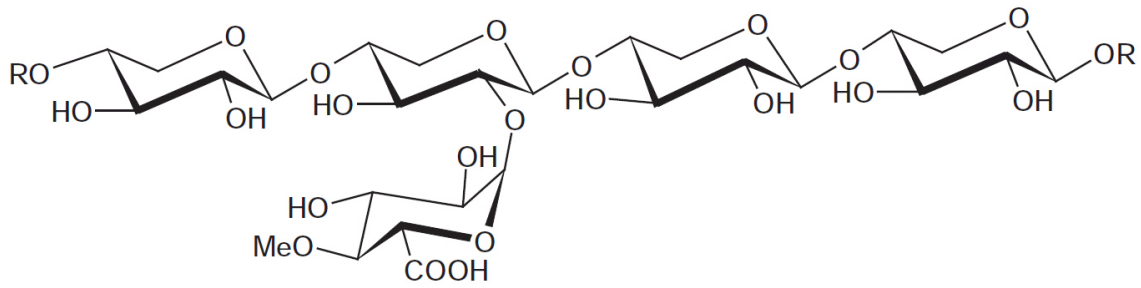
Cellulose microfibrils are attached to each other by hemicelluloses and polymers of other sugars as well as by pectin, and they are covered by lignin. The cellulose microfibrils are often associated with macrofibrils in the form of bundles (Menon and Rao, 2012). The tight packaging of the microfibrils leads to a crystalline cellulose structure (the main segment of cellulose), where not

only enzymes but also small molecules such as water cannot enter, rendering natural cellulose insoluble in water. However, there are amorphous regions, which are areas of less ordered, non-crystalline structures (Arantes and Saddler, 2010). The amorphous regions of the microfibrils are areas of lower polymerisation and are therefore more accessible to cellulolytic enzymes (Lynd et al., 2002; Hallac and Ragauskas, 2011).

2.3.2. Hemicellulose

Hemicellulose is a branched amorphous heteropolymeric chain consisting of different sugar moieties such as D-xylose and L-arabinose as pentoses, D-mannose, D-glucose and D-galactose as hexoses, minor amounts of D-glucuronic acid and of other saccharides. The main hemicellulose chain (backbone) has either a homopolymeric structure (e.g. xylans) or heteropolymeric structures (e.g. glucomannans) with short junctions linked by β-(1,4)- and in some cases by β-(1,3)-glycosidic bonds. They can be classified into four groups: xylans (β-1,4-linked D-xylose units), mannans (β-1,4-linked D-mannose units), arabinans (α-1,5-linked L-arabinose units), and galactans (β-1,3-linked D-galactose units; da Silva et al., 2012). In contrast to cellulose, the polymers in hemicellulose are more accessible (Kumar et al., 2009). Significant differences in the contents of the different hemicellulose components in hardwood and softwood exist. Examples of complex hemicellulose structures are presented in Figure 2.3-3.

A:



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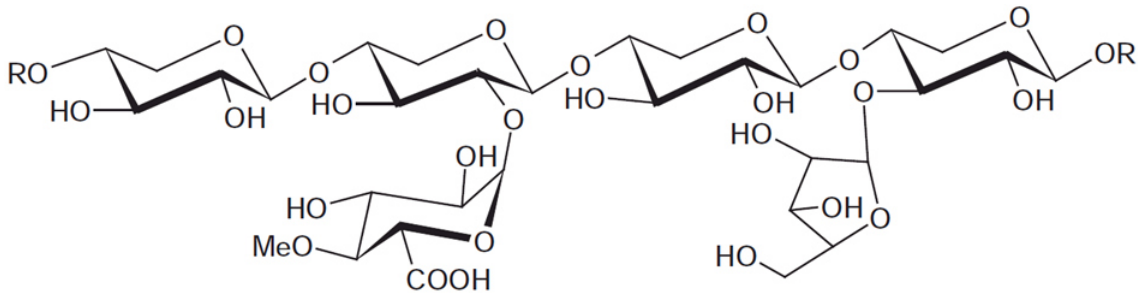
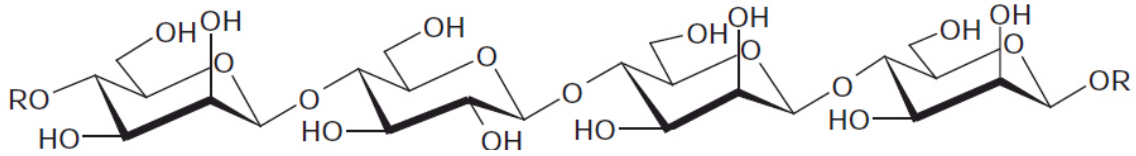


C:



D:

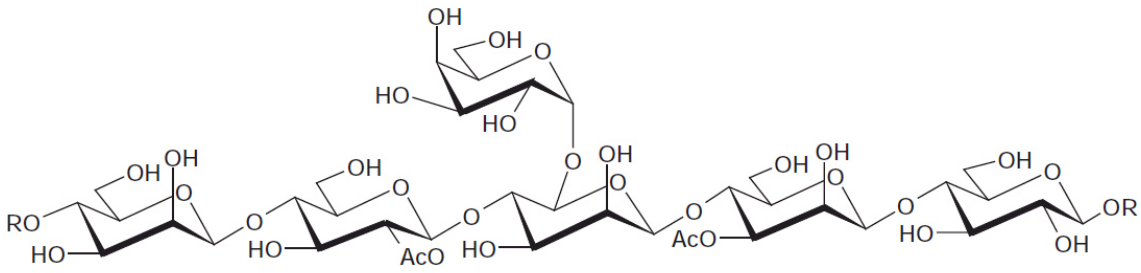


Figure 2.3-3: Main components and molecular structure of xylenes and glucomannans in hardwood and softwood.
A: Hardwood xylan; B: Softwood xylan; C: Hardwood glucomannan; D: Softwood glucomannan
Adopted from: Fibers in Wood Matrix (Herbert Sixta, Handbook of Pulp, 1, 41, 2006, 3-527-30999-3)

Xylan is the most common polymer from hemicellulose. Its polysaccharides include mainly linear chains of β-(1,4)-D-xylanopyranosyl residues. Various agricultural plants consist of xylans, such as straw, sorghum, sugar cane, corn stalks, cobs, hulls, and husks, as well as forest and pulping waste products from hardwoods and softwoods. According to Silva et al. (2012), the following xylan groups can be distinguished according to their structure: arabinoxylans, glucuronoxylans, glucuronoarabinoxylans, heteroxylans, and unsubstituted homoxylans.

2.3.3. Lignin

Lignin is a hydrophobic structure of cross-linked phenolic components (Figure 2.3-4), namely p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Naturally lignocellulosic biomass contains a variable quantity of lignin (25%–33% in hardwood; 20%–25% in softwood) and the ratio of the phenolic component lignin differ between different plants, wood tissues, and cell wall layers (Menon and Rao, 2012). Lignin is the most rigid component of the plant cell wall, which protects the plant against biological degradation. Furthermore, the lignin binds covalently to hemicellulose, which leads to a more complex lignocellulosic structure.

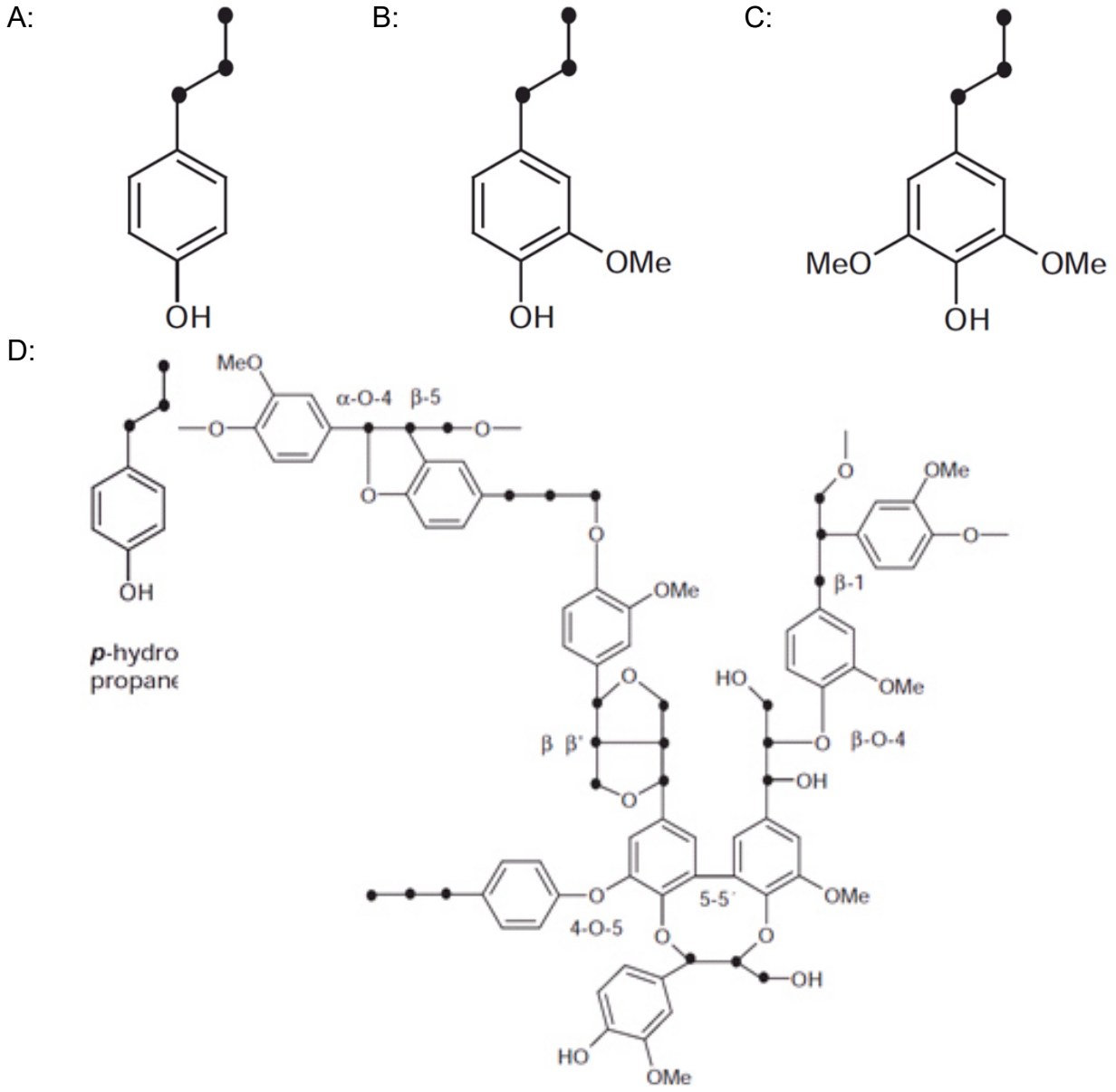


Figure 2.3-4: Molecular structure of the basic phenyl-propane building blocks of lignin and its linkages. A: p-hydroxyphenylpropane; B: guaicylpropane; C: syringylpropane; D: Model of important lignin linkages. Adopted from: Fibers in Wood Matrix (Herbert Sixta, Handbook of Pulp, 1, 41, 2006, 3-527-30999-3).

2.4. Lignocellulose bioconversion

An effective pre-treatment is the first and most crucial step for the efficient production of biochemicals from heterogeneous sources of lignocellulosic biomass. Pre-treatment affords the solubilisation, separation, or conversion of the component cellulose, hemicellulose, and lignin. Numerous methods of pre-treatment are available (Mosier et al., 2005a, 2005b; Sánchez and Cardona 2008; Sun and Cheng 2008; Sánchez 2009; Hendriks and Zeeman, 2009). The type and effectiveness of the pre-treatment have an immense impact on the overall costs of the whole production process, independent of the final target product (Mupondwa et al., 2017). The first impediment that must be resolved for the implementation of a bio-based production process is the removal of lignin during the pre-treatment step (Menon and Rao, 2012; Yang and Wyman, 2008). Lignocellulosic biomass pre-treatment methods include physical, chemical, physicochemical, and biological methods and various combinations of them (Wyman et al., 2005; Mosier et al., 2005a, 2005b; Sánchez and Cardona 2008; Sun and Cheng 2008; Tao et al., 2011). Several major criteria characterise an effective pre-treatment: reducing particle size (PS), limiting the formation of inhibitors and degradation of by-products, minimising energy input, recovering co-products and catalysts, and being cost-efficient (Banerjee et al., 2010). An excerpt of the widely discussed pre-treatment methods is given in Table 2.4-1.

Table 2.4-1: Widely discussed and used pre-treatment methods for lignocellulose biomass

Method	Short description	Main targeted component	Advantages	Disadvantages and limitations
Mechanical	> Size reduction and reduction of cellulose crystallinity by milling or irradiation	> crystallinity	> Increased impact and performance of enzymes	> High power consumption
Acidic	> Chemical pre-treatment using (diluted) acid	> Cellulose > Hemicellulose	> Alteration of the lignin structure	> Formation of inhibitory compounds such as furfural, hydroxymethylfurfural
Alkaline	> Chemical pre-treatment using (diluted) base	> Hemicellulose > Lignin	> Mild process conditions > Potentially higher yields > Low utility cost	> Rates of enzymatic hydrolysis have been the subject of extensive study due to solved inhibitory lignin
Liquid hot water	> Using of water at high temperatures and high pressure	> Hemicellulose > Xylose	> Recovery of high amount of hemicellulose and xylose > No inhibitor or by-products > No washing or neutralisation steps > Generation of high-grade products > No need for intense mechanical pre-treatment	> Higher water usage than steam explosion processes
Steam explosion	> Use of water vapour at high temperatures and high pressure > Instant pressure release to atmospheric pressure results in sudden explosive decompression of the materials and reduced polymerization grade	> Hemicellulose	> Minimal use of chemicals > Applicable to various feedstocks	> Not applicable to softwoods > Steam pre-treated biomass of low bulk density needs washing before hydrolysis and fermentation to remove inhibitors
AFEX	> Physico-chemical alkaline treatment uses periodic exposition of lignocellulose biomass to liquid ammonia at high temperatures and high pressure followed by a rapid release of the pressure	> Hemicellulose > Lignin	> Ammonia recycling is possible > No inhibitors formed > No washing step needed	> Not applicable to biomass with a high lignin content > Recycling of ammonia needed to achieve economically feasible process costs
Organosolv	> Use of hot aqueous organic solvents (e.g. ethanol, methanol, acetone, ethylene glycol, and phenol)	> Cellulose > Hemicellulose > Lignin	> High selectivity to achieve three separate lignocellulosic fractions > Organic solvents are easy to recover by distillation and recycled for pre-treatment; > Chemical recovery can isolate lignin as a solid material and carbohydrates as syrup	> Complex recovery system
Biological Enzymatic	> Cellulases and hemicellulases are used to hydrolyse lignocellulose	> Cellulose > Hemicellulose > Lignin	> Easy and save method > Recovery of enzymes possible	> Other pre-treatment is needed prior to enzymatic hydrolysis

2.4.1. Physical pre-treatment

The physical or mechanical pre-treatment for lignocellulosic biomass is required for reducing the size of particles. Physical methods such as cutting, grinding, and irradiation (e.g. microwave irradiation) are meant to reduce the crystallinity and degree of polymerisation and further increase the surface area, to improve enzymatic hydrolysis or biodegradability (Sánchez and Cardona 2008). Other advantages include lower transportation costs (Sánchez 2009).

The energy required for this treatment depends on the desired final PS, crystallinity, moisture content, bulk density, and the type of lignocellulose biomass (Mani et al., 2004, 2006; Adapa et al., 2011). Once the lignocellulose is exposed to the milling to achieve the needed particle sizes, it can be assumed that the required energy for particle size reduction will become too great for a cost-efficient process. Analyses of comminution in respect to chopping, hammer milling, and physical characteristics of four lignocellulosic feedstocks–barley, oat, wheat straw, and canola (untreated and treated with steam explosion)–show that bulk and PS density increases with a decrease in hammer mill screen size. Adapa et al. (2011) reported that the PS of pre-treated straw was smaller than that of untreated straw. They suggested that milling prior to or following other pre-treatment methods may be beneficial in several ways: a) decreased energy consumption during milling; b) decreased costs for solid/ liquid separation through the converted ratio of solids into liquids; c) decreased energy requirements for mixing pre-treatment slurries; and d) avoidance of inhibitor formation (Zhu and Pan, 2010).

An increased surface area and a reduced degree of crystallisation were shown to lead to an enhancement of biogas production from 5% to 25% (Hartmann et al., 2000). Furthermore, a reduced conversion time from 23% to 59% was shown to be an effect of comminution for different substrates, such as maple leaves and hay stems (Palmowski et al., 2000). Ball milling improved the accessibility of chemicals and catalysts to β-1,4-glycosidic bonds (Zhao et al., 2006). Within the impact of PS, the use of pelletized softwood (Spruce, Pine, and Fir) was examined. Enzymatic treatment (see Chapters 2.4.4. & 2.4.5. for further reference) on pelletized softwood showed that the pellets were as hydrolysable as pre-treated biomass chips (Kumar et al., 2012).

Microwave irradiation is a type of pre-treatment that improves later hydrolyses using cellulosic biomass immersed in an aqueous environment. Microwave treatment is based on the direct interaction between the heated object and an electromagnetic field, which is necessary to create heat (Gabhane et al., 2011). Microwave irradiation leads to an increase in porosity and specific surface area, a decrease in cellulose crystallinity, increased hemicellulose hydrolysis, and partial depolymerisation of lignin (Kashaninejad and Tabil 2011; Zhu et al., 2005; Saha et al., 2008).

2.4.2. Chemical pre-treatment

Chemical pre-treatments involve the use of chemicals: acids, alkali, organic solvents, oxidising agents, and ionic liquids (Fu and Mazza 2010, 2011a, 2011b). In the following chapters, the chemical pre-treatments such as the dilute acid, alkaline, and organosolv methods are further described.

2.4.2.1. Acidic pre-treatment

An acidic pre-treatment uses concentrated or diluted acids to break down the structure of lignocellulose biomass and aims to hydrolyse hemicellulose (80% to 90%; Saha et al., 2005a). A common acidic pre-treatment uses dilute sulphuric acid (H_2SO_4), such as for the hydrolysis of biomass types: switchgrass, corn stover, spruce, rye straw, and poplar (Digman et al., 2010; Li et al., 2010; Du et al., 2010; Xu et al., 2009; Saha et al., 2005b; Shuai et al., 2010; Sun and Cheng 2005; Wyman et al., 2009; Kumar and Wyman, 2009). Other acids, such as hydrochloric acid (HCl), phosphoric acid (H_3PO_4), and nitric acid (HNO_3), are used as well (Wang et al., 2010a; Zhang et al., 2007; Marzialetti et al., 2008).

Owing to their ability to hydrolyse hemicellulose, acids can be used for fractionation of the components of lignocellulose (Zhang et al., 2007). A treatment with acid, which removes hemicellulose, can be done after an alkaline treatment, which removes lignin. Such an approach can result in a relatively clean cellulose fraction. Different conditions for acid pre-treatment are described for several biomass types (Sassner et al., 2008; Silverstein et al., 2007).

2.4.2.2. Alkaline pre-treatment

Alkaline pre-treatment of lignocellulosic biomass uses bases, such as hydroxides (e.g. sodium-, potassium-, calcium-, and ammonium-hydroxide). The alkali method leads to the structural alteration of lignin, cellulose swelling, and partial decrystallisation of cellulose through the degradation of ester and glycosidic side chains and partly solvated hemicellulose (Cheng et al., 2010; Ibrahim et al., 2011; McIntosh and Vancov 2010; Sills and Gossett 2011). One of the most studied bases is sodium hydroxide, which has been shown to break down lignin structures. That breakdown of the lignin structures improves the access of cellulase and hemicellulase enzymes for further hydrolysis of the compound cellulose and hemicellulose (Zhao et al., 2008; Zhu et al., 2010, Hu et al., 2008).

Another alkaline pre-treatment that has been intensively studied is pre-treatment using lime. In lime pre-treatment, the biomass is treated with calcium hydroxide and water under different temperature and pressure conditions (Sierra, Liang et al., 2010; Park et al., 2010). Alkaline

treatment can be performed under ambient conditions. If higher temperatures are used, the treatment times can be reduced. However, an additional step is required prior to enzymatic treatment to remove lignin and inhibitors such as salts, phenolic acids, furfural, and aldehydes (Zhao et al., 2008).

Sierra et al. (2009) have offered the following overview regarding lime pre-treatment, which can be divided into three main utilised methods: (1) a short-term treatment lasting up to 6 hrs using high temperatures over 100°C with a high pressure around 200 psi. (2) A long-term pre-treatment of up to 8 weeks at temperatures in the range of 55°C–65°C. This pre-treatment method can occur under atmospheric pressure with or without air. (3) The simplest pre-treatment requires 1 hr of boiling in water. For lignocellulose biomass with low lignin content (below approximately 18% lignin), non-oxidizing conditions are effective, whereas for feedstock with higher lignin content (above approximately 18% lignin), oxidative conditions are more effective.

2.4.2.3. The organosolv pre-treatment

The organosolv method aims to extract lignocellulose components using hot aqueous organic solvents such as ethanol, methanol, acetone, ethylene glycol, and phenol (Mupondwa et al., 2017). The typical type of alcohol used is a normal primary alcohol. Zhao et al. (2009) found that these are better suited for delignification than secondary or tertiary alcohols. Furthermore, using primary alcohols leads to lower process costs, owing to improved recovery. Therefore, methanol and ethanol are favoured agents for alcohol-based organosolv pre-treatment (Zhao et al., 2009).

Owing to the high selectivity of the organosolv process, it is possible to achieve three separate lignocellulosic fractions: a cellulose-rich solid fraction; an ethanol organosolv sulphur-free low molecular weight lignin fraction; and a water-soluble fraction that contains aqueous hemicellulose sugars and breakdown products of sugars, as well as degraded lignin and other by-products (Pan 2005; Pan et al., 2005, 2006b). With those processes, residual lignin can be recovered during the organophilic stage (Mabee et al., 2006). A further advantage of organosolv processes is that the reduction in size of biomass is not necessary, in contrast to acid or alkaline pre-treatments (Pan 2005). This saves money and time that would otherwise be needed in physical pre-treatments. One more advantage of organosolv over chemical pre-treatments is the easy recovery of the organic solvents by distillation. A flowchart showing an example of an ethanol and water based organosolv pre-treatment is shown in Figure 2.4-1.

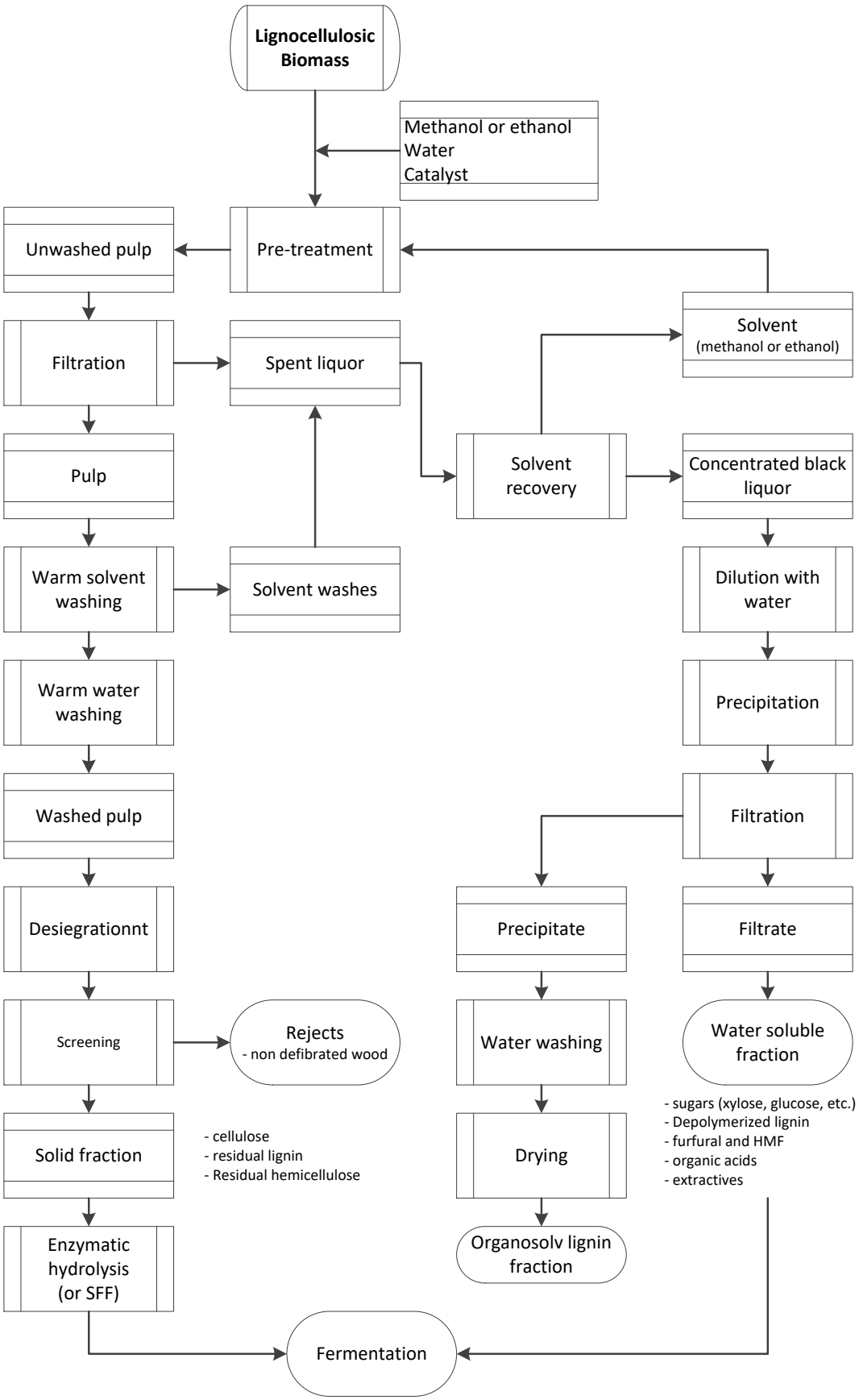


Figure 2.4-1: Flowchart of an ethanol/water based organosolv process.
(adopted from Pan et al., 2006a and Zhao et al., 2009)

2.4.3. Physicochemical pre-treatment

Combined pre-treatments of physical and chemical methods are referred to as physicochemical pre-treatments. The principal methods of this group of pre-treatments include catalysed steam explosion (CO_2; SO_2), ammonia fibre expansion (AFEX), and ammonia recycle percolation (ARP)–an AFEX variant (Kim and Lee 2005), liquid hot water, and microwave-chemical pre-treatment (Wyman et al., 2005; Mosier et al., 2005a, 2005b; Sánchez and Cardona 2008; Sun and Cheng 2008; Tao et al., 2011; Brodeur et al., 2011).

2.4.3.1. Steam explosion pre-treatment

Steam explosion pre-treatment processes for biomass (Mosier et al., 2005a; Sánchez and Cardona 2008; Sun and Cheng 2008) are also referred to as auto-hydrolysis (Chandra et al., 2007), and involve the use of high-pressure saturated steam. Such a process is designed to solubilise hemicellulose, which results in higher accessibility of cellulose for enzymatic hydrolysis. During the treatment, the high pressure is held for a few seconds and then suddenly released. This pressure reduction leads to a sudden decompression of the materials.

Typical process parameters for steam explosion are temperatures of 160°C–260°C, with a pressure range of 0.69–4.83 MPa prior to the exposure to atmospheric pressure (Sun and Cheng 2008). The interval time ranges from a few seconds to a few minutes prior to exposure to atmospheric pressure, which leads to the explosive decompression and the end of the process (Varga et al., 2004; Ruiz et al., 2006; Kurabi et al., 2005). Steam explosion processes have several advantages when compared to other pre-treatments: lower energy requirements than mechanical pre-treatments; lower chemical use and therefore less environmental impact; lower dilution of released carbohydrates; lower recycling costs; and applicability to various feedstocks except for softwood (Sun and Cheng 2008). They can be further improved through the addition of catalysts, such as H_2SO_4, CO_2, or SO_2, enhancing hemicellulose recovery (Mosier et al., 2005b; Sassner et al., (2008); Sun and Cheng 2008; Mabee et al., 2006). Negative aspects of the steam explosion include the formation of inhibitory compounds and the need for washing low bulk density steam-pre-treated biomass prior to fermentation to remove inhibitory compounds such as phenolic acids, furfural (a pentose by-product), and hydroxymethyl-furfural (a hexose by-product; Palmqvist and Hahn-Hägerdal 2000a).

2.4.3.2. Liquid hot water pre-treatment

Liquid hot water pre-treatment processes can also be described as hydrothermal methods, as they involve hot water at high pressure (Yu G. et al., 2010a, 2010b; Kobayashi et al., 2009; Ingram et al., 2009; Yu Q. et al., 2010, Mosier et al., 2005a; Sánchez and Cardona 2008). Liquid hot water pre-treatment methodologically differs from steam pre-treatment because of the state in which the water is used. The steam explosion uses water in a gaseous phase, while the liquid-hot water treatment uses the water in an aqueous phase. The process variables range from 170°C to 230°C, with a pressure higher than 5 MPa (Sánchez and Cardona 2008). Owing to the higher amount of water used in liquid hot water pre-treatment, the yield concentration is lower, despite a higher amount of usable hydrolysed biomass in this process (Hendriks and Zeeman, 2009; Laser et al., 2002). Negatively, this process requires a large amount of water. Positively, this method can increase enzymatic digestibility of cellulose that leads to improved sugar extraction (xylose recovery from 88% up to 98%), and improved hemicellulose recovery (80% to 100%), while having lower titres of inhibitory compounds (Kim et al., 2009, Mosier et al., 2005b; Hendriks and Zeeman 2008; Sánchez and Cardona 2008; Perez et al., 2007, 2008).

Positively, the resulting hydrolysate can be directly fermented, since no additional chemicals are needed for the pre-treatment, and no inhibitors are formed during the process that might affect the fermentation (Mosier et al., 2005b; Kobayashi et al., 2009; Kim et al., 2009). A further advantage of this method is that an additional pre-treatment step for PS reduction is not required due to the structural breakdown that occurs as a result of the process itself.

2.4.3.3. Ammonia fibre expansion

Ammonia fibre expansion (AFEX) is a physicochemical alkaline pre-treatment. In this process, the lignocellulosic biomass is periodically exposed to liquid ammonia at high temperatures and pressures for a short period prior to a sudden release (Teymouri et al., 2005). AFEX pre-treatment leads to a de-crystallisation of cellulose and partial depolymerisation of hemicellulose. Furthermore, acetyl groups, mainly from hemicellulose, are removed, and lignin-carbohydrates complexes and lignin C-O-C linkages are cleaved. These effects lead to increased surface accessibility due to the breakdown of the biomass structure (Gollapalli et al., 2002; Kumar et al., 2009). An advantage of the AFEX method is that there is a possibility of recovering ammonia, which is crucial for the reduction of costs and of environmental impact of this process. Further advantages are the high reallocation of lignin and the fact that no inhibitory by-products are formed that can affect further processing steps (Sun and Cheng 2008). However, AFEX is known as an inefficient process in biomass with high lignin contents such as woods and nutshells (Chandra et al., 2007; Taherzadeh and Karimi, 2008).

Typical process parameters for an AFEX process are 1–2 kg of ammonia per kilogram of dry biomass. The temperature should range from 70–200°C with a pressure holding time of 30 mins at 100–400 psi (690–2,760 kPa; Bals et al., 2010; Zheng et al., 2009). The ammonia percolation method ARP is also worth mentioning. This method uses aqueous ammonia (10–15%wt) at temperatures ranging from 150–170°C, with a residence time of 14 min and a fluid velocity of 1 cm/min (Galbe and Zacchi, 2007).

2.4.4. Biological pre-treatment

Biological pre-treatment involves white-, brown-, and soft-rot fungi, and bacteria such as lignocellulose-degrading MOs. They secrete enzymes that can increase the accessibility for hydrolysing enzymes, or directly degrade biomass to oligo- and monosaccharides (Sánchez 2009; Talebnia et al., 2010).

Fungi show specific characteristics for lignocellulose handling. While brown-rot fungi mainly attack cellulose, minor lignin modifications have been described. White- and soft-rot fungi access lignin components using their lignin- and cellulose-degrading enzymes, which leads to a better hydrolysis performance (Sun and Cheng, 2002; Sánchez, 2009).

The main advantage of biological treatment using fungi is the safety of the handling, due to a smaller chance of explosion and toxicity, and the fact that it requires less energy input. The method does not produce inhibitory compounds or chemical residues due to the use of chemicals for hydrolysation, such as sulfuric acid. Thus, it can be considered as an environmentally friendly method. However, the method is slower than chemical treatments and requires a high level of control and effort for processing and a lot of space (Salvachúa et al., 2011; Mupondwa et al., 2017). Current research aims to find organisms for improved efficacy and specific lignin degradation, and for improved enzymes, where white-rot fungi are mainly considered (Eggeman and Elander 2005).

Using the three families of enzymes–cellulases, hemicellulases and lignin-modifying enzymes–in the form of concentrated enzyme mixtures, therefore alkalinising or acidifying the lignocellulose pre-treatment method, has been described as an ideal alternative (Hong et al., 2013). However, enzymatic hydrolysis of lignocellulose biomass is limited by economic considerations arising from enzyme production costs and specific enzyme activity (Goldbeck et al., 2016). Performance improvements have been achieved by optimising the enzyme production strains through genetic engineering and through the optimisation of enzyme production processes (e.g. protein purification). Further improvements have been made in the development and optimisation of pre-treatment methods to limit inhibitory effects, and in the optimisation of the enzyme mixture (Goldbeck et al., 2016, Zhang et al., 2010).

2.4.4.1. Cellulases saccharification systems for cellulose hydrolysis

Cellulases are a group of enzymes with catalytic activity towards cellulose. They have a central role in enzymatic pre-treatment because they catalyse the hydrolysis of cellulose to water-soluble and fermentable sugars. Fungi, bacteria, and plants produce cellulases. Menon and Rao (2012) provide references related to MOs that can produce cellulases. Three types of cellulase activity have been described as participating in the cellulose hydrolysis process (Figure 2.4-2). The degradation of cellulose and hemicellulose polymeric structures requires two different types of enzymes: (1) Endo-enzymes, which cut the bonds inside a polymeric chain, resulting in lower molecule mass polymers, and (2) exo-enzymes, which degrade the ends of polymer chains, hydrolysing oligomers to monomer sugars. These three types of cellulase activity are

(1) Endoglucanases, also known as 1,4-β-D-glucan-4-glycanhydrolases (EC 3.2.1.4), which break the inner glycosidic-linkages of the amorphous cellulose regions, resulting in freed polysaccharides of lower polymerisation and soluble oligosaccharides.

(2) Exoglucanases such as 1,4-β-D-glucan glucanohydrolases (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91), which lead to glucose and cellobiose. The glucanhydrolase liberates glucose monomers from the ends of the cellulose chain. Cellobiohydrolases liberate cellobiose from the reducing end (cellobiohydrolase I) and the non-reducing end (cellobiohydrolase II) of the cellulose chain.

(3) The third type of cellulase includes the β-glucoside glucohydrolases (β-1,4-glucosidases) (EC 3.2.1.-21), which degrade cellobiose to glucose and break down the cellulose chain by hydrolysing β-1,4- bonds (Henrissat et al., 1998; Lynd et al., 2002).

These three different types of cellulase enzymes must be used together in a mixture for complete hydrolysis of cellulose into glucose. However, it is known that the cellobiohydrolases and glucanohydrolases and β-1,4-glucosidases suffer end-product inhibition by glucose (de Castro et al., 2010; Lynd et al., 2002). This means that with increasing glucose concentration in the hydrolysis medium, the activity of the enzymes will decrease.

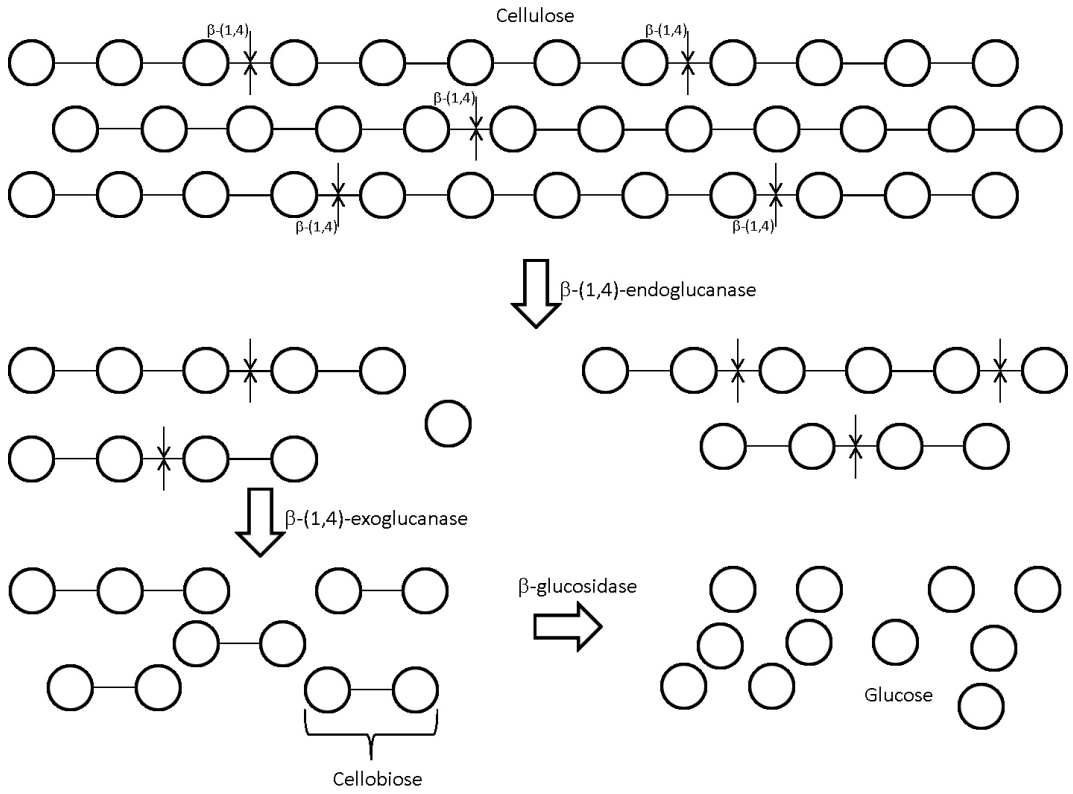


Figure 2.4-2: Efficiency in the cooperation of members of the cellulase enzyme system. (adopted from Mussatto and Teixeria, 2010)

The catalytic enzymes in a mixture, as shown in Figure 2.4-2, can be used separately as individual enzymes, used as a mixture, or linked together as a multi-enzymatic complex (named 'cellulosome'). The single enzymes consist of a catalytic domain that is responsible for the hydrolysis reaction, while a cellulose-binding domain mediates the binding of the enzyme to the substrate. Both domains are conjugated by a linker peptide that enables effective orientation and function. Enzymes that are conjugated as cellulosome are noncovalently bound to the cellulosome-integrating protein that carries a carbohydrate-binding domain (also named as a carbohydrate-binding module; Carrad et al., 2000). There are 64 families of the carbohydrate-binding domain, and they are defined by their specific amino acid sequences (Cantarel et al., 2009).

Lignin is known to inhibit cellulose activity due to irreversible binding to the enzyme (Kumar et al., 2010, 2011). Therefore, a process for lignin separation from cellulose and hemicellulose is used prior to enzymatic hydrolysis for a higher hydrolysis activity. Research has been focused on the improvement of the enzymatic hydrolysis process. Strategies for improvements are based on enhancing cellulase activities, increasing thermostability, lowering vulnerability to inhibition, resisting shear force, increasing acidic tolerance, optimising the reaction conditions and enzyme and substrate mixture compositions, using enzymes from different sources, recovering enzymes, and developing recycling strategies (Maki et al., 2009; Gusakov et al., 2007, Miller and Blum 2010; Carvalho 2011; Zhou et al., 2009).

2.4.4.2. Hemicellulases

Hemicellulases are multi-domain proteins that are comparable to the cellulosome. They generally consist of structurally separated catalytic and non-catalytic domains (Shallomv and Shoham, 2003). Hemicellulases can be either glycoside hydrolases, which hydrolyse glycoside bonds, or carbohydrate esterase, which hydrolyse ester linkages of acetate or ferulic acid (FA) side chains (Kulkarni and Shendye, 2003).

Hemicellulases improve cellulose hydrolysis by increasing the accessibility of cellulose through a higher exposition of cellulose fibres. However, the enzymatic hydrolysis of hemicellulose also requires the cooperation of several enzymes. Like cellulase enzymes, hemicellulose-degrading enzymes are categorised into endo- and exo-acting enzymes as well as into additional accessory enzymes. Endo-acting enzymes have low efficiency on short oligomers, while exo-acting enzymes are effective on both short- and long-chain substrates. The accessory enzymes are required for the breakdown of lignin-linked glycosidic bonds (Himmel et al., 2010).

Xylan is the main component of the hemicellulose complex in lignocellulose, and it mainly consists of xylopyranosyl residues linked by β-1,4-glycosidic bonds. The structure of xylan and activities of the xylan-degrading enzymes are shown in Figure 2.4-3. Hydrolysis requires the action of an intact enzyme system (Decker et al., 2009). Endo-xylanases hydrolyse the glycosidic bonds of the β-1,4- or β-1,3- linked, unsubstituted xylan backbone to produce β-D-xylopyranosyl oligomers. The enzymes β-D-xylosidases cleave xylooligosaccharides such as xylobiose from the non-reducing terminus, liberating β-D-xylopyranosyl residues. Specifically, the endo-1,4-β-D-xylanase (β-1,4-D-xylan, xylan hydrolase; EC 3.2.1.8) cleaves β-1,4-glycosidic bonds to unbranched xylooligosaccharides, such as xylotriose and xylobiose, and β-xylosidases (β-1,4-D-xyloside xylohydrolase; EC 3.2.1.37) cleave short-chain xylooligosaccharides and xylobiose to release xylose. The endo-1,3-β-D-xylanosidase (β-1,4-D-xylan, xylanohydrolase; EC 3.2.1.32) cleaves β-1,3-glycosidic bonds. The acetylxylan esterase (EC 3.1.1.72) and FA esterase (EC 3.1.1.73) and p-coumaric acid esterase are required to liberate O-acetyl groups from β-D-xylopyranosyl of acetylated xylan, and aromatic acids FA, and p-coumaric acid linked to arabinose units. These enzymes are important in the degradation of xylan because the acetyl and aromatic groups can inhibit the approach of endo-xylanases that digest the backbone. For the breakdown of the side chains, the enzymes α-L-arabinofuranosidase (EC 3.2.1.55) release α-arabinofuranosyl residues from the β-D-xylopyranose component. The enzyme α-glucuronidase (EC 3.2.1.139) degrades the α-1,2-bonds between β-D-xylopyranosyl units and glucuronic acid (and also their O-methyl ethers) (Saha et al., 2005a, 2005b; Flores et al., 1997; Biely 2003; Gilbert and Hazlewood 1993 Biely et al., 2003; Polizeli et al., 2005).

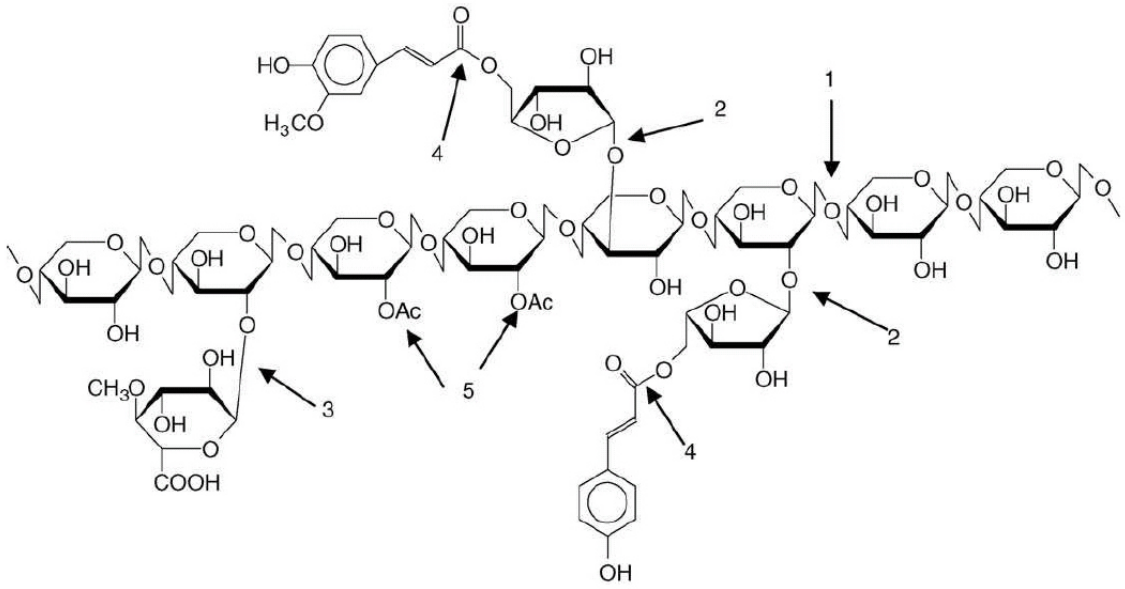


Figure 2.4-3: The structure of xylan and activities of the xylan-degrading enzymes.
1: endoxylanases; 2: α-L-arabinofuranosidases; 3: α-glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases (Chavez et al., 2006)

A further main sugar component in hemicellulose is galactoglucomannan, which consists of galactose, glucose, and mannose. For the complete breakdown of the galactoglucomannan, a combination of enzymes is required: endo-1,4-β-mannanases (EC 3.2.1.78) and exo-acting β-mannosidases (EC 3.2.1.25), as well as additional enzymes, such as β-glucosidases (EC 3.2.1.21), α-galactosidases (EC 3.2.1.22), and acetyl mannan esterase (Dhawan and Kaur 2007; Wyman 2003; Paulechka et al., 2003). Various organisms can produce these enzymes: fungi, bacteria, yeast, marine algae, protozoans, and more (Collins et al., 2005; Polizeli et al., 2005). Menon and Rao (2012) provide more information regarding the MOs that produce hemicellulases.

2.4.4.3. Lignin-modifying enzymes

Lignin-modifying enzymes (also known as ligniases or lignases) catalyse the breakdown of lignin biopolymers. These enzymes use an oxidative mechanism. The family of the lignin-modifying enzymes includes peroxidases, such as lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16), and phenoloxidases of the laccase type. Lignin-modifying enzymes are mainly produced by species of white-rot fungi. Some peroxidases have been reported for species of filamentous bacteria. However, combinations of fungal lignin-modifying enzymes, peroxidases, and laccases (multicopper oxidase) have only achieved effective lignin degradation with organic charge-transfer mediators. For more information on current research related to this topic, please see Lundell et al. (2010).

2.4.5. Enzyme characterisation

Enzymatic hydrolysis is one of the main strategies for the release of sugars from lignocellulosic biomass. Because of the complexity of the lignocellulose and its recalcitrance to hydrolysis, the polysaccharide-degrading enzymes lack efficiency in lignocellulose biomass hydrolysis. Therefore, cellulases with higher performances must be discovered or engineered and characterised. Efforts to improve the efficacy and efficiency of the cellulases and the whole process to increase yields and ensure competitive prices are required.

The large-scale cultivation and strain improvement by genetic manipulation in prokaryotes has focused its attention on the isolation of newer lignocellulose-hydrolysing enzymes. Different fungi and bacteria (e.g. *Aspergillus niger*, Fusarium spp. *Paenibacillus sp., Bacillus sp. and Aeromonas sp. Paenibacillus sp*.) have been used generally for the synthesis of cellulases and hemicellulases using diverse substrates and conditions (Cattaneo et al., 2018; Pachauri et al., 2017; Farjana and Narayan, 2018, deb Dutta et al., 2018, Imran et al., 2018; Listyaningrum et al., 2018). To provide an optimised process for enzyme production, several culture parameters such as pH, temperature, substrate concentration, incubation period, and carbon sources must be optimised. Cellulases can be identified through sequencing or tests for cellulolytic activities.

However, the function of enzymes and mixtures of enzymes must be validated under different environmental conditions. The performance of lignocellulose-degrading enzymes differs based on their optimal conditions for temperature. Herein, enzymes usually are characterised on different artificial substrates, simulating their main substrate with the type of molecular binding (e.g. β-1,4-glycosidic bonds). Common substrates are filter paper (FP), used in the filter paper assay (FPA) for saccharifying cellulase, carboxymethyl-cellulose, used in the CMC-ase assay for endo-3-l,4-glucanase, cellobiose, salicin, or p-nitrophenyl-β-D-glucopyranoside for the characterisation of β-glucosidase (Ghose, 1987; Tian et al., 2011). The activity of hemicellulases is typically tested on xylan derivates such as birch wood xylan.

2.5. Fermentation from hydrolysed lignocellulose biomass-derived soluble mono- or oligosaccharides

Pre-treated lignocellulosic biomass can be processed using process configurations, such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated biomass processing (CBP).

SHF can be described as a conventional serial two-step process: in the first step, the hydrolysis of the lignocellulose biomass is performed, while in the second step, the fermentation is finished. Owing to this, the two steps can be run with the conditions that are optimal for each individual step.

In SSF, the saccharification of the lignocellulosic raw material occurs using direct supplementation of cellulases in the lignocellulosic growth medium, while the fermentation process of the sugars simultaneously takes place in the same bioreactor. The challenge of the SSF process lies in optimising the compatibility of the saccharification and the fermentation processes in terms of process conditions such as pH, temperature, and substrate concentration (Ansul et al., 2007; Wee and Ryu 2009; Shi et al., 2015; Bai et al., 2016; Hu et al., 2016; Overbeck et al., 2016; Wang et al., 2017; Grewal and Khare 2018). An advantage for enzymatic hydrolysis is its enhanced rate, due to the removal of the hydrolytic end-product, which thus decreases end-product inhibition.

SSCF is an option for the production from xylose-rich hydrolysates (Eiteman et al., 2008). The co-fermentation of the glucose and xylose have been reported to occur in genetically engineered microbes for ethanol production but can also be achieved by choosing the most suitable wild-type MOs, such as *B. coagulans* for LA production (Glaser and Venus 2017). SSF and SSCF both result from the integration of processes that offer cost reduction benefits (Hahn-Hagerdal et al., 2006).

CBP uses MOs equipped with heterologous cellulase systems (naturally available, available by artificial laboratory evolution (ALE), or genetically engineered) to utilise lignocellulosic directly (Mazzoli et al., 2014) while product formation occurs such as LA or ethanol. This leads to the possibility that the hydrolytic cellulases can be directly produced by fermenting hydrolysed sugars. Another approach is to co-cultivate the producing MO with cellulolytic MOs (Shahab et al., 2018) as a strategy to allow lignocellulose biomass fermentation (Hasunuma et al., 2012).

In the work discussed here, a hybrid approach between the SHF and SSF/SSCF strategies is used. The addition of enzymes was done at least one day before the inoculation with MO, to initiate the hydrolysation of the lignocellulose. This approach allowed for the collection of information regarding the enzyme performance on the substrates during the start of the process. The data

was used to optimise the mathematical model derived by enzymatic hydrolyses screening tests for yield prediction.

2.5.1. Lactic acid-producing microorganisms using lignocellulose sugars

With a long history in industrial processes, LA bacteria (LAB) are also used extensively in food fermentation applications, including fermentative production of LA. Additionally, LAB are used as biocontrol agents (e.g. acidifier and flavour-enhancing agents) and as probiotics (e.g. as food starters) in the cosmetics industry (as an emulsifying and moisturising agent) and in the pharmaceutical industries. LAB are potential candidates to produce ethanol, polyhydroalkanoates, sweeteners, and exopolysaccharides (Mazzoli et al., 2014; Tarraran and Mazzoli, 2018). In general, LAB have complex requirements; most LAB are auxotrophic for several amino acids, nucleotides, and vitamins, which must be provided as supplements in the growth media for their nutrition. Therefore, with a few exceptions, LAB seemingly cannot directly ferment inexpensive feedstocks such as lignocellulose (Tarraran and Mazzoli, 2018). These properties are limitations for the use of LAB in economically feasible biorefinery processes, since most LA is produced by the bioconversion of dedicated crops.

LA is a multipurpose component chemical and nowadays is used as a monomeric building block for the biocompatible and biodegradable plastic polylactic acid (PLA). The chemical synthesis of LA is feasible, but results in a racemic mixture of D- and L-lactic acid. The racemic LA mixture is not suitable to produce PLA, leading to chain breaks in the polymer. Racemic LA is also not suitable for use as a food or pharmaceutical supplement, as it causes metabolic disturbances (Jem, van der Pol and de Vos 2010). In biological production, one must depend on LAB strains with specific genes to produce either L- or D-lactic acid, or if desired, a mixture of both. The biotechnologically produced PLA is expected to provide a potentially sustainable plastic alternative to petroleum-derived plastics (Abdel-Rahman et al., 2011, 2013, 2016; Pornkamol Unrean, 2018). Owing to the use of LA as a multi-purpose chemical, the global demand for LA has increased rapidly (with an expected annual growth of 16.2%; deOliveira et al., 2018). Producing LA by the bioconversion of dedicated crops is a threat to food crops. Accordingly, the increasing global demand for LA requires the development of fermentation processes that are based on second-generation raw materials (e.g. lignocellulosic feedstocks). This remains a high priority for the extensive application and development of LAB use in biorefineries.

Lignocellulosic material, cellulose, and hemicellulose can be hydrolysed into hexoses (D-glucose, D-mannose, D-galactose) and pentoses (D-xylose, D-arabinose) for fermentation (Bezerra and Ragauskas, 2016). From an economic perspective, strains capable of fermenting mixtures of hexoses and pentoses derived from lignocellulosic feedstocks are needed. Currently, a high number of LAB strains have been isolated and identified from plant environments, such as

fermented vegetables, leaves, soil, or the gastrointestinal tracts of herbivores. Many of these LAB possess the ability to utilise a variety of soluble sugars, including both hexoses (e.g. glucose and galactose) and pentoses (e.g. xylose and arabinose; Kandler 1983), which are also common components derived from lignocellulose hydrolysis.

LA-producing MOs can be classified by their metabolic characteristics into homofermentative and heterofermentative organisms (Kandler, 1983). While homofermentative LABs catabolise sugars through glycolysis (Figure 2.5-1) and convert these through pyruvate reduction to LA, the heterofermentative conversion of sugar involves the phosphoketolase pathway. In heterofermentative metabolism, equimolar mixtures of LA and ethanol or acetic acid are produced (Kandler 1983), an adverse outcome that makes the production process inefficient. The efficient metabolisation of hexose and pentose sugars is essential for hemicellulose fermentation (Jordan et al., 2012). Studies have shown that *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Leuconostoc lactis* can metabolise xylose and arabinose through heterofermentative metabolism (Tanaka et al., 2002; Okano et al., 2009a, 2009b). Another pathway for pentose metabolism was identified as a shift from xylose catabolism of the phosphoketolase pathway to the pentose-phosphate pathway (Figure 2.5-2), which results in a homo-lactic conversion (Tanaka et al., 2002; Okano et al., 2009a, 2009b). Another possibility for the utilisation of sugars would be the metabolism of oligosaccharides that are available because of partial or incomplete hydrolysis of cellulose and hemicellulose (Galazka et al., 2010; Lane et al., 2015). Oligosaccharides are less effectively hydrolysed in an extracellular environment and are transported into the cytoplasm for further metabolism (Desvaux 2006), as has been described for starch and sucrose metabolism (Figure 2.5-3). Figures 2.5-1, 2.5-2, and 2.5-3 show the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps based on the KEGG Orthology (KO) database. The KEGG pathway maps are designed as networks of KO nodes, representing high-level functions of the organism and cell (Kanehisa et al., 2000, 2016, and 2017).

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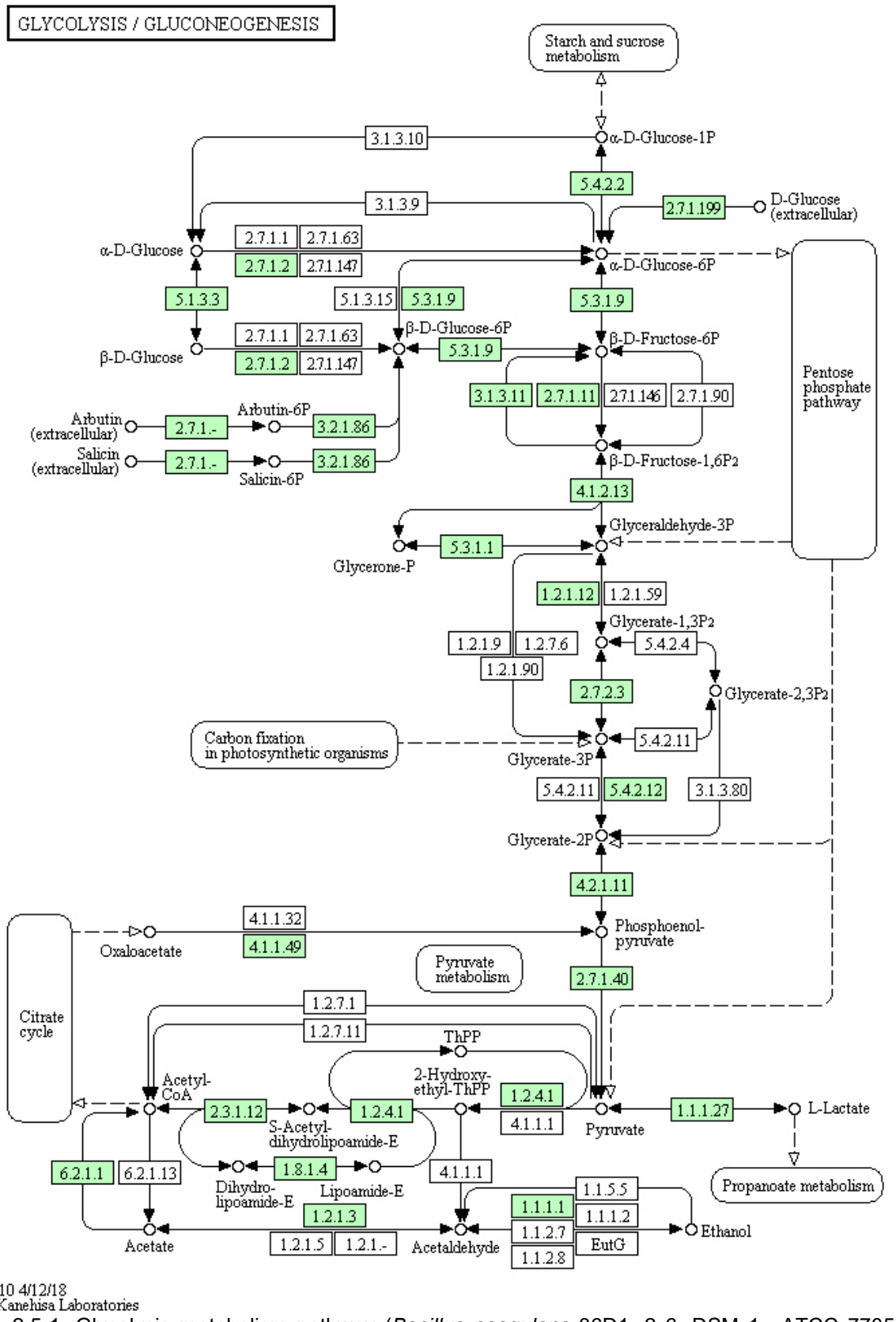

Figure 2.5-1: Glycolysis metabolism pathway (*Bacillus coagulans* 36D1, 2 6, DSM 1= ATCC 77050)
Glycolysis is the process of converting glucose into pyruvate and generating small amounts of ATP (energy) and NADH (reducing power). It is a central pathway that produces important precursor metabolites: six-carbon compounds of glucose-6P and fructose-6P and three-carbon compounds of glycerone-P, glyceraldehyde-3P, glycerate-3P, phosphoenolpyruvate, and pyruvate. Acetyl-CoA, another important precursor metabolite, is produced by oxidative decarboxylation of pyruvate. When the enzyme genes of this pathway are examined in completely sequenced genomes, the reaction steps of three-carbon compounds from glycerone-P to pyruvate form a conserved core module, which is found in almost all organisms and which sometimes contains operon structures in bacterial genomes. Gluconeogenesis is a synthesis pathway of glucose from noncarbohydrate precursors. It is essentially a reversal of glycolysis with minor variations of alternative paths. https://www.genome.jp/kegg-bin/show_pathway?bag00010

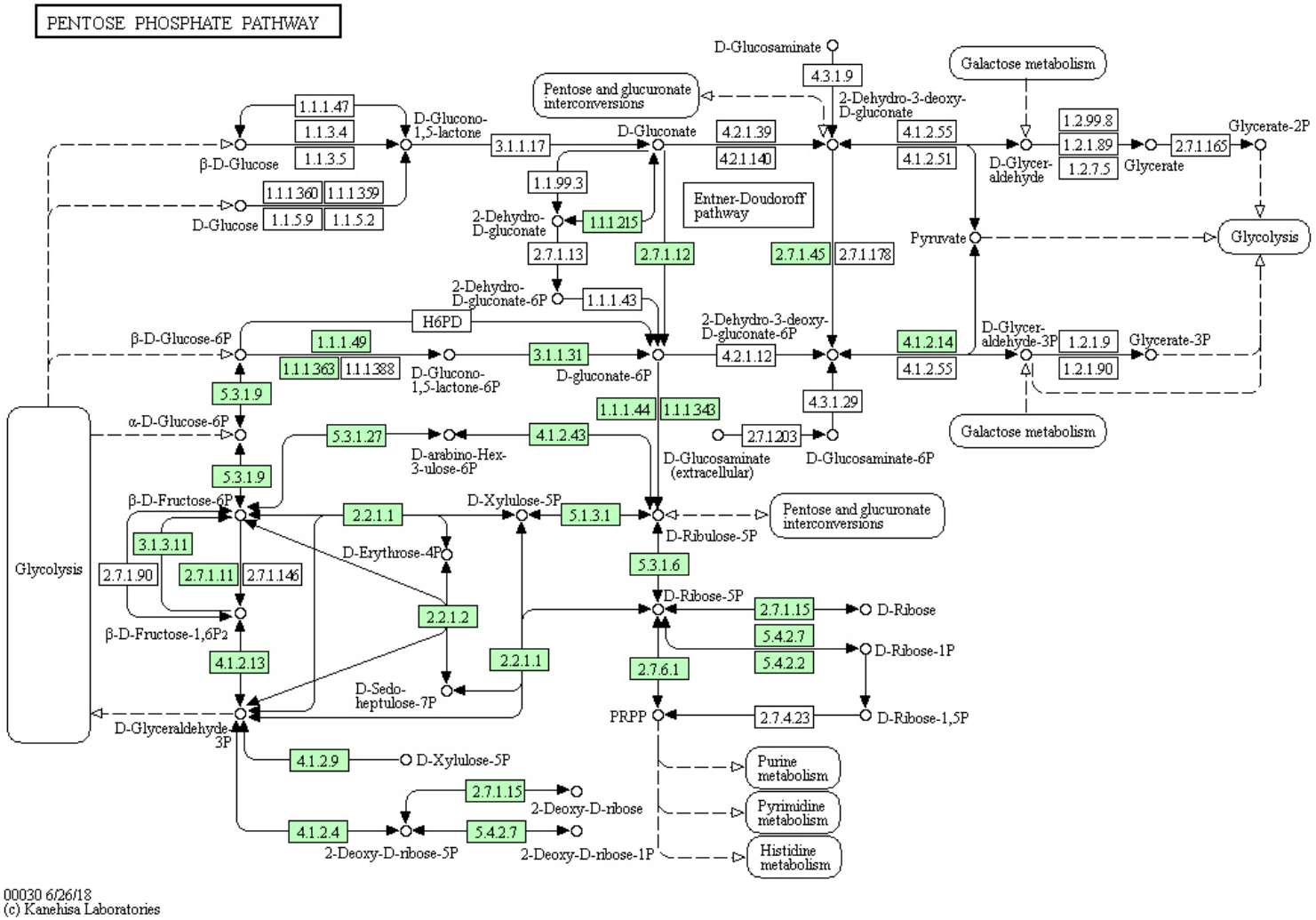

Figure 2.5-2: Pentose phosphate metabolism pathway (*Bacillus coagulans* 36D1, 2-6, DSM 1= ATCC 77050) The pentose phosphate pathway is a process of glucose turnover that produces NADPH as reducing equivalents and pentoses as essential parts of nucleotides. There are two different phases in the pathway. One is the irreversible oxidative phase, in which glucose-6P is converted to ribulose-5P by oxidative decarboxylation, and NADPH is generated. The other is the reversible non-oxidative phase, in which phosphorylated sugars are interconverted to generate xylulose-5P, ribulose-5P, and ribose-5P. Phosphoribosyl pyrophosphate (PRPP) formed from ribose-5P is an activated compound used in the biosynthesis of histidine and purine/pyrimidine nucleotides. This pathway map also shows the Entner-Doudoroff pathway, where 6-P-gluconate is dehydrated and then cleaved into pyruvate and glyceraldehyde-3P. https://www.genome.jp/kegg-bin/show_pathway?bag00030

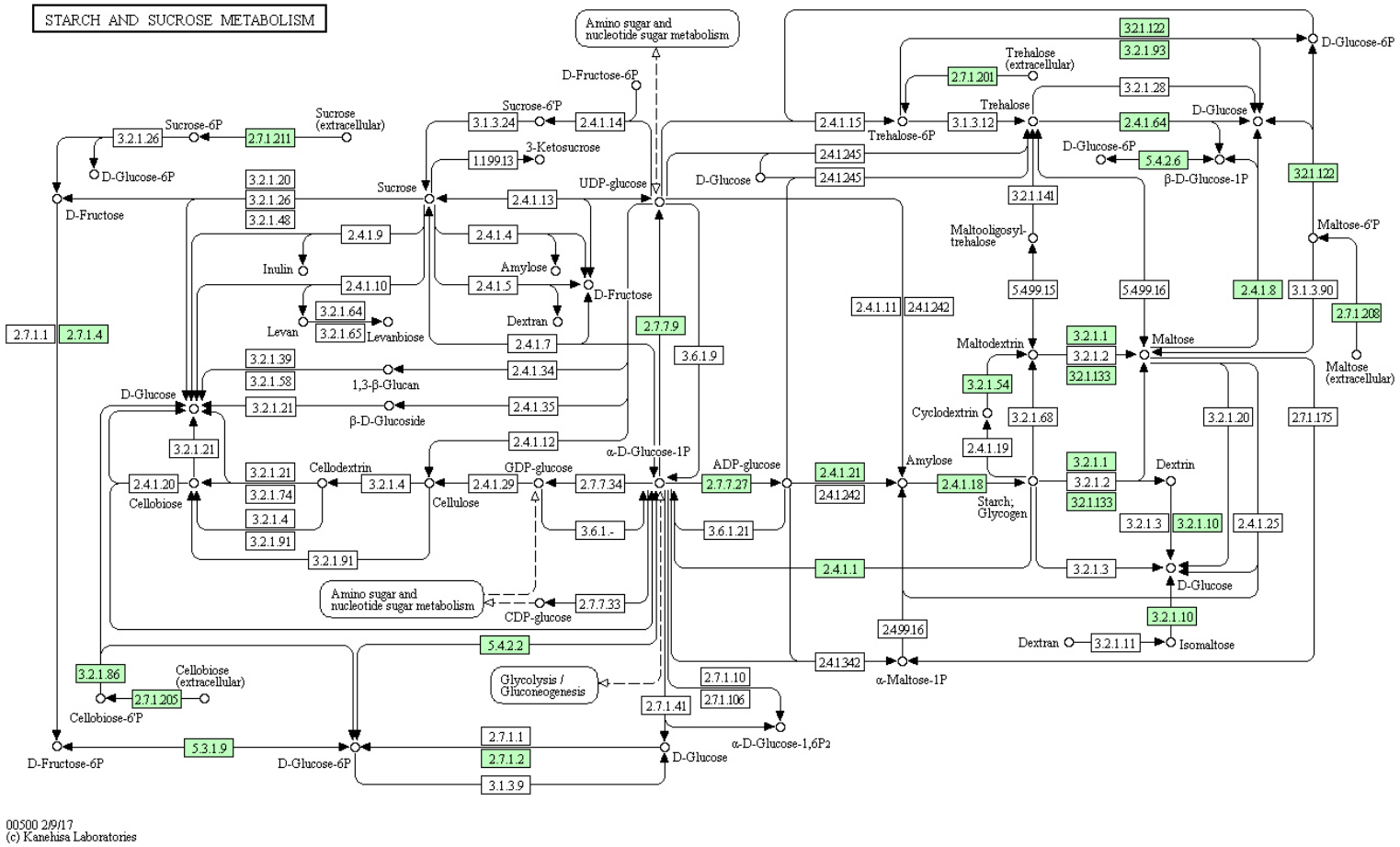

Figure 2.5-3: Starch and sucrose metabolism pathway (*Bacillus coagulans* 36D1, 2-6, DSM 1= ATCC 77050)
https://www.genome.jp/kegg-bin/show_pathway?map00500

For a better overview of how the shown metabolic pathways fit together, the metabolic pathway of the Carbone metabolism is shown in Figure 2.5-4.

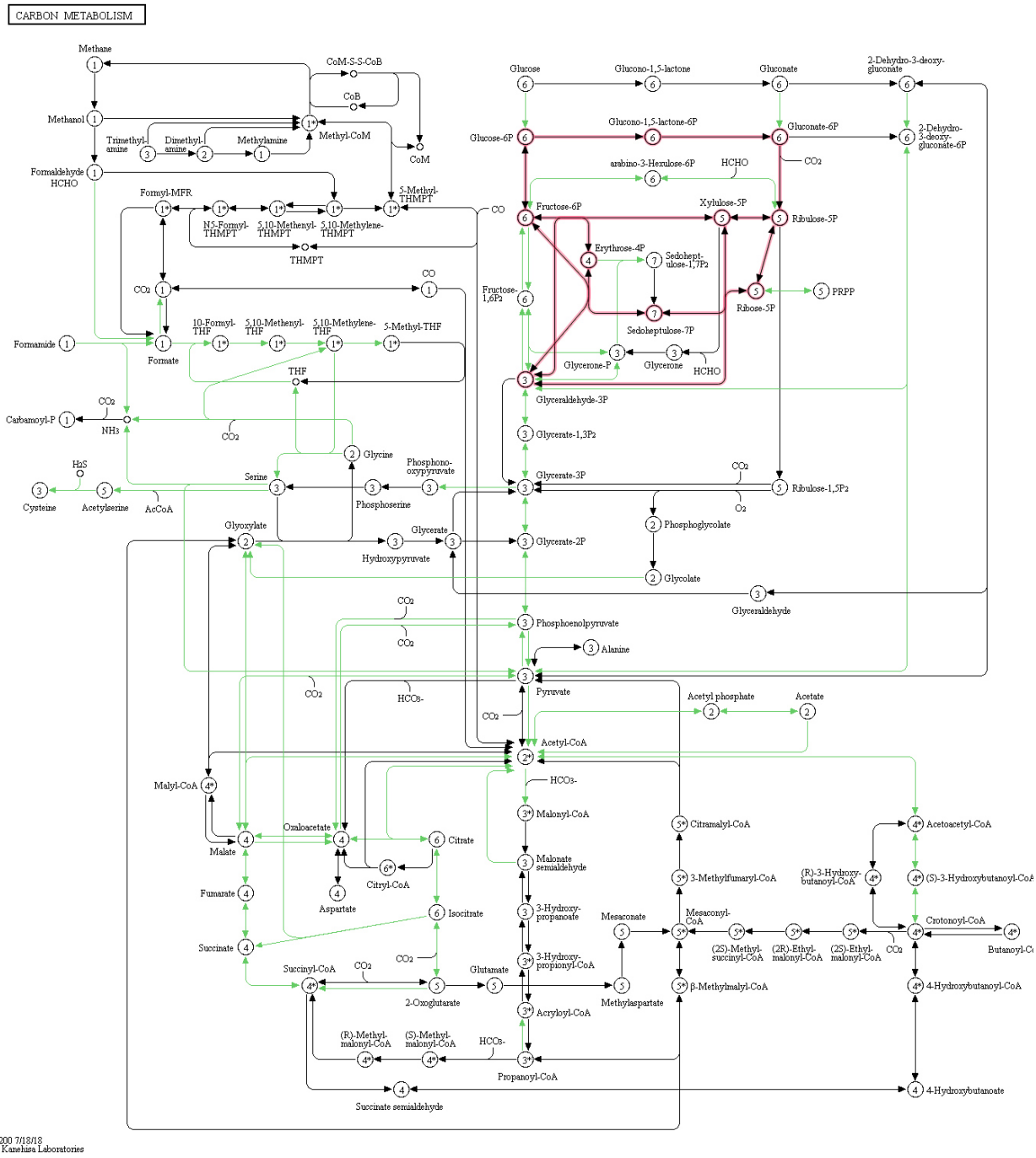

Figure 2.5-4: Carbon Metabolism Pathway (*Bacillus coagulans* 36D1, 2-6, DSM 1= ATCC 77050) Pentose-phosphate-pathway is highlighted in red) Carbon metabolism is the most basic aspect of life. This map presents an overall view of central carbon metabolism, where the number of carbons for each compound is denoted by a circle, excluding a cofactor (CoA, CoM, THF, or THMPT) that is replaced by an asterisk. The map contains carbon utilisation pathways of glycolysis, pentose phosphate pathway, and citrate cycle, and six known carbon fixation pathways as well as some pathways of methane metabolism. The six carbon fixation pathways are (1) reductive pentose phosphate cycle (Calvin cycle) in plants and cyanobacteria that perform oxygenic photosynthesis, (2) reductive citrate cycle in photosynthetic green sulphur bacteria and some chemolithoautotrophs, (3) 3-hydroxypropionate bi-cycle in photosynthetic green nonsulphur bacteria, two variants of 4-hydroxybutyrate pathways in *Crenarchaeota* (4) this is called hydroxypropionate-hydroxybutyrate cycle and (5) dicarboxylate-hydroxybutyrate cycle, and (6) reductive acetyl-CoA pathway in methanogenic bacteria. https://www.genome.jp/kegg-bin/show_pathway?org_name=bag&mapno=01200&mapscale=&show_description=hide&show_module_list=

2.5.2. *Bacillus coagulans* and lactic acid

Bacillus coagulans is a Gram-positive, thermophilic, spore-forming, and facultative anaerobe soil bacterium. It was first isolated and described in 1915 by B. W. Hammer within the genus *Bacillus* (Hammer, 1915). Among its probiotic effects (Khalighi et al., 2014), it can be effectively used for the fermentation of lignocellulosic sugars into LA due to its capability to homofermentatively convert both hexose and pentose sugars to L-(+)-lactic acid. It possesses a unique pentose phosphate pathway (Figure 2.5-2) that reaches its optimal functionality at 50–55°C and pH 5.0. These are also optimal conditions for commercially available fungal cellulases (Hasunuma et al., 2012). With hydrolysis and fermentation both in thermophilic temperature ranges, processes such as SSF can be achieved at optimal temperature conditions.

B. coagulans DSM 2314 has shown its potential as an L-(+)-lactic producer from lignocellulose sugar fermentation derived by lime-pre-treated wheat straw. Bischoff et al. (2010) have described the enrichment of xylose-fermenting thermophilic bacteria from dairy manure compost using elevated temperatures. While wild-type strains of *B. coagulans* are producers of optically pure L-(+)-lactic acid from both glucose and xylose (Ou et al., 2011), no natural strains have yet been identified as capable of the synthesis of D-(–)-lactic acid. Using genetically engineering methods, a derivative *B. coagulans* strain, P4-102B, was designed to produce D-(±)-lactic acid at 50°C and pH 5.0 via the expression of a mutated form of a glycerol dehydrogenase (Wang et al., 2011).

3. Research questions and aims of the project

For a forward-looking, sustainable, bio-based economy that aims to produce biobased products, research focusing on biochemicals, production and product life cycles, and industrial applications is essential. New strategies to guarantee the supply of energy and resources must be investigated to replace fossil fuel resources. The activation of regenerative resources by bioconversion is a fundamental part of this approach.

Besides the increasing demand for basic chemicals, organic acids, and solvents such as LA and ethanol, there are also the ethical challenges that have to be dealt with, such as determining the economic utilisation of space and deforestation for energy-crop plants. To handle these challenges, biorefineries of newer generations tend to increasingly use forestry and agriculturally managed lignocellulosic raw materials, biomass by-products and biomass wastes. The large-scale application of biomass feedstocks will produce innovative research that will lead to the development and implementation of technologies, and biorefineries will be used as multi-feedstock and multi-product facilities.

This work discusses two different aspects of lignocellulosic bioconversion into biochemicals: the use of enzymatic systems for lignocellulose hydrolysation and the co-fermentation of lignocellulosic sugars by *Bacillus coagulans* into L-(+)-lactic acid. This should enable and improve the conversion processes in industrial (non-food) applications, by supplying fermentable sugars from lignocellulosic non-starchy plants, such as soft- and hardwoods, grass, and straw. Using commercially available and new enzymes–kindly provided by Dr. Arkady Sinitsyn, Moscow State University–hydrolyses of different lignocellulosic raw materials are conducted.

One goal of this work is the development and optimisation of methods to characterise enzymes and hydrolyses. Fermentations are conducted with the prepared hydrolysates. Here, the potential of MOs, according to the utilisation performance of different lignocellulosic sugars (hexoses, pentoses, oligosaccharides), and their tolerance against inhibitory compounds (soluble lignin), are characterised.

The following key aspects will be addressed: the characterisation of used lignocellulosic substrate to achieve quality control parameters and the identification of the hydrolysis potential of usable enzyme mixtures of cellulases and hemicellulases on basic and industrial feasible substrates. Further key aspect addressed is the characterisation of LA-producing MOs in terms of their sugar utilisation and inhibitor tolerance, providing key performance parameters by optimising and defining mathematical models and simulations.

The thesis' central point of view might be more useful and profitable for small and mid-sized industry, such as agricultural family businesses, with a lignocellulose-based biorefinery for production of bioenergy or biochemicals. The questions to be discussed are

Q1. *Are the current approaches to measure the standard kinetic units–FPU, CMU, CBU XU–enough to characterise cellulases or cellulase mixtures?*

The background for this question is the significance of researchers' use of 25 FPU as a standard amount of cellulases for their processes. In these studies, a critical examination of the real effect of the cellulases on the lignocellulosic substrate did not take place. To get a reliable quality parameter, the characterization and preparation of lignocellulose feedstock prior an enzymatic hydrolysis process is crucially needed.

Q2. *Is it possible to define a set of kinetic units to be able to predict a yield on an actual lignocellulose substrate prior to hydrolysation?*

The current set of standardised kinetic units are not suitable to predict hydrolyses yields of actual lignocellulose subtracts. The standard kinetic units are based on standardised substrates and are used to characterise and achieve information on the diverse enzymes in a cellulase mixture.

Q3. *Is it possible to use LA-producing bacteria and lignocellulose hydrolysates, with their different sugar components and occurring inhibitors, to produce LA?*

This question is aimed at analysing the sugar components–glucose, xylose, arabinose, and cellobiose–which should be fermented to LA. Furthermore, the inhibitory component of lignin should be considered.

Q4. *Is it possible that the results of the work can be used to facilitate the work of small and medium-sized enterprises?*

It is not easy for small and mid-sized enterprises, such as family business, to optimize their processes: to use the right amount of cellulases, further pre-treatment of the lignocellulose raw materials and yield prediction.

4. Results

The first part of this work examines how to derive quality parameters based on the physical properties of milled lignocellulose raw materials with different PS distributions. It is shown that the specific knowledge about physical parameters, such as of the PS distribution–determined by the Sauter mean diameter (SMD)–can significantly improve the enzymatic hydrolysation process. Different lignocellulose raw materials are covered in the tests, such as wheat straw, aspen wood (AW), pinewood, and dried grass. Continuing to the subject of lignocellulose hydrolysation, the performance of cellulases and hemicellulases are evaluated. The evaluation gives information on the expected performance and yield prediction based on the actual substrate characteristics and available cellulase mixtures. Furthermore, the evaluation of how effective the fermentation process of the lignocellulosic sugars is for creating a product of value–in this case, the production of L-(+)-lactic acid produced by *Bacillus coagulans* strains–is discussed. Therefore, the second part of the work focuses on the evaluation of utilisable sugars, such as glucose, arabinose, xylose, and cellobiose. Concerning growth performance, the resistance to inhibitors, such as lignin, FA, or vanillin (VAN), and their possible utilisation was reviewed. This was accompanied by an estimation of kinetic process parameters for process evaluation using mathematical model equations.

4.1. Enzyme-based lignocellulose hydrolyzation–Sauter mean diameter of raw materials as a basis for cellulase performance characterisation and yield prediction. [1]

4.1.1. Abstract

The characterisation of cellulase performance for industrial-scale processes holds special challenges. A huge gap exists between the enzyme characterisation in a laboratory and in large-scale process performances. Common cellulase mixtures, from Novozymes and enzymes provided by Moscow State University, were used to hydrolyse wheat straw, grass, pine wood (PW), and AW. Glucose yields from the enzymatic hydrolysis of the raw materials were investigated as a function of cellulase enzyme loading and of PS with different solid loading. The PS had a significant effect on glucose yield, while the enzyme concentration had a smaller effect. Hydrolyses of sets of wheat straw particles were used to introduce a substrate-specific kinetic enzyme unit. The data was used to generate an empirical model to predict the glucose yield based on the SMD of the feedstocks.

4.1.2. Introduction

The limited fossil resources will bring social challenges in the short term. Various attempts have been made to reduce humans' dependency on fossil resources by substituting fossil-based chemicals with chemicals that are manufactured from renewable biomass, like lignocellulose. A wide range of lignocellulosic raw materials (e.g., corn stover, cereal straw), forestry residues (e.g., logging residues, sawmill waste), and purpose-grown crops (e.g., short-rotation poplar, switchgrass) have been considered as possible feedstocks (Apprich et al., 2014; Baker et al., 1997; Boussaid et al., 1999; Dinus, 2000; Pan et al., 2005; Prückler et al., 2014; Kim and Dale, 2004; Tirpanalan et al., 2014; Ahokas et al., 2014). Lignocellulosic raw materials contain cellulose, hemicellulose, and lignin (Jäger and Büchs, 2012). Cellulose and hemicellulose, such as polymeric carbohydrates, formed from glucose (Glc), xylose (Xyl), and arabinose, amongst others, can be depolymerized by hydrolyzation through acid, alkali, or enzymatic treatments (Camesasca et al., 2015; Hahn-Hägerdal et al., 2007; Pedersen et al., 2011; Saha, 2003). Therefore, lignocellulosic raw materials can serve as a potential substrate to produce basic chemicals like LA or ethanol through biotechnological microbial fermentation (Chandel et al., 2010). The aforementioned chemical pre-treatment processes (e.g. dilute acid, alkali, or organosolv treatments) have been evaluated in terms of their hydrolyzation, but the formation of complex sugars and inhibitory compounds was also noted (Pan et al.,

[1] Based on: Robert Glaser, 2015. Enzyme-based lignocellulose hydrolyzation – Sauter mean diameter of raw materials as a basis for cellulase performance characterization and yield prediction. Journal of Biotechnology 214; 9–16. http://dx.doi.org/10.1016/j.jbiotec.2015.08.024

2005; Rasmussen et al., 2014; Wyman et al., 2005; Zheng et al., 2013; Tu et al., 2009; Guo et al., 2014). These compounds have a negative influence on the further enzymatic treatment and biotechnological fermentation process (Glaser and Venus, 2014; Klinke et al., 2004; Kumar et al., 2009; Pedersen et al., 2011; Palmqvist and Hahn-Hägerdal, 1996).

For the hydrolysis of lignocellulose, most research efforts have been recently focussed on the development of enzyme-catalysed processes. On the one hand, the benefit of enzymatic hydrolyzation of lignocellulosic raw materials are the low inhibitory compound concentration. On the other hand, using enzymes creates special challenges, such as how to characterise the mixtures of cellulase enzyme performance for industrial processes. A standard tool for cellulase characterisation is the determination of the filter paper units (FPU) through the FPA (Ghose, 1987). However, the FPA appears to be an unsatisfying reference for cellulase performance, because it is based on a sole cellulose substrate and neglects the properties of natural lignocellulosic raw materials. For instance, accessory enzymes influence the performance of the cellulase mixtures. Enzymes that hydrolyse hemicellulose (xylanases) or lignin-degrading enzymes (lignin peroxidases) assist in the hydrolysis of complex lignocellulose materials (Baker et al., 1997; Berlin et al., 2005a, 2005b, 2006; Menon and Rao, 2012; Tenkanen et al., 1999). Additional enzymes allow for the adaption of cellulase mixtures, with the aim of improving their performance on lignocellulosic raw materials and their different compositions of cellulose, hemicellulose, and lignin. Therefore, it is necessary to know more about the mode of action of cellulase enzymes in its complex substrates.

Among other things, the performances of enzymes depend on the physical properties of cellulose and hemicellulose fibres, like crystallinity and PSs (Hall et al., 2010). A pre-treatment was set to produce a more susceptible substrate to the enzyme, for example, the reduction of the crystallinity of lignocellulose by reducing the PS through mechanical pre-treatment of the raw materials (Pedersen and Meyer, 2009).

The presented data in this work focus on the characterisation of cellulase performance for industrial purposes. A kinetic parameter will be introduced based on the PS distribution of wheat straw (WS). The most important parameter is the SMD. It provides a possible method to approximate the hydrolysis performance of inhomogeneous substrates. The presented data in this work is based on WS particles of five different sets of PS: 630–800 µm, 315–630 µm, 250–315 µm, 125–200 µm, and below 125 µm. This set of PS reflects a possible range of PS of milled WS. After enzymatic hydrolyses are done with sets of commercially available enzymes from Novozymes and cellulase provided by Moscow State University, glucose yields are compared. The results are used to establish a mathematical model to predict the performance of hydrolyses based on the SMD of inhomogeneous substrates.

4.1.3. Materials and methods

4.1.3.1. Cellulase preparations

Novozymes (Novozymes, Denmark) provided Cellic CTec2 (CT2) and Cellic HTec2 (HT2), which are both commercial cellulase preparations. The cellulase activity of CT2 cellulase lies in the range of 100 FPU/mL and 3,950 U/mL of cellulase and β-glucosidase, respectively, according to Alvira et al. (2013), 128 FPU/mL and 4,465 U/mL β-glucosidase according to Erdei et al. (2012), and 150 FPU/mL according to Lan et al. (2012). According to Cannella et al. (2012), CT2 has a protein amount of 161 mg/mL and activities of 120.5 FPU/mL and 2,731 U/mL β glucosidase units. According to Alvira et al. (2013), HT2 has an activity of 1,300 U/mL of xylanase activity. Dr. Arkady Sinitsyn of Moscow State University kindly provided four newly developed cellulase preparations, hereinafter marked as R1, R2, R3, and R4. R1 had a cellulase activity of 640 FPU/g_P, CMC-ase activity of 22,320 U/g_P, and a xylanase activity of 27,658 U/g_P. R2 as a β-glucosidase had an activity of 128,000 U/g_P. R3 had a cellulase activity of 740 FPU/g_P, CMC-ase activity of 19,741 U/g_P, and a xylanase activity of 26,205 U/g_P. The β-glucosidase R4 had an activity of 98,000 U/g_P.

4.1.3.2. Protein determination

The protein concentration of the enzyme mixtures was determined using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich Chemie GmbH, Germany), according to the manufacturer's recommendations.

4.1.3.3. Hydrolyses for the determination of wheat straw units

The following method describes the determination of the WS-based kinetic activity units (WSU) based on an altered FPA. In contrast to the FPA, the FP was exchanged by milled WS. Sets of milled WS of different PS had been prepared (630–800 µm, 315–630 µm, 250–315 µm, 125–250 µm, and lower than 125 µm). A four-point determination was done twice, using different concentrations of enzymes. Stock solutions of cellulases were prepared with 2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL protein amount of cellulases. The amount of 200 mg of WS was weighed out directly in the 15 mL tubes (Carl Roth GmbH + Co. KG) and charged with 2 mL of 50 mM sodium acetate buffer at pH 5. The sodium acetate buffer was prepared with sodium acetate (Carl Roth GmbH + Co. KG) and was set to pH 5 with sodium hydroxide (Carl Roth GmbH + Co. KG). The mixture of WS and buffer were preheated to 52°C, as were the enzymes. Next, 2 mL of the enzyme solution was put into the WS-filled tubes. The used concentrations of cellulase protein solution were 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL. The closed tubes were then

placed on a planar shaker at 100 rpm in combination with an incubator at 52°C for 60 min (Certomat U, B. Braun Diessel Biotech). To stop the hydrolysis, the tubes were placed in a boiling water bath for 10 min. Afterwards, they were cooled down to -20°C and stored for further use.

4.1.3.4. Technical-scale hydrolyses

Naturally dried and milled WS, AW, PW, and dried grass were selected as lignocellulosic raw materials. AW and PW were kindly provided by Dr. Arkady Sinitsyn of Moscow State University. The hydrolyzation medium was autoclaved at 121°C for 60 min. After resting for 12 hrs, a second autoclavation was done to prevent process contaminations that might come from capsuled MOs in the feedstock. For hydrolyses, several enzyme loadings were used (ranging from 25 FPU/g_s to 75 FPU/g_s). Hydrolyses were run for 48 hrs at pH 5.0. The temperature during this time was kept constant at 52°C.

Hydrolyses took place in Erlenmeyer flasks and in stirred tank reactors. Shaking flask experiments were carried out in an Erlenmeyer flask charged with 200 mL at pH 5, 52°C and 5% w/v of solid loading. The flasks were shaken at 100 rpm with a planar shaker (Certomat U, B. Braun Diessel Biotech) with a longitudinal motion of 0.025 m. In comparison to this system, hydrolyses were carried out in 2 L and 10 L stirred tank reactors, Biostat B. (Braun/Satorius AG). The WS was used at 5% w/v and 7.5% w/v, and the AW and PW were used at 5% w/v. Working volumes were 1 L for the 5% w/v or 1.5 L and 8 L for hydrolyses at 7.5% w/v of solid loading. The 2 L reactor was equipped with two six-blade Rushton turbines and its physical parameters were d = 0.054 m, d/D = 0.42, h_{low}/D = 0.08, h_{high}/D = 0.32, and H_{fill}/D = 0.71 at 300 rpm. The stirrer setting had a power number of 6.6. The reactor of the 10 L scale was also equipped with two six-blade Rushton turbines with a combined power number of 7.2 and parameters of d = 0.07 m, d/D = 0.42, h_{low}/D = 0.71, h_{high}/D = 1.05, and H_{fill}/D = 1.58 at 300 rpm. The torque data for the estimation of the power number is given in Glaser (2015).

To inactivate the samples, they were placed in a hot water bath at 92°C for 30 min. After inactivation, they were stored in the refrigerator at -8°C for further use. After thawing the samples, they were centrifugated (Sigma 4k15 centrifuges) at 5000 rpm for 20 min. The supernatant was filtered with a 20 µm cellulose acetate membrane micro filter (Th. Geyer GmbH & Co. KG). The filtrate was used for the detection of fermentable sugars on high-pressure liquid chromatography (HPLC).

4.1.3.5. Detection of sugars

The quantitative determination of sugars was done by anion-exclusion HPLC with a EurokatH column (300 mm × 8 mm, 10 lm, eluent: 0.01 N H_2SO_4, RI 75 detector; KNAUER). The column was used at a constant temperature of 35°C under constant acidic pH conditions (0.005 mol/L H_2SO_4) in the mobile phase. The injection volume was 10 µL at a pressure of 1.5 MPa.

4.1.4. Theory and calculations

4.1.4.1. Calculation of the α-cellulase units

The following equations are used to adjust the different enzyme amounts and different used amounts of substrate to calculate the kinetic units for comparison. They are also described in Glaser (2015a). Calculations described are based on calculations described by Decker et al. (2003). The standard FPA uses a mass of 50 mg (m_{FP}) of FP in a test volume of 1.5 mL (V_{FP}) of assay solution. The described α-cellulase assay uses 4 mL per reaction tube (V_{RT}), and therefore the mass of α-cellulase for the new assay can be calculated as follows:

$$m_{cellulose} = \frac{m_{FP}}{V_{FP}} \cdot V_{RT} \qquad /4.1\text{-}1/$$

$$m_{cellulose} = \frac{50\ mg_{FP}}{1.5\ mL} \cdot 4\ mL = 133.\overline{3}\ \frac{mg_{cellulose}}{per\ reaction\ tube} \qquad /4.1\text{-}2/$$

Despite this, the amount targeted was at 100 mg, 200 mg, and 300 mg per reaction tube. The results of 133.3 mg per reaction tube were calculated by regression. The standard FPA targets 2 mg of reducing sugars from 50 mg FP. This equals to a 3.6% conversion. Because the standard FPA applies to the 2 mg reducing sugar released, and not to the concentration of a product, a conversion factor is needed to adjust the 2 mg glucose equivalents to the hydrolysis factor (hf_{FPA}) of 3.6%. One must take into account that the product mass has to be adjusted by an adjustment factor (af_{Glc}), because 1.11 mg of glucose is formed by a 100% hydrolysis of 1 mg cellulose. The required conversion of substrate mass (m_{Glc}) in the reaction volume can be determined according the equations 4.1-3 to 4.1-9.

$$hf_{FPA} = \frac{m_{Glc}}{m_{cellulose}} \cdot \frac{1}{af_{Glc\_cellulose}} \qquad /4.1\text{-}3/$$

$$hf_{FPA} = \frac{2\ mg_{Glc}}{50\ mg_{cellulose}} \cdot \frac{1 mg_{cellulose}}{1.11 mg_{Glc}} = 0.\overline{036} \qquad /4.1\text{-}4/$$

$$m_{Glc\_i} = hf_{FPA} \cdot m_{cellulose} \cdot af_{Glc} \qquad /4.1\text{-}5/$$

$$m_{Glc\_100mg} = 0.036 \cdot 100 mg_{cellulose} \cdot \frac{1.11\ mg_{Glc}}{mg_{cellulose}} = 4.0\ mg_{Glc} \qquad /4.1\text{-}6/$$

$$m_{Glc\_133mg} = 0.036 \cdot 133.33 mg_{cellulose} \cdot \frac{1.11\ mg_{Glc}}{mg_{cellulose}} = 5.3\ mg_{Glc} \quad /4.1\text{-}7/$$

$$m_{Glc\_200mg} = 0.036 \cdot 200 mg_{cellulose} \cdot \frac{1.11\ mg_{Glc}}{mg_{cellulose}} = 8.0\ mg_{Glc} \quad /4.1\text{-}8/$$

$$m_{Glc\_300mg} = 0.036 \cdot 300 mg_{cellulose} \cdot \frac{1.11\ mg_{Glc}}{mg_{cellulose}} = 12\ mg_{Glc} \quad /4.1\text{-}9/$$

For direct comparison, the equation for the 133.3 mg is also given. Based on the results that are available for different amounts of used enzyme protein, a regression can be used to evaluate the amount of enzyme protein, which in turn determine the defined amounts of reducing sugar to be released. The standard FPA is based on the total product at a specific time in a nonlinear reaction. Therefore, a conversion factor is used to determine a specific activity-type unit. The conversion in the standard FPA examines the release of 2 mg of glucose and is based on the use of 0.5 mL of enzyme (V_{Enzyme}), despite the fact that the total amount of reaction solution is 1.5 mL (V_{FPA}). The conversion rate (r_c) and FPU are calculated (equations 4.1-10 to 4.1-12) as follows for the standard FPA:

$$r_{c_i} = m_{Glc\_i} \cdot \frac{1}{M_{Glc}} \cdot \frac{1}{t_R} \quad /4.1\text{-}10/$$

$$r_{c_{FPA}} = 2\ mg_{Glc} \cdot \frac{\mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{60\ min} \equiv 0.37 \frac{\mu mol_{Glc}}{min} \quad /4.1\text{-}11/$$

$$FPU = \frac{r_{c\_FPA}}{V_{Enzyme}(2\ mg)} = \frac{0.37}{V_{Enzyme}(2\ mg\ glucose\ release)} \frac{Units}{mL_{Enzyme}} \quad /4.1\text{-}12/$$

In the assay for the determination of α-cellulase units, the premixed enzyme buffer with known enzyme protein amount results in a different conversion rate (r_c; equations 4.1-13 to 4.1-16).

$$r_{c_{100mg}} = 4\ mg_{Glc} \cdot \frac{\mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{60\ min} = 0.368 \frac{\mu mol_{Glc}}{min} \quad /4.1\text{-}13/$$

$$r_{c_{133mg}} = 5.3\ mg_{Glc} \cdot \frac{\mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{60\ min} = 0.492 \frac{\mu mol_{Glc}}{min} \quad /4.1\text{-}14/$$

$$r_{c_{200mg}} = 8\ mg_{Glc} \cdot \frac{\mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{60\ min} = 0.740 \frac{\mu mol_{Glc}}{min} \quad /4.1\text{-}15/$$

$$r_{c_{300mg}} = 12\ mg_{Glc} \cdot \frac{\mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{60\ min} = 1.108 \frac{\mu mol_{Glc}}{min} \quad /4.1\text{-}16/$$

The mass of enzyme protein that releases x mg glucose ($m_{Enzyme}[m_{Glc}]$)–in this case, 1.0 mg glucose for 100 mg α-cellulose, 2.0 mg glucose for 200 mg α-cellulose, and 3.0 mg glucose for 300 mg α-cellulose–can be determined by regression through the enzyme concentration and their glucose yields. The calculation of the αCU is as follows (equations 4.1-17 to 4.1-22) for the different α-cellulose amounts:

$$\alpha CU_i = \frac{r_{c\_i}}{m_{Enzyme}(m_{Glc})} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}17/$$

For the test with 100 mg

$$\alpha CU_{100mg} = \frac{0.368}{m_{Enzyme}(4.0\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}18/$$

For a test if 133.3 mg were used

$$\alpha CU_{133mg} = \frac{0.492}{m_{Enzyme}(5.3\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}19/$$

For the test with 200 mg

$$\alpha CU_{200mg} = \frac{0.740}{m_{Enzyme}(8\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}20/$$

For the test with 300 mg

$$\alpha CU_{300mg} = \frac{1.108}{m_{Enzyme}(12\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}21/$$

4.1.4.2. Calculation of cellobiase units

The calculation of the cellobiase units is based on Ghose's (1987) calculations. The standard cellobiase assay (CBA) uses 1 mL of 15 mM of cellobiose (CB) solution in a volume of 2 mL of assay solution. The CBA assay described here uses 4 mL assay mixture per reaction tube with 2 mL of 7.5 mM, 15 mM, and 30 mM cellobiose solution and 2 mL of the 2.5 mg, 5 mg, 10 mg, and 20 mg enzyme solution. As in the αCU and FPA, the concentration of enzyme that would have released 1.0 mg of glucose has to be determined. The critical liberated glucose amount to define one unit is 1.0 mg (0.18016 mol). This correlates to 0.5 mg converted cellobiose amount. If this amount of cellobiose was converted by the 1 mL of enzyme solution in 30 min, then 1.0 mg_{Glc} = 0.5 mg_{CB}/(0.18016 mg/µmol * 1.0 mL_{enzyme} * 30 min). The amount of converted cellobiose is therefore 0.0926 µmol/(min * mL). Following this approach, the estimated amount of enzyme, which releases 1 mg of glucose or hydrolyses 0.5 mg of cellobiose, contains 0.0926 units. The required conversion of substrate can be determined accordingly (equations 4.1-22 to 4.1-27):

$$hf_{CBA} = c_{m\_Glc} \cdot \frac{1}{c_{M\_CB}} \cdot \frac{1}{af_{Glc\_CB}} \cdot \frac{1}{M_{Glc}} \quad /4.1\text{-}22/$$

$$hf_{CBA} = 1\ \frac{mg_{Glc}}{mL} \cdot \frac{mL}{15\ \mu mol_{CB}} \cdot \frac{\mu mol_{CB}}{1.05 \mu mol_{Glc}} \cdot \frac{\mu mol_{CB}}{0.18016\ mg_{CB}} \cong 0.35 \quad /4.1\text{-}23/$$

$$m_{Glc\_i} = hf_{CB} \cdot c_{M_{CB}} \cdot af_{Glc} \cdot M_{Glc} \cdot V_{RT} \quad /4.1\text{-}24/$$

$$m_{Glc\_7.5mM} = 0.35 \cdot 7.5 \frac{\mu mol_{CB}}{mL} \cdot 1.05 \frac{\mu mol_{Glc}}{\mu mol_{CB}} \cdot 0.18016 \frac{mg_{Glc}}{\mu mol_{Glc}} \cdot 4\ mL \cong 2\ mg_{Glc} \quad /4.1\text{-}25/$$

$$m_{Glc\_15mM} = 0.35 \cdot 15 \frac{\mu mol_{CB}}{mL} \cdot 1.05 \frac{\mu mol_{Glc}}{\mu mol_{CB}} \cdot 0.18016 \frac{mg_{Glc}}{\mu mol_{Glc}} \cdot 4\ mL \cong 4\ mg_{Glc} \quad /4.1\text{-}26/$$

$$m_{Glc\_30mM} = 0.35 \cdot 30 \frac{\mu mol_{CB}}{mL} \cdot 1.05 \frac{\mu mol_{Glc}}{\mu mol_{CB}} \cdot 0.18016 \frac{mg_{Glc}}{\mu mol_{Glc}} \cdot 4\ mL \cong 8\ mg_{Glc} \quad /4.1\text{-}27/$$

The concentration of the amount of enzyme that releases 1.0 mg glucose for the three different amounts of cellobiose concentration can be calculated referring to the αCU. The calculation of the conversion rates factor is as follows (equations 4.1-28 to 4.1-31) for the different cellobiose amounts:

$$r_{c\_i} = m_{Glc\_i} \cdot \frac{1}{M_{Glc}} \cdot \frac{1}{t_R} \quad /4.1\text{-}28/$$

$$r_{c\_7.5mM} = 2\ mg_{Glc} \cdot \frac{1\ \mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{30\ min} = 0.184 \frac{\mu mol_{CB}}{min} \quad /4.1\text{-}29/$$

$$r_{c\_15mM} = 4\ mg_{Glc} \cdot \frac{1\ \mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{30\ min} = 0.37 \frac{\mu mol_{CB}}{min} \quad /4.1\text{-}30/$$

$$r_{c\_30mM} = 8\ mg_{Glc} \cdot \frac{1\ \mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{30\ min} = 0.74 \frac{\mu mol_{Glc}}{mL \cdot min} \quad /4.1\text{-}31/$$

The calculation of the CBU is as follows (equations 4.1-32 to 4.1-34) for the different cellobiose amounts based on equation 4.1-17:

For the test with 7.5 mM

$$CBU_{7.5\ \mathrm{mM}} = \frac{0.184}{m_{Enzyme}(2.0\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}32/$$

For the test with 15 mM

$$CBU_{15\ \mathrm{mM}} = \frac{0.37}{m_{Enzyme}(4.0\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}33/$$

For the test with 30 mM

$$CBU_{30\ \mathrm{mM}} = \frac{0.74}{m_{Enzyme}(8.0\ mg\ glucose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}34/$$

4.1.4.3. Calculation of the xylanase units

The calculation of the xylanase units (XU) in the xylan assay (XA) is based on the definition that one unit of enzyme activity equals the amount of enzyme that is required to produce 1 µmol of

xylose per minute at 52°C, from defined concentrations of 1% w/v, 2% w/v, and 3% w/v of a solution of birch wood xylan, dissolved in 50 mM sodium acetate with a pH of 5.0. As with the αCU and CBU, the concentration of enzyme that would have released 1.0 mg of xylose has to be determined. The critical liberated xylose amount to define one unit is 1.0 mg (0.15013 mol). This correlates to 0.5 mg converted xylan amount. The required conversion of substrate can be determined accordingly (equations 4.1-35 to 4.1-41):

$$XU = \frac{1\ \mu mol_{Xyl}}{\min * mg_E} = \frac{0.15013\ mg}{\min * mg_E} \equiv \frac{4.5\ mg}{in\ 30\ \min * mg_E} = \frac{1.125\ mg}{in\ 30\ \min\ * in\ 4\ mL * mg_E} \quad /4.1\text{-}35/$$

The required hydrolysis factor of substrate can be determined as

$$hf_{XA} = c_{m\_Xyl} \cdot \frac{1}{c_{M\_Xyl}} \cdot \frac{1}{af_{Xyl}} \cdot \frac{1}{M_{Xyl}} \quad /4.1\text{-}36/$$

$$hf_{XA} = 1.125\ \frac{mg_{Xyl}}{mL} \cdot \frac{mL}{133.2\ \mu mol_{Xylan}} \cdot \frac{\mu mol_{Xylan}}{1.13\ \mu mol_{Xyl}} \cdot \frac{\mu mol_{Xyl}}{0.15013\ mg_{Xyl}} \cong 0.05 \quad /4.1\text{-}37/$$

$$m_{Xyl} = hf_{XA} \cdot c_{M_{Xyl}} \cdot af_{Xyl} \cdot M_{Xyl} \cdot V_{RT} \quad /4.1\text{-}38/$$

$$m_{Xyl} = 0.05 \cdot 66.6 \frac{\mu mol_{XL}}{mL} \cdot 1.13\ \frac{\mu mol_{Xyl}}{\mu mol_{XL}} \cdot 0.15013 \frac{mg_{Xyl}}{\mu mol_{Xyl}} \cdot 4\ mL \cong 2.26\ mg_{Xyl} \quad /4.1\text{-}39/$$

$$m_{Xyl} = 0.05 \cdot 133.2 \frac{\mu mol_{Xl}}{mL} \cdot 1.13\ \frac{\mu mol_{Xyl}}{\mu mol_{XL}} \cdot 0.15013 \frac{mg_{Xyl}}{\mu mol_{Xyl}} \cdot 4\ mL \cong 4.5\ mg_{Xyl} \quad /4.1\text{-}40/$$

$$m_{Xyl} = 0.05 \cdot 199.8 \frac{\mu mol_{XL}}{mL} \cdot 1.13\ \frac{\mu mol_{Xyl}}{\mu mol_{XL}} \cdot 0.15013 \frac{mg_{XL}}{\mu mol_{XL}} \cdot 4\ mL \cong 6.75\ mg_{Xyl} \quad /4.1\text{-}41/$$

The conversion rate factors for the three different amounts of xylan can be calculated as follows (4.1-42 to 4.1-45):

$$r_{c\_i} = m_{Xyl} \cdot \frac{1}{M_{Xyl}} \cdot \frac{1}{t_R} \quad /4.1\text{-}42/$$

$$r_{c\_1\%} = 2.26\ mg_{Xyl} \cdot \frac{\mu mol_{Xyl}}{0.15013\ mg_{Xyl}} \cdot \frac{1}{30\ min} = 0.5\ \frac{\mu mol_{Xyl}}{min} \quad /4.1\text{-}43/$$

$$r_{c\_2\%} = 4.5\ mg_{Xyl} \cdot \frac{\mu mol_{Xyl}}{0.15013\ mg_{Xyl}} \cdot \frac{1}{30\ min} = 1.0\ \frac{\mu mol_{Xyl}}{min} \quad /4.1\text{-}44/$$

$$r_{c\_3\%} = 6.75\ mg_{Xyl} \cdot \frac{\mu mol_{Xyl}}{0.15013\ mg_{Xyl}} \cdot \frac{1}{30\ min} = 1.5\ \frac{\mu mol_{Xyl}}{min} \quad /4.1\text{-}45/$$

The calculation of the XU is as follows (equation 4.1-46 to 4.1-48), based on equation 4.1-18:

For the test with 1% w/v

$$XU_{1\%} = \frac{0.5}{m_{Enzyme}(2.25\ mg\ xylose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}46/$$

For the test with 2% w/v

$$XU_{2\%} = \frac{1}{m_{Enzyme}(4.5\ mg\ xylose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}47/$$

For the test with 3% w/v

$$XU_{3\%} = \frac{1.5}{m_{Enzyme}(6.75\ mg\ xylose\ release)} \frac{Units}{mg_{Enzyme}} \quad /4.1\text{-}48/$$

4.1.4.4. Calculation of the glucose-based wheat straw units

WS was used to discuss the behaviour of the cellulase mixtures, while a WS-based assay (WSA) was introduced for the determination of the WSU. For this purpose, the FPA has to be adapted as Decker et al. (2003) have described it. Subsequently, the mathematical determination of the WSU was elucidated alongside the FPA. The standard FPA used 50 mg of FP in 1.5 mL of assay solution. The new method presented in this work was carried out in a 4 mL reaction mixture with 200 mg WS. The target of the standard FPA assay was to release 2 mg of glucose from 50 mg FP. This equals 3.6% hydrolysis. The FP hydrolysis factor hf_{FPA} is calculated according to equations 4.1-3 to 4.1-4. In respect to the hf_{FPA}, the WSA glucose release can be calculated as follows:

$$hf_{WS\_Glc} = hf_{FPA} \cdot m_{WS} \cdot cf_{WS\_cellulose} \cdot af_{Glc\_cellulose} \quad /4.1\text{-}49/$$

$$hf_{WS\_Glc} = 0.036 \cdot 200\ mg_{WS} \cdot \frac{0.47\ mg_{cellulose}}{1.0\ mg_{WS}} \cdot \frac{1.11\ mg_{Glc}}{1.0\ mg_{cellulose}} = 3.4\ mg_{Glc} \quad /4.1\text{-}50/$$

The factor cf_{cWS} with cf_{cWS} = 0.47 $mg_{cellulose}/mg_{WS}$ represents the content of 47% cellulose (Table 4.1-4) fraction in the used WS sample. The conversion of the FPA was based on 0.5 mL of enzyme given to 1 mL cellulose suspension. In the assay used here, the enzyme and buffer solutions were premixed, and different concentrations of enzymes were used. The determination of 1 FPU on the basis of the FPA was given in equations 4.1-10 to 4.1.12. In comparison to the calculation of the FPU, the determination of 1 WSU by the WSA can be calculated as

$$r_c = 3.4\ mg_{Glc} \cdot \frac{1.0\ \mu mol_{Glc}}{0.18016\ mg_{Glc}} \cdot \frac{1}{60\ min} = 0.315 \frac{\mu mol_{Glc}}{min} = 0.315\ Units \equiv 3.4 \frac{g_{Glc}}{h} \quad /4.1\text{-}51/$$

$$WSU = \frac{0.315}{amount\ of\ enzyme\ protein\ which\ releases\ 3.4\ mg\ glucose} \frac{Units}{mg_{enzyme}} \quad /4.1\text{-}52/$$

The method used for the WSA can be adjusted easily to other substrates and substrate combinations.

4.1.4.5. Calculation of the xylose-based wheat straw units

The calculation of the xylose-based wheat straw units (WSU_{Xyl}) was based on the definition of the glucose-based WSUs, and it is comparable to the determination of the αCU described previously. The calculation method was adjusted according to the characterisation of the xylanases and xylose yield:

$$hf_{WS\_Glc} = hf_{FPA} \cdot m_{WS} \cdot cf_{WS\_Hemicellulose} \cdot af_{Xyl} \quad /4.1\text{-}53/$$

$$hf_{WS\_Xyl} = 0.036 \cdot 200 mg_{WS} \cdot \frac{0.21\ mg_{Hemicellulose}}{1.0\ mg_{WS}} \cdot \frac{1.13\ mg_{Xyl}}{mg_{Hemicellulose}} = 1.71\ mg_{Xyl} \quad /4.1\text{-}54/$$

The factor $cf_{WS\_hemicellulose}$ = 0.21 $mg_{hemicellulose}/mg_{WS}$ represents the content of 21% (Table 4.1-4) hemicellulose fraction in the WS sample. The WSU_{Xyl} is calculated as follows:

$$r_c = 1.71 mg_{Xyl} \cdot \frac{1\ \mu mol_{Xyl}}{0.15013\ mg_{Glc}} \cdot \frac{1}{60\ min} = 0.191 \frac{\mu mol_{Xyl}}{min} \quad /4.1\text{-}55/$$

$$WSU_{Xyl} = \frac{0.191}{amount\ of\ enzyme\ protein\ which\ releases\ 1.71\ mg\ xylose} \frac{Units}{mg_{Protein}} \quad /4.1\text{-}56/$$

4.1.4.6. Calculation of the yield prediction based on the Sauter mean diameter

Due to the previously described behaviour of the cellulase performance, an empirically derived power function was used to investigate the process parameters that have an impact on the sugar yield. The equation can be used for the prediction of cellulase performance on inhomogeneous raw materials for different scales, enzymes, and solid loadings. The relation between the actual hydrolysis performance and the coded values can be described according to the following mathematical equations (equations 4.1-57 to 4.1-59):

$$Y(t) = S_{cellulose} \cdot cf_{PS} \cdot \left(a - \exp\left(\mu_p \cdot t\right)\right) \quad /4.1\text{-}57/$$

$$Y(t) = [g_{cellulose}] \cdot \left[\frac{g_{glc}}{g_{cellulose}}\right] \cdot [-] = [g_{glc}]$$

$$\mu_p = \frac{M_{Glc}}{hf_{WS\_Glc}} \cdot WSU \cdot E_p \equiv 1/hf_{WS_{Glc}} \cdot WSU \cdot E_p \quad /4.1\text{-}58/$$

$$\mu_p = \frac{[g_{Glc}/mol_{Glc}]}{[g_{glc}/h]} \cdot \left[\frac{mol_{Glc}}{h \cdot g_p}\right] \cdot [g_P] \equiv \left[\frac{h}{g_{Glc}}\right] \cdot \left[\frac{g_{Glc}}{h \cdot g_P}\right] \cdot [g_p] = \left[\frac{1}{h}\right]$$

$$cf_{PS} = a \cdot PS^{-b} \quad /4.1\text{-}59/$$

The parameter μ_P describes the predicted maximum activity in the context of glucose release per hour, dependent on the previously determined WSU, using 1 WSU [$g_{Glc}/(h*g_p)$] as pre-factor corresponds to 0.0034 g_{glc}/h. E_p [g_{Enzmye}] as set by the protein amount of the enzyme. The factor cf_{PS} [$g_{glc}/g_{cellulose}$] is based on the conversion yield shown in Figure 4.1-1 and can be calculated for the different PS. The conversion factor cf_{PS} depends on the PS and on the fitting parameters *a* and *b* of the power function, which can describe an efficiency of the hydrolyses depending on a

certain PS such as SMD or PS distribution. $S_{cellulose}$ defines the cellulose available for hydrolysis into glucose bonds in the lignocellulose raw material of the relevant process. The time *t* represents the run time of the process. The parameter *α* provides a possible method for an offset of the whole yield prediction: for example, in the case that, due to a prior treatment such as alkaline, low basal concentrations of sugars ($Y(t=0) > 0$) are available before the enzymatic hydrolysis. If the basal concentration influences the maximum conversion, the PS-based conversion factor cf_{PS} (equation 4.1-59) may be adjusted for the problem in the following form: $Y(t) = S \cdot (cf - \beta) \cdot (\alpha - exp(\mu_{P \cdot t}))$. For β, the following form of $\beta = \alpha \cdot exp(\alpha)$ is appropriate. At the prediction test, the yields of the processes were normalised to $\alpha = 1$. For the identification of the basic parameters and for the reproduction of the actual experimental data, the accuracy and general ability of the equation can be evaluated by the analysis of variance (ANOVA) and the regression coefficient R^2.

4.1.5. Results and discussion

The cellulose structure of the pulverised α-cellulose is easier to hydrolyse due to the higher accessibility. The calculated αCU activity per gram protein is shown in Table 4.1-1.

Table 4.1-1: α-cellulose units (αCU) derived by hydrolyses of pulverized α-cellulose using different enzymes.

α-cellulose amount	R1	R2	R3	R4	CT2
100 g	367.09	326.72	332.15	437.21	452.00
133 g	397.19	320.63*	374.22*	420.57*	529.67
200 g	458.31	308.83	458.88	387.75	686.50
300 g	549.87	403.25	437.21	524.33	746.35

*derived by regression

The αCU of the enzyme mixtures provided by the Moscow State University was lower than the measured activity for the industrially available enzyme mixture CT2. For HT2, a αCU activity could not be determined. No significant product release could be measured. An unexpected result is the high αCU of the cellobiase enzyme mixtures R2 and R4. Here, it is expected that the physical structure of the pulverised α-cellulose allows for a good hydrolysation by the cellobiase mixtures R2 and R4. The determined β-cellobiase activity, referred to as CBU (Table 4.1-2), of CT2 was lower than described by Alvira et al. (2013), Erdei et al. (2012), and Cannella et al. (2012). The activity of the β-cellobiase R4 was higher than the cellobiase activity of CT2, but the β-cellobiase activity of R2 was lower. The β-cellobiase activity of the cellulase mixtures R1 and R3 are comparable.

Table 4.1-2: Cellobiase units (CBU) derived by hydrolyses of cellobiose using different enzymes.

Cellobiose amount	R1	R2	R3	R4	CT2	HT2
1%; 7.5 mM	131.82	182.42	175.61	410.39	383.36	198.67
2%; 15 mM	225.98	338.53	278.61	754.66	626.39	227.15
3%; 30 mM	353.50	714.66	410.39	1571.70	1080.37	294.38

When examining the xylanase activity, referred to as XU (Table 4.1-3), an unexpected behaviour becomes visible when comparing CT2 and HT2. Here, CT2 has a higher xylanase activity than the hemicellulose mixture HT2. However, the XU of the enzyme mixture R1 and R3 is much lower than the activity of the enzymes R3 and R4.

Table 4.1-3: Xylanase units (XU) derived by hydrolyses of pulverized birchwood xylan using different enzymes.

Xylose amount	R1	R2	R3	R4	CT2	HT2
1% (w/v)	41.96	198.49	28.42	68.98	447.98	101.80
2% (w/v)	100.14	660.71	68.98	1381.33	1210.44	375.74
3% (w/v)	107.85	756.70	77.82	1675.95	1301.11	439.14

According to this behaviour, the enzyme mixtures are used as combinations of R1 and R2 and of R3 and R4 to achieve the best possible results in hydrolyses using lignocellulosic feedstocks.

4.1.5.1. Wheat straw assay and wheat straw units

The monosaccharide compositions of the different raw materials were analysed using the Weender and van Soest analysis. Results are shown in Table 4.1-4. The WS consists mostly of 47.6% cellulose, 20.6% hemicellulose, and 7.6% of acid soluble lignin (ASL) by weight of the dry matter of the milled raw material.

Table 4.1-4: Composition of the dry lignocellulosic raw materials (%) and Sauter mean diameter (µm).

	Cellulose	Hemicellulose	ASL	SMD
Wheat straw	47.6	20.6	7.6	435
Wheat straw, pulverized	27.1	21.6	4.0	202
Aspen wood	43.1	5.7	18.4	513
Pinewood	53.6	8.3	10.9	483
Grass	32.5	25.7	5.2	153
Rice straw	23.8	15.8	7.7	169

The substrates were characterised through their SMD, which is given in Table 4.1-5.

Table 4.1-5: Particle size distribution of a set of milled wheat straw, pulverized wheat straw (pWS), pine wood (PW), aspen wood (AW), rice straw (RS), and grass (data was published in Glaser [2015]).

$d_{m,i}$ [µm]	WS	pWS	AW	PW	grass	RS
900	6.31	0.04	42.40	36.05	0.07	0.00
715	35.83	0.08	24.08	28.02	0.22	0.02
472.5	42.56	13.08	19.94	20.82	2.55	9.83
282.5	4.19	27.86	5.73	6.47	3.28	13.99
225	3.40	31.76	2.85	2.87	28.14	25.75
162.5	6.61	20.48	4.03	3.82	44.22	34.58
107.5	0.84	3.91	0.46	0.52	15.93	10.59
85	0.14	0.69	0.16	0.40	3.67	2.90
75.5	0.03	0.30	0.11	0.71	0.62	0.55
67	0.05	0.35	0.14	0.30	0.24	0.28
31.5	0.03	1.46	0.10	0.02	1.05	1.52
SMD [µm]	435	202	513	483	153	169

The data from the wheat straw hydrolyses (WSH) of the different particle sets that were carried out in the 15 mL tubes indicated that the performance of the enzymes strongly depended on the substrate and their PS. The summation curve of the particles is shown in Figure 4.1-1A as a percentage of bound sugar per particle fraction. The high increase of the curve at lower PS for the grass and the pulverized wheat straw (pWS) shows the base of a low SMD and, therefore, most

particles that were available for hydrolysis had a low PS. The late and small increase of the curves for PS of WS, AW, and PW could possibly explain the higher SMD. Smaller particles were more amenable to cellulase enzymes than the larger substrate particles, as shown in the monosaccharide release (Figure 4.1-1B; Figure 4.1-1D). The conversion of the lignocellulose increased when the PS of the straw substrate was reduced. The release from the smallest WS particles (lower than 125 µm) increased between 27% and 37% of the theoretical maximal release of glucose after 60 min of hydrolysis. The mean values of hydrolysis raw data are given in Table 2 in Glaser (2015).

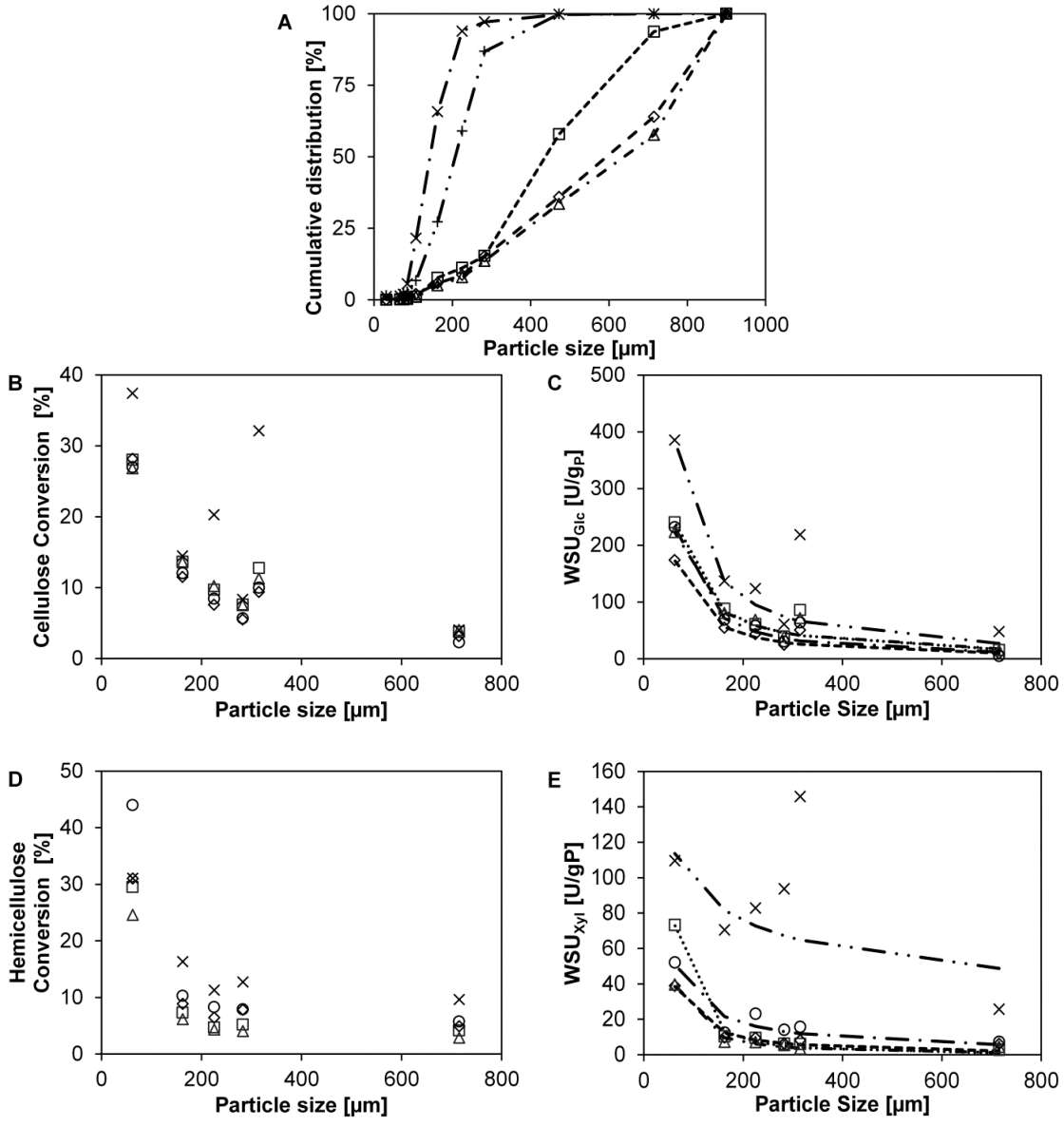

Figure 4.1-1: Comparison of substrate particle size and conversion by introducing the wheat straw units.
A: Cumulative distribution of the bound sugar per particle fraction; µm (□ WS, ◊ PW, Δ AW, x grass, + PWS). B: Conversion yield of glucose depending on the PS of the WS (□ R1, ◊ R2, Δ R3, ○ R4, x CT2). C: Enzyme activity as a result of a hydrolysis of different sets of milled wheat straw displayed as WSU (□ R1, ◊ R2, Δ R3, ○ R4, x CT2). Regression curves indicated as lines (▪ ▪ ▪ R1, - - - R2, − ▪ − ▪ R3, — ▪ — R4, — ▪ ▪ CT2).

The data that had been obtained from the WSH of the different fractions of substrate PS was used for the calculations of the WSU (Figure 4.1-1C; 4.1-1E). Ghose (1987) and Decker et al. (2003)

have used FP disks to characterise cellulase enzymes in the FPA. They found that the FPA is insufficient for estimating the performance of cellulase formulations for the hydrolyzation of complex inhomogeneous lignocellulosic raw materials. The method that is described here represents an advance in the characterisation of the behaviour of cellulase preparations and predicted yields that depend on the PS distribution of the raw material. For the calculation of the WSU (Section 4.1.4.5.), FPUs in the range of 4.8 FPU to 38.5 FPU (24–192 FPU/g_s) were used. As expected, the cellulase activity, determined by the WSA, did not reach the activity values given by the FPUs per gram protein due to different substrates and different kinds of physical properties of PS. The yield of glucose and xylose increased with decreasing PS (Figure 4.1-1B; 4.1-1D). In this context, the WSU increased with a decrease of the PS (Table 4.1-6; Table 4.1-7; Figure 4.1-1D; Figure 4.1-1E). For the smallest fraction of particles below 125 µm, 38%, 31%, and 41% of the FPU could be achieved for R1, R3, and CT2, respectively. The cellulase R1 and R3 achieved only 2% of the activity, while CT2 could achieve only slightly higher levels of activity, with 5% of the FPU. Of importance are the high yields solely with the β-glucosidases R2 and R4. Here, R4 achieved nearly the same yield as the mixtures R1 or R3. The particle set of 315–630 µm showed an extremely high yield of glucose, in contrast to the neighbouring fractions. It is possible that impurities accumulated in this fraction, because the WS samples were not washed before use. These impurities have a significant effect on hydrolyses. A regression curve of the form of $y=a \cdot WSU^{-b}$ fit. The RMS values (Table 4.1-6) were in the range of 4.1±1.3% of the lowest WSU value. The parameter optimisation for xylan hydrolyses showed higher RMS values (Table 4.1-7). It has been seen that the xylan hydrolyses have a higher overall deviation also regarding wheat straw hydrolyses in technical scales.

Table 4.1-6: Protein amount and glucose-based WSU as a result of regression. Regression data of experiments with an enzyme loading range of 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL were used for this.

	Protein	$WSU_{800-630}$	$WSU_{630-315}$	$WSU_{315-250}$	$WSU_{250-200}$	$WSU_{200-125}$	$WSU_{125\ and\ lower}$	Regression		RMS
	g_P/g	U/g_P	U/g_P	U/g_P	U/g_P	U/g_P	U/g_P	a	b	
R1	0.984	15	85	39	60	87	240	23149	1.10	0.002
R2	0.825	8	50	24	43	54	173	24034	1.19	0.004
R3	0.947	14	71	38	68	80	222	16997	1.04	0.004
R4	0.821	4	63	30	57	68	231	39210	1.24	0.006
CT2	0.283	47	218	60	123	136	385	35115	1.09	0.004

Table 4.1.7: Protein amount and xylose based WSU_{Xyl} as a result of regression. Regression data of experiments with an enzyme loading range of 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL were used for this.

	Protein	$WSU_{800-630}$	$WSU_{630-315}$	$WSU_{315-250}$	$WSU_{250-200}$	$WSU_{200-125}$	$WSU_{125\ and\ lower}$	Regression		RMS
	g_P/g	U/g_P	U/g_P	U/g_P	U/g_P	U/g_P	U/g_P	a	b	
R1	0.984	2.9	6.1	6.7	8.2	10.9	25.6	1014	0.89	0.58
R2	0.825	2.4	6.0	6.8	8.8	12.8	38.4	4394	1.15	2.12
R3	0.947	1.1	3.7	4.3	6.0	9.7	39.3	16846	1.47	1.82
R4	0.821	5.7	11.8	13.0	16.0	21.5	50.1	2112	0.90	4.79
CT2	0.283	45.6	61.7	64.3	69.9	78.9	112.3	519	0.37	14.94
HT2	0.283	6.4	11.8	12.8	19.4	19.4	39.8	886	0.75	0.00

4.1.5.2. Yield prediction through Sauter mean diameter

A possible mathematical approach for yield prediction for a hydrolysis pre-treatment of lignocellulose was given in Section 4.1.4.6. The model equation, with the parameters derived by the WSA, was able to predict a WSH process that was done in shaking flasks (Figure 4.1-2A). However, the WSH hydrolysis showed a nearly linear development of glucose release in several shaking flask experiments. The model equation made it possible to distinguish between the three enzyme combinations used for the experiments. The discrepancies in the behaviours of the different cellulase mixtures became evident. While the mixtures of R1 and R2 and of R3 and R4 have nearly the same prediction results, CT2 had a higher increase of the conversion, which was represented in the higher conversion factor *C*. Table 4.1-8 shows the identified key parameters of the prediction. For a comparison, a parameter estimation of the model equation was undertaken. The prediction of the cellulase mixture performance was done autonomously with the individual enzymes R_i/R_j. It can be examined to which extent the predicted values might be cumulative with each other, which could not be proven in the current state.

For the prediction of the hydrolysis of the pWS (Figure 4.1-2A), between experimental data and prediction, the coefficient for correlation R^2 was 0.85 to 0.97. The statistical significance of the equation was checked using ANOVA. The *F*-value was 1.62, with a critical *F*-value of 2.53. Since $F < F_{critical}$, it could be stated that populations of experimental data and predicted data had the same mean value and variances. Within a 95% confidence interval, the model was statistically able to predict the yields. The statistical values for hydrolyses are given in Table 4.1.-8.

A hydrolysis of dried milled grass (Figure 4.1-2B) showed a basal level of 0.25 g of glucose. Here, α allowed for the adjusting of the model to a basal level of glucose, depending on a previous pre-treatment process. The data was adapted to α = 1. Therefore, the ANOVA gave an *F*-value of 0.38 and an $F_{critical}$ value of 2.53. The coefficients for correlation R^2 were 0.96 to 0.97. A yield between 8% and 13% could be achieved in hydrolyses of the pWS and 9% and 11% of the grass hydrolyses. Pedersen and Meyer (2009) achieved about 50% of Glc of the cellulose fraction in a WSH of particles of the range of 53 µm to 149 µm within 24 hrs with their hydrolysis method. An additional hydrolysis of rice straw and PW (Figure 4.1-2C, Figure 4.1-2D) showed the substrate-dependent behaviour of the enzyme performance and therefore of the model. For RS, the model predicted 12% to 14% Glc yield, while the actual yield was between 20% and 25%. While the model predicts a conversion yield from 5% to 7% for PW due to the higher SMD of 483 µm, the actual Glc yield ranges from 12% to 15%. Here, the performance of the enzymes on those two substrates was better than the prediction with the parameters for the WSU. Raw data is given in Glaser (2015).

Table 4.1-8: Parameter prediction and yields of lignocellulose hydrolyses in shaking flask experiments. Prediction of the cellulase mixture performance was done autonomously with the individual enzymes R_i and R_j. The glucose yield was given without basal concentration.

	Predicted values		**Statistics**		**Fitted values**			**$Yield_{Glc}$**	**WSU**	
	$\mu_{p,i}$ & $\mu_{p,j}$	**C_i & C_j**	**R^2**	**$F < F_{critical}$**	**μ_p**	**C**	**C (t = 1h)**	**$g_{Produkt}$**	**U/g_P**	**U**
pWS										
R1&R2	0.276 & 0.065	0.108 & 0.088	0.97	1.62 < 2.53	0.061	0.083	0.0049	0.20	108	100
R3&R4	0.231& 0.090	0.109 & 0.090	0.85		0.054	0.071	0.0038	0.16	119	95
CT2	0.164	0.118	0.89		0.049	0.135	0.0064	0.29	106	48
Grass										
R1&R2	0.375 & 0.091	0.135 & 0.115	0.97	0.38 < 2.53	0.588	0.090	0.482	0.60	149	137
R3&R4	0.390 & 0.128	0.133 & 0.117	0.96		0.429	0.114	0.453	0.67	163	128
CT2	0.222	0.115	0.96		0.339	0.109	0.302	0.71	144	65
RS										
R1&R2	0.336 & 0.081	0.124 & 0.104	0.77	4.05 > 2.63	0.613	0.219	0.118	0.60	133	122
R3&R4	0.278 & 0.119	0.124 & 0.107	0.91		1.702	0.199	0.129	0.67	146	115
CT2	0.199	0.140	0.82		8.408	0.250	0.244	0.71	129	58
PW										
R1&R2	0.105 & 0.023	0.052 & 0.037	0.97	3.71 > 2.47	0.291	0.153	0.0388	0.96	40	37
R3&R4	0.092 & 0.030	0.055 & 0.040	0.95		0.319	0.132	0.0363	0.80	45	36
CT2	0.063	0.050	0.96		0.256	0.123	0.0278	0.75	41	18

To screen the behaviour in a scaled-up system and get an overview of the dynamic behaviour, the cellulase enzymes were tested in a stirred tank reactor. The prior results of the shaking flask test showed a variance of Glc yield between the different substrates that were used. Figure 4.1-3A and Figure 4.1-3B show the dynamics of the three WSHs, which also displayed a variance.

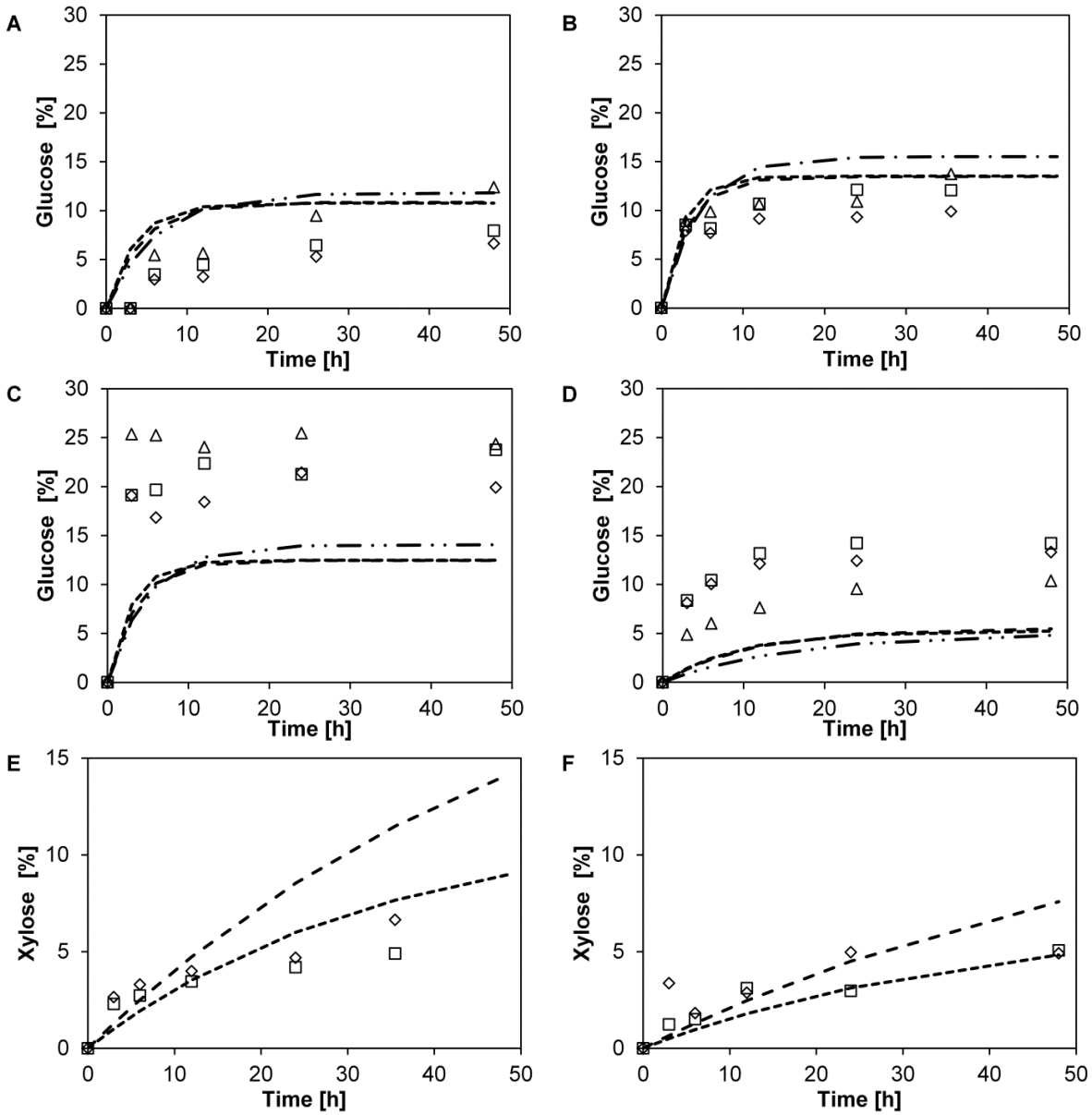

Figure 4.1-2: Hydrolysis profiles and model prediction for shaking flask experiments with A: Glucose released using pulverized wheat straw at 5% w/v; B: grass at 5% w/v; C: rice straw at 5% w/v; D: pine wood at 5% w/v. Xylose released using E: grass at 5% w/v; F: rice straw at 5% w/v. Experimental data shown as marks: □ R1&R2, ◊ R3&R4, Δ CT2. Model prediction curves are shown as lines ----- R1&R2, − − − R3&R4, — ▪ ▪ CT2.

Upscaling the hydrolysis process to the 2 L stirred tank reactor, a scale factor (*sf*), as described in equation /4.1-57/, was needed to adjust the prediction of the model. No further adjustment was needed for the yield prediction for the 10 L scale stirred reactor.

$$Y(t) = sf \cdot S \cdot cf_{PS} \cdot (\alpha - \exp(t \cdot \mu_P)) \qquad /4.1\text{-}61/$$

A mean scale factor of 2.15 was determined with 10 WS hydrolyses for the stirred tank system. Different results of the pine hydrolysis established a mean scale parameter of 2.33. The results of the WS and PW of the shaking flask experiments were directly compared to each other, revealing also a better enzyme performance on PW. The need of the scale factor may be caused by the short reaction time of 60 min at the determination of the WSU units (Table 4.1-8).

Differences in the prediction might result from different mixing environments and hydrodynamic conditions. The Reynolds number (*Re*) gives information about laminar or turbulent flow properties. If $Re > 10^4$, the presence of a turbulent flow pattern can be assumed (Kraume, 2003, 2012). In shacked vessels, such as flasks and reaction tubes, *Re* is defined as $Re = N*R^2/\nu$ (Dardik et al., 2005; Büchs et al., 2000a, 2000b; Tianzhong et al., 2010). Here, *N* is the shaking frequency; *R* defines the amplitude, and ν is the kinetic viscosity, in this case, of water (1.012x10-6 m2/s). The *Re* in a stirred vessel is defined by $Re = N*d^2/\nu$. N is the number of stirrer revolutions per second and *d* is defined by the diameter of the stirrer.

The maximum *Re* in shaking flasks in this study was 1,040, indicating that the flow was expected to remain in the laminar flow regime (Salek et al., 2011). The laminar flow properties were not able to keep the WS in suspension, leading to a stagnant layer of WS at the bottom of the flask. In the stirred bioreactor of the 2 L and 10 L scale, the *Re* were about 14,400 and 24,200, respectively. The more turbulent flow system seemed to have a positive influence on the cellulase performance. If a constant *Re* is used as a scale-up criterion (190 rpm in 10 L scale), then the WS particles will not be held in flotation. A better scale-up criterion was the stirrer speed (if kept constant), which minimised the stagnant material.

Murthy et al. (2007) have also described the prediction of impeller speed for hydrolysis processes with high solid loadings for the solid suspension as a critical step. Ye et al. (2012), who have specified an increased cellulase activity for 250 rpm shaking after 24 hrs incubation time, investigated the correlation of an increased activity of cellulase mixtures and the mixing speed. Kadić et al. (2014) have analysed the effect of agitation on enzymatic hydrolysis at varying agitation rates for Norway spruce. In their study, the conversion of glucan to glucose in spruce at 13% water insoluble solid loading was described as strongly affected by agitation. They described conversions after 48 hrs reaching 20%, 32%, and 37% for 100 rpm, 300 rpm, and 600 rpm, respectively. They observed stagnant zones throughout the hydrolysis at 100 rpm and explained the lower yield due to mass transfer limitations. Palmqvist et al. (2011) have also suggested that the impeller speed strongly affects the hydrolysis rate of pre-treated spruce. With the information above, increased cellulase activity and glucose yield can be assumed because of the change from laminar to a lower turbulent flow regime.

A significant increase in the concentration of Glc (Figure 4.1-3A, Figure 4.1-3B) was visible for the hydrolysis process in the 2 L scale stirred reactor. However, this increase happened only during the first 6 hrs. After 6 hrs, the release of sugar decreased steadily, until the increase of the yield was nearly undetectable. Increasing the enzyme concentration did not result in a satisfying higher yield. Hydrolysis yields of around 10% did correspond to the amount of glucose that was bound within a PS up to 250 µm. The match of the predicted process and the experimental data is shown in (Table 4.1-9), as most coefficients had a correlation of $R^2 > 0.9$. PW and AW were also used as enzyme substrates in the 2 L reactor (Figure 4.1-3C, Figure 4.1-3D), describing the same

hydrolysis behaviour. WSH results of R1 and R3 in the 10 L scale are shown in Figure 4.1-4, while the parameters and statistical values are given at the end of Table 4.1-9. As in the previous hydrolyses, a large increase in the glucose concentration occurred within the first 6 hrs. There were no considerable differences in the performances of the cellulase mixtures. The prediction of hydrolyses showed a clear correlation with the experimental data. Raw data is given in (2015).

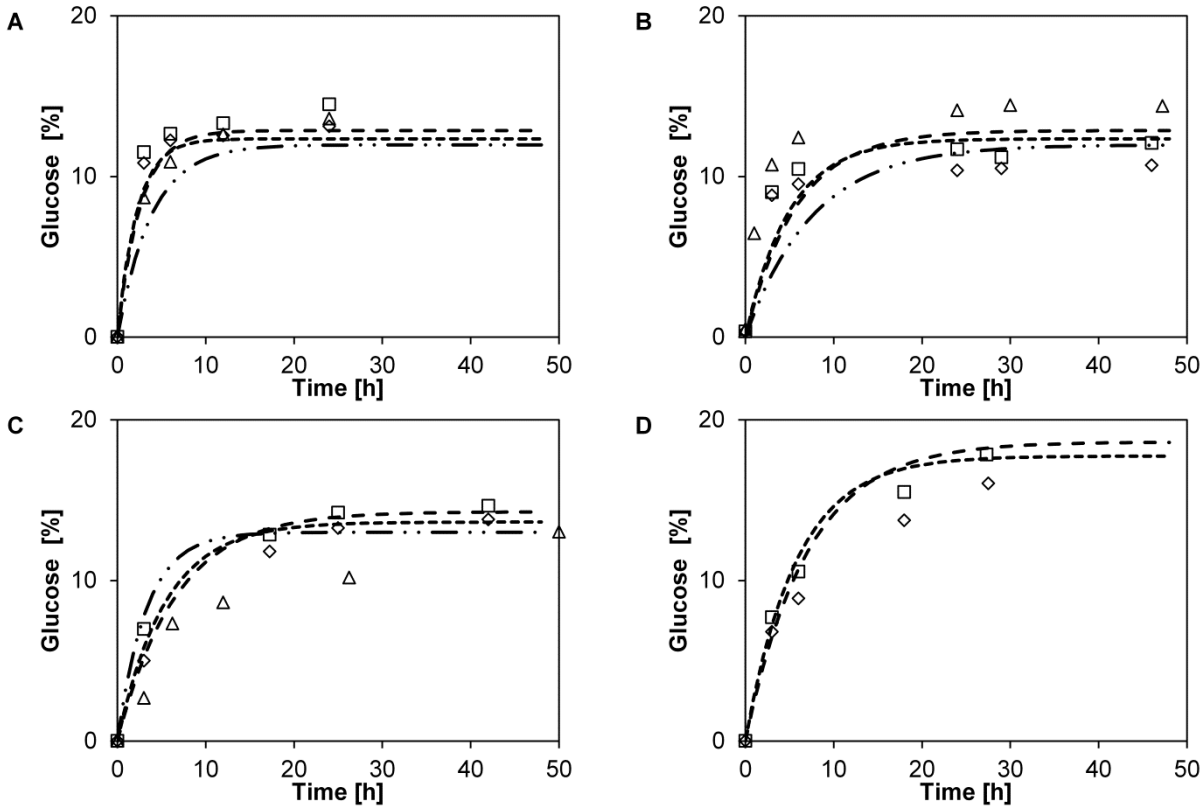

Figure 4.1-3: Hydrolysis profiles and model prediction for yield comparison of A: wheat straw at 7.5% w/v; B: wheat straw 5% w/v; C: pine wood 5% w/v; D: aspen wood 5% w/v; experimental data shown as marks: □ R1&R2, ◊ R3&R4, Δ CT2. Model prediction curves are shown as lines ----- R1&R2, − − − R3&R4, — ▪ ▪ CT2.

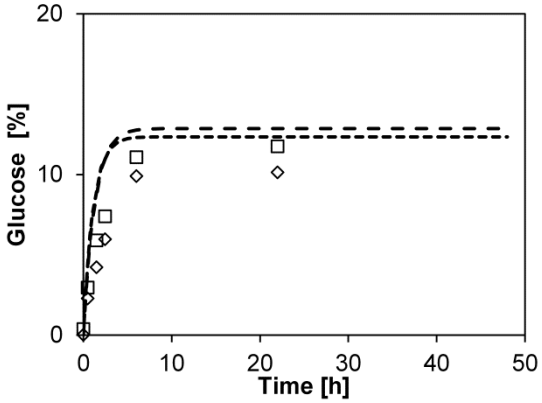

Figure 4.1-4: Hydrolyses profiles and model prediction for yield comparison of A: Wheat straw at 7.5% w/v in a 10 L bioreactor; Experimental data shown as marks: □ R1, ◊ R3. Model prediction curves are shown as lines ----- R1, − − − R3.

Table 4.1-9: Parameter prediction and yields of lignocellulose hydrolyses in stirred tank reactor experiments. Prediction of the cellulase mixture performance was done autonomously with the individual enzymes R_i/R_j. The glucose yield is given without basal concentration.

	Predicted values			Statistics		Fitted values			$Yield_{Glc}$	WSU	
	$\mu_{p,I}$ & $\mu_{p,j}$	C_i & C_j	C_i &C_j sf	R^2	$F < F_{critical}$	μ_p	C	C(t=1)	$g_{Produkt}$	U/g_P	U
2 L scale stirred bioreactor											
WS 5% w/v											
R1&R2	0.203&0.028	0.057&0.040	0.123&0.088	0.94	0.11 < 2.53	0.477	0.115	0.044	3.10	45	68
R3&R4	0.174&0.035	0.059&0.044	0.128&0.094	0.89		0.592	0.103	0.046	2.73	50	61
CT2	0.131	0.055	0.119	0.97		0.517	0.140	0.057	3.78	46	39
WS 7.5% w/v											
R1&R2	0.457&0.011	0.057&0.040	0.123&0.088	0.99	2.56 < 2.62	0.593	0.133	0.059	8.58	45	137
R3&R4	0.390&0.013	0.059&0.044	0.128&0.094	0.98		0.807	0.128	0.053	7.86	50	118
CT2	0.260	0.055	0.119	0.99		0.334	0.116	0.039	8.08	46	77
PW 5% w/v											
R1&R2	0.185&0.042	0.052&0.036	0.136&0.095	0.99	0.42 < 2.49	0.211	0.142	0.027	4.51	40	67
R3&R4	0.156&0.001	0.055&0.040	0.142&0.103	0.99		0.124	0.163	0.016	4.34	44	45
CT2	0.305	0.502	0.130	0.92		0.109	0.121	0.012	3.92	41	90
AW 5% w/v											
R1&R2	0.173&0.039	0.050&0.034	0.177&0.122	0.98	1.59 < 3.09	0.202	0.228	0.041	5.47	37	62
R3&R4	0.142&0.047	0.052&0.037	0.186&0.133	0.97		0.168	0.216	0.033	5.74	41	55
10 L scale stirred bioreactor											
WS 7.5% w/v											
R1	0.930	0.057	0.123	0.95	0.56 < 3.23	0.426	0.114	0.039	37.10	28	273
R3	0.807	0.059	0.128	0.94		0.377	0.104	0.032	32.99	29	237

The hydrolysis results give an insight into the importance of the physical properties of the feedstock. A mentionable hydrolysis occurred for particles with a size below 200 µm. For industrial purposes, it is suggested that a sufficient hydrolysis takes place with PS below 200 µm. The obtained hydrolysis titres of feedstocks with PS over 200 µm were insufficient for an industrial-scale process. Despite variations in the outcome, the determination of WSU in combination with the model equations produced satisfactory results for the yield prediction. Nevertheless, the process of parameter estimation was highly dependent on the substrate and on the fluid dynamic properties in the higher scales.

4.1.6. Conclusion

A possible characterisation of cellulase enzymes for industrial purposes was introduced through an enzyme unit that is based on WS and its physical properties. Hydrolyses of WS, aspen, and PW demonstrated that the PS should be below 200 µm for efficient processes. The investigated possibility of the model-based process prediction gave proper results. The introduced determination of a particle-size-dependent enzyme activity unit for wheat straw, in combination with the proposed equations, was able to predict hydrolysis yields for different enzyme concentrations and different substrates and solid loadings. This allows for the prediction of process behaviours on different scales.

4.2. Screening of *Bacillus coagulans* strains in lignin supplemented minimal medium with high throughput turbidity measurements.[2]

4.2.1. Abstract

To extend the options for screening and characterisation of MO through kinetic growth parameters, automated turbidimetric measurements were accomplished in order to obtain data about the response of strains of *Bacillus coagulans*. For the characterisation, the influence of varying concentrations of lignin with respect to bacterial growth were examined. Different mathematical models are used for comparison: Richards and Stannard, logistic, Gompertz, and Baranyi. The growth response was characterised by parameters such as maximum growth rate, maximum population, and the lag time. In this short analysis, a mathematical approach towards a comparison of different MOs is presented. Furthermore, it is demonstrated that lignin in low concentrations can have a positive influence on the growth of *Bacillus coagulans*.

4.2.2. Introduction

LA is widely used in the food processing, cosmetics, pharmaceutical and chemical industries. Increasing prices of fossil fuels leads to an increasing interest in LA, as a component for the production of the biodegradable polymer poly LA (Venus, 2011). There have been various attempts to produce LA efficiently in biorefineries from inexpensive feedstock such as lignocellulosic raw materials (e.g., wheat straw or hard- and softwood; Betts et al., 1991, Jäger and Büchs, 2012). Lignocellulose, as part of the secondary cell wall of rooted plants, is one of the most abundant natural materials. It contains cellulose, hemicellulose, and lignin (Anuj et al., 2011). Cellulose and hemicellulose represent polymeric carbohydrates formed from glucose, xylose, and arabinose, amongst other sugars (Saha, 2003; Hahn-Hagerdal et al., 2007). Therefore, lignocellulose is also the most abundant carbonate storage. After a hydrolysation process, lignocellulose can serve as a potential substrate in a biotechnological microbial fermentation for the formation of valuable products such as LA (Candel et al., 2010; Carvalheiro et al., 2005, Venus, 2011). Unfortunately, a non-specific chemical hydrolysis
treatment, such as high temperature acid or alkali pre-treatments, leads to solvation of lignin and to the formation of complex sugars and inhibitory compounds such as furfural (Klinke et al., 2004; Kumar et al., 2009; Pederson et al., 2011; Palmqvist and Hahn-Hagerdal, 1996). One way to reduce the inhibitory effect of lignin for process optimisation is through the reduction of the lignin concentration in the fermentation medium. Another option is to use MOs that are inhibited by lignin only to a small extent, or those that can transform lignin into another compound like VAN (Barthelmebs et al., 2001; Curiel et al., 2009).

[2] Based on: Robert Glaser and Joachim Venus, 2014. Screening of Bacillus coagulans strains in lignin supplemented minimal medium with high throughput turbidity measurements Biotechnology Reports 4 (2014) 60–65. http://dx.doi.org/10.1016/j.btre.2014.08.001

To improve the screening of MOs usable in complex and inhibitory media, such as lignocellulosic hydrolysates, it is necessary to characterise their growth behaviour. High throughput methods for kinetic analysis of the lignin inhibition are useful to achieve information about the lag time (λ) and the maximum growth rate (μ_m). These screening methods provide the chance to investigate the growth behaviour under different working conditions. To access lignin stable natural MOs, it is crucial to screen interesting bacteria in an inhibitory environment.

Here, a rapidly automated optical density (OD) measurement was applied to determine the growth response of *Bacillus coagulans* strains. The used strains are thermophilic bacteria, frequently utilised in these processes at a technical scale. In studies that take place in unsterile conditions, *Bacillus coagulans* was shown to be the most predominant species (Akao et al., 2007). Furthermore, the *Bacillus coagulans* strains are known for their tolerance to inhibitors (Kenneth et al., 2010) and their ability to utilise pentose sugars from the hemicellulose fraction of lignocellulose (Walton et al., 2010). These facts provide for the possibility to ferment difficult media under semi-sterile condition. Prior to the fermentation in technical and pilot scales, kinetic data is needed to gain a basic understanding of the characteristics of the MOs for later fermentation processes and their design. Growth models are used to obtain the basic growth parameters, such as specific growth rate and duration of lag phase, in order to classify and differentiate MOs in respect to their behaviour towards diverse lignin concentrations. Numerous models were developed for the representation of growth curves. Widely known models include the logistic (Zwietering et al., 1990), Gompertz (Gompertz, 1825; Winsor, 1932, Yamanao, 2009; Zwietering et al., 1990), Champbell-Richards and Stannard (Zwietering et al., 1990), and the model offered by Baranyi (Baranyi et al., 1993a). These models have been established to fit the equations to the sigmoidal shape of a typical growth curve.

4.2.3. Materials and methods

4.2.3.1. Microbes and media

Bacillus coagulans strains were isolated from different environmental areas. They were stored in cryogenic vials (VWR, 822074ZA) at -70°C and reactivated on MRS broth (Merck, 1.10661.0500) at 52°C for 24 hrs). After reactivation, the MOs were cultivated on slant culture tubes with MRS agar (Merck, 1.10660.0500) and stored at 4°C for further use in inoculum. The used strains were officially microbiologically characterised through the Leibniz Institute's German Collection of microorganisms and Cell Cultures (DSMZ). DSM No. 2314 was isolated from potato washing water, DSM ID 14-298 was isolated from chicken feed, and DSM ID 14-301 was isolated from rotten foliage.

4.2.3.2. Inoculum culture conditions

Inoculum was cultivated on 60 mL MRS (Merck, 1.10661.0500) broth in shaking flasks (52°C, 100 rpm, 15 hrs). These were put into 5 mL tubes for centrifugation (5,000 rpm, 15 min, 4°C). Centrifuged bacteria were resuspended in minimal medium for the lignin test (60 g/L D-(+)-Glucose, 5 g/L yeast extract, 0.025 mol/L sodium-acetate-buffer at pH 6.0). A set of five different lignin concentrations (Sigma, 471003; 0.0 g/L, 0.2 g/L, 0.4 g/L, 0.6 g/L, and 0.8 g/L) was applied.

4.2.3.3. Optical density measurement

A Bioscreen C from Oy Growth Curves Ab Ltd. was used for the optical density experiments. Measurements were taken with a wide band filter (wavelength 420-580 nm).

4.2.3.4. Calibration curve

For the calibration curve, Bioscreen C micro-array Honeycomb plates were prepared as follows: all wells, except the wells of the 10th row, were filled with 250 µl of the minimal medium. The wells of the 10th row were filled with 500 µl inoculum. 250 µl were removed from these wells and transferred into the next upper row. Appropriate serial 2-fold dilutions were made up to the 2nd row and were mixed by repeated syringing. The 1st row was used as medium blank. The filled plates were placed in the Bioscreen C, followed by a short measurement. The OD from the non-inoculated wells was subtracted from the growth data to minimise the effect of the signal draft. The concentrations of the CFUs were determined by an Abbe counting chamber. On demand, additional 10-fold dilutions were prepared for counting.

4.2.3.5. Optical density measuring of bacterial growth

The Honeycomb plates were prepared as described in Chapter 2.3.1. The incubation temperature was set to 52°C with interval shaking, changing to medium and slow intensity for 30 sec prior to and after OD reading. Measurements were taken every 5 min for 32 hrs. At least two replicate wells were used in one experiment for the determination of maximum growth rate for each lignin concentration.

4.2.3.6. Models and parameter estimation

Presupposing that the cell concentration increases in sigmoidal shape, different models were used to simulate the bacterial growth curve (Baranyi et al., 1993a; Grijspeerd and Vanrolleghem, 1999;

Yilmaz, 2011). Although these models had the same key parameters, they differed in shape and number of parameters. A logistic, a Gompertz, and the Richards and Stannard model were used in a modified and re-parameterised shape, as has been offered by Zwietering et al. (1990). The Baranyi equation was used as a two (μ_m, λ) and three (μ_m, λ, v) parametrical model (Baranyi, 1997; Buchanan et al., 1997).

- natural logarithm of the quotient of the cell concentration (N) and minimal cell concentration (N_{min})

$$y = ln\left(\frac{N}{N_{min}}\right) \quad \text{with } [y] \equiv 1 \qquad /4.2\text{-}1/$$

- natural logarithm of the quotient of the initial cell concentration (N_0) and minimal cell concentration (N_{min})

$$y_0 = ln\left(\frac{N_0}{N_{min}}\right) \quad \text{with } [y_0] \equiv 1 \qquad /4.2\text{-}2/$$

- natural logarithm of the quotient of the asymptotic cell concentration (N_{max}) and the initial cell concentration (N_{min})

$$y_{max} = ln\left(\frac{N_{max}}{N_{min}}\right) \text{ with } [y_{max}] \equiv 1 \qquad /4.2\text{-}3/$$

- difference of logarithmic cell concentrations

$$\Delta y = y_{max} - y_o \text{ with } [\Delta y] \equiv 1 \qquad /4.2\text{-}4/$$

- maximum specific growth rate

$$\mu_m \quad \text{with } [\mu_m] \equiv 1/h \qquad /4.2\text{-}5/$$

- lag time

$$\lambda \quad \text{with } [\lambda] \equiv h \qquad /4.2\text{-}6/$$

The models were implemented in MATLAB©. A simulated annealing algorithm was used to obtain the statistical global solution with standard properties. The Euclidean distance was used as an optimisation criterion.

4.2.4. Results and discussion

4.2.4.1. Calibration curve

The relationship between a certain concentration of CFUs per millilitre medium (*cfu/ml*) and the resulting measurable OD can be used to construct a calibration curve. The calibration curve is used to equate the concentration of the cells at any given time in the experiment. The calibration

curve is shown in Figure 4.2-1 and described with a regression of a 3rd order binomial equation in equation /4.2-7/. Using the calibration curve, the values of the measured OD can be directly converted into the microbial concentration.

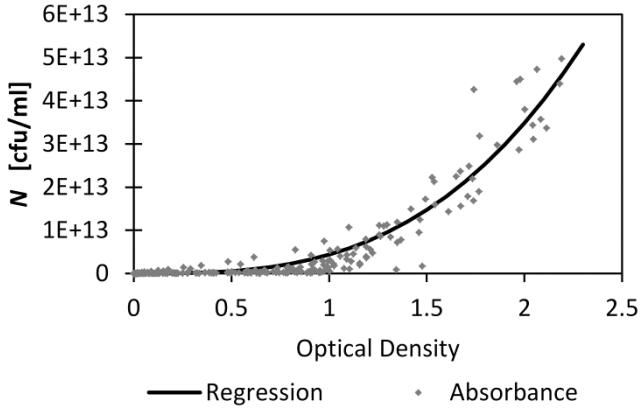

Figure 4.2-1: 3rd order calibration curve between cell concentration and optical density of the different *Bacillus coagulans* strains.

$$cfu/ml = 4.3555*10^{12} \cdot OD^3 + 6.9824*10^{-2} \cdot OD^2 \cdot 4.8828*10^{-4} \cdot OD \qquad /4.2\text{-}7/$$

$R^2 = 0.92601$

4.2.4.2. Data Analysis

The general shape of the bacterial growth curve is known and characterised by the lag phase, the exponential growth phase, and the stationary phase. In this study, the simulated annealing algorithm is used, and the models are matched to growth data already published by Grijspeerdt and Vanrolleghem (1999) and Baranyi (1993b). This step is important to check the discrepancy of the optimisation results between the key parameters μ_m and λ as compared to the mentioned published results and to each other. Table 4.2-1 is a summary of the results of this test. Based on the simulation results, the average value of μ_m and λ of the different models are used.

Table 4.2-1**:** Comparison of the estimated parameters with prior published data from other sources. The used model equations show differences from the estimated parameters. Due to this, the average value of the parameters will be used to quantify the growth of the MOs.

	Baranyi & Roberts 3-Parameter	**Baranyi & Roberts 2-Parameter**	**Gompertz 2-Parameter**	**Logistic 2-Parameter**	**Richard & Stannard 3-Parameter**		
Grijspeerdt & Vanrolleghem (1999) in Table 1 μ_m = 1.089 λ = 2.364						**AM**	**e**
μ_m	1.063	1.089	1.255	1.338	0.441	1.037	2.178
λ	2.825	3.154	3.719	6.026	1.791	3.503	7.956
e	2.748	3.014	3.156	3.792	4.880		
Grijspeerdt & Vanrolleghem (1999) in Table 2 μ_m = 1.11 λ = 2.781						AM	e
μ_m	1.093	1.096	1.585	1.452	0.834	1.212	2.553
λ	2.613	2.731	3.891	5.827	3.891	3.791	8.467
e	1.255	1.507	1.230	1.517	2.389		
J. Baranyi et al. (1993b) μ_m = 0.56 λ = 2.58						AM	e
μ_m	0.541	0.538	0.687	0.682	1.030	0.696	1.512
λ	1.493	1.587	4.322	3.806	9.954	4.232	11.60
e	2.220	2.764	2.679	3.165	4.574		

e = Euclidian distance; AM = average mean

4.2.4.3. Experimental Data

Growth curves for different initial inoculum of DSM No. 2314 are shown in Figure 4.2-2. Each individual curve shows the same growth characteristics. Independent from the inoculum dilutions, they reached nearly the same maximum cell concentration. Obviously, the lag time and the maximum growth rate differ from dilutions (DL) in a dependent way. This effect is also described by Baranyi and Roberts (1995) and by Baranyi and Pin (1999) with specified mathematical background. Furthermore, the data leads to the assumption that there exists a minimum lag time with an optimal cell concentration. That means that the lag time cannot be reduced by a further increase of the cell concentration (Figure 4.2-2A).

A slight decrease of the cell density within the first hours of the experiments can be noticed (Figure 4.2-2B). This is possibly due to a lysis process during the adaptation period of the MOs to the new environment. Also, a reduction of the cell density can be detected at the end of the final cell concentration. If the inoculum concentration is about $\ln(N_0)$ = 25 ln[*cfu/mL*], no increase of OD is detected (1:2 DL in Figure 4.2-2A and 1:2, 1:4, and 1:8 DL in Figure 4.2-2B). The other DL leads to the same final concentration of DSM No. 2314 about $\ln(N_{max})$ = 28.913±0.049 ln[*cfu/mL*] without lignin and $\ln(N_{max})$ = 26.103±0.396 ln[*cfu/mL*] with 0.4 g/L of lignin. Consequently, a threshold exists for the highest achievable concentration that depends on the lignin concentration.

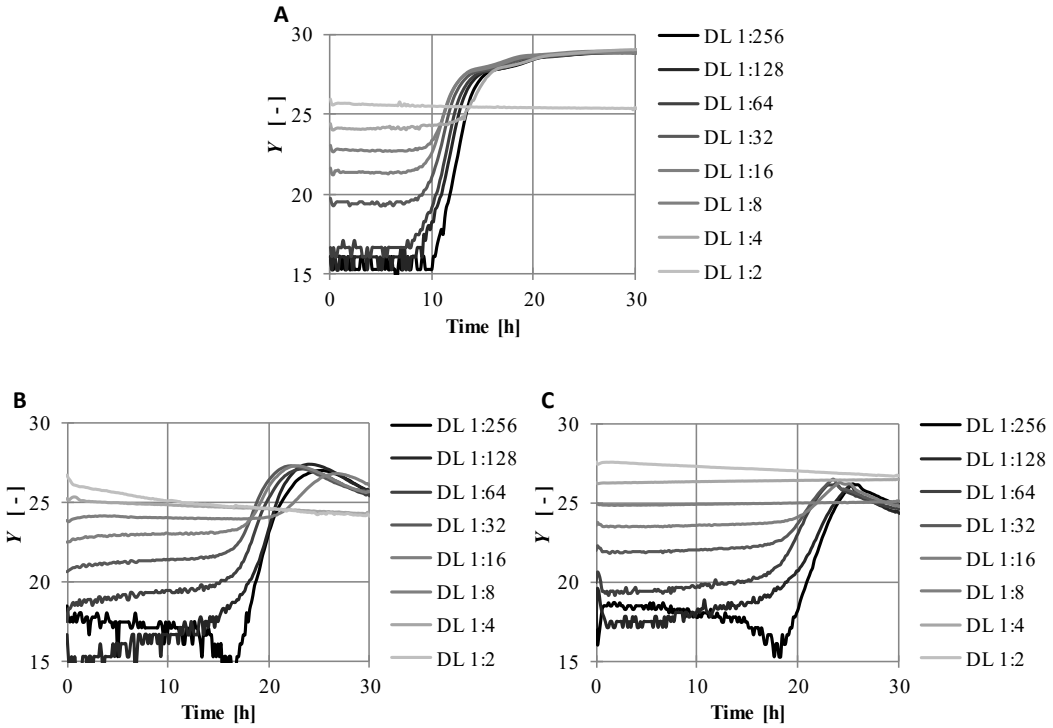

Figure 4.2-2: Incubation time plot of multiple initial inoculum dilutions of DSM No. 2314. Each curve represents the growth of a single initial inoculum dilution. A: Growth without lignin; B: Growth with 0.2 g/L supplement of lignin; C: Growth with 0.4 g/L supplement of lignin.

4.2.4.4. Parameter Estimation

The parameters of growth characteristics, μ_m and λ, were estimated and the average values were taken. In Figure 4.2-3, an exemplary survey of the parameters for the different inoculum dilutions of DSM ID 14-301 and DSM ID 14-298 is shown. All parameters show direct dependence on the initial inoculum. With increasing inoculum concentration μ_m, λ, and the differences in the maximum of the achieved cell concentration, Δy decreases, as can be expected. In Figure 4.2-3A, a general lower μ_m of DSM ID 14-301 compared to DSM ID 14-298 (Figure 4.2-3B) is visible. Likewise, DSM ID 14-301 does not vary much in the value of μ_m and λ about 0.6 g/L of lignin. Also Δy (Figure 4.2-3C) is very low and does not indicate any growth. The high cell density only leads to small growth of the MOs and might be the reason for the growth impulse at the point of higher inoculum. Unexpectedly, DSM ID 14-301 shows a slightly higher value of μ_m and a decrease in lag time concerning 0.2 g/L and 0.4 g/L of lignin. Growth is detected only with higher inoculum concentrations.

DSM ID 14-298 shows growth on all indicated lignin concentrations, with a steady decrease of μ_m (Figure 4.2-3D). The parameter λ of DSM ID 14-298 (Figure 4.2-3E) also shows little variance, just like Δy (Figure 4.2-3F). Because of this aspect, the estimated parameter cannot be used directly to distinguish between the capabilities of the MOs to withstand higher concentrations of lignin.

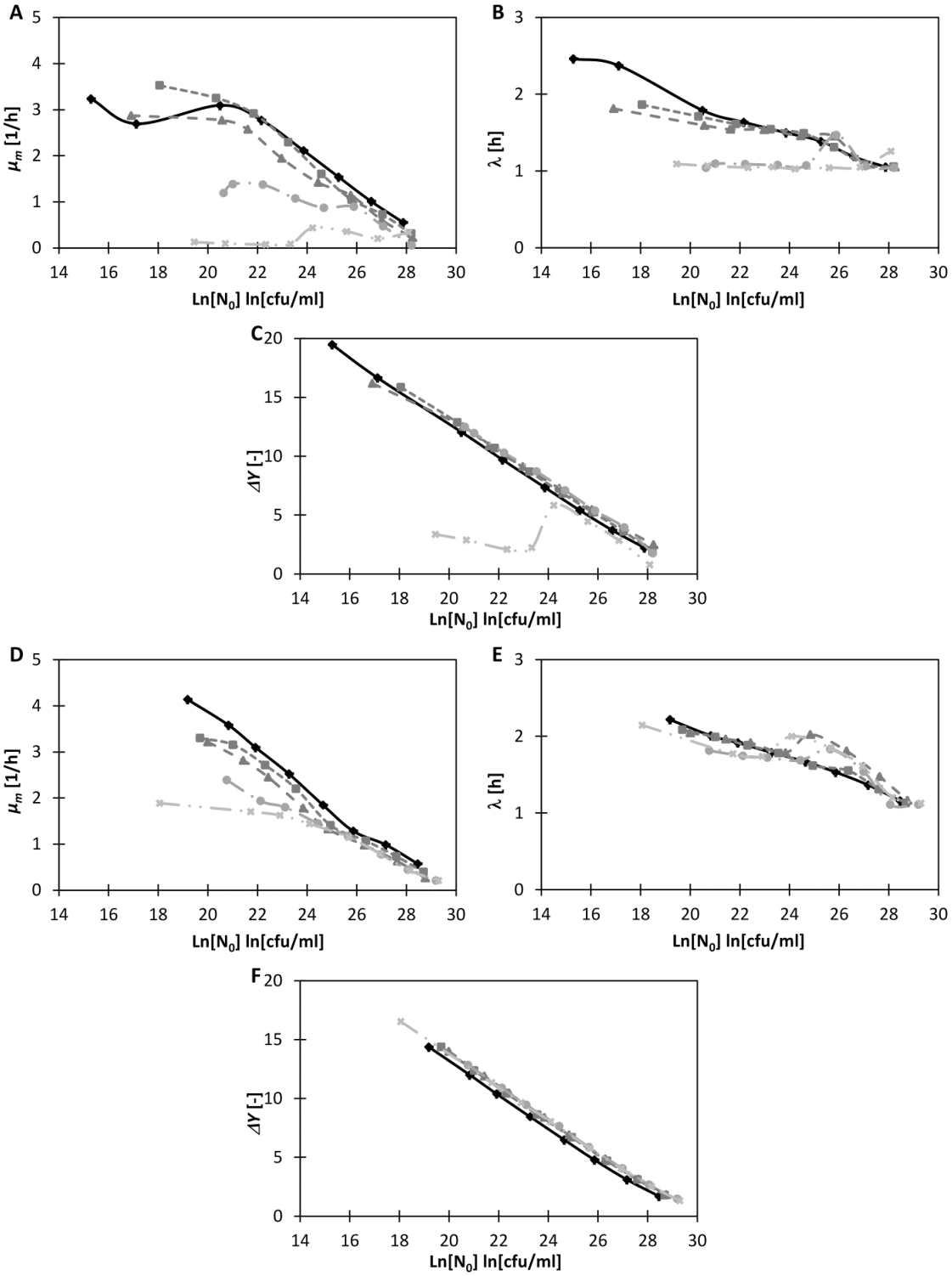

Figure 4.2-3: Average of estimated parameters as a result of using different lignin supplement concentrations (0.0 g/L, 0.2 g/L, 0.4 g/L, 0.6 g/L, 0.8 g/L) on different inoculum concentrations of DSM ID 14-301 and DSM ID 14-298. Increasing lignin concentration from black (0.0 g/L) up to light gray (0.8 g/L)
DSM ID 14-301: A: Maximum growth rates μ_m; B: lag time λ; C: Difference Δy;
DSM ID 14-298: D: Maximum growth rates μ_m; E: lag time λ; F: Difference Δy;

The maximum growth rates shown in Figure 4.2-3 display values up to 4 1/h, leading to a doubling time of around 10 min. In comparison, the bacterium *Vibrio natriegens* can double with a generation time of less than 10 min (Eagon, 1962) and is known as the only bacterium with such

a high growth rate. *Vibrio natriegens* is a genus of bacillus shaped, Gram-negative, facultatively aerobic, halophilic bacteria (Delpech, 2001). Such a growth rate requires an extremely high rate of protein synthesis and therefore *V. natriegens* contains a large number of rRNA operons, and its rRNA promoters are extremely strong (Aiyar et al., 2002). In a later, more advanced version of the screening method, an average maximum growth rate on glucose without lignin of 1.1±0.5 1/h was derived by parameter fitting of the models (Table 4.3-1). Lab-scale fermentation results revealed an average maximum growth rate on glucose of 0.48 ± 0.09 1/h (Table 4.3-3).

A dimensionless parameter $\alpha = exp(-\mu_m \lambda)$ is described by Baranyi and Roberts (1995) and by Baranyi and Pin (1999) to quantify the physiological state of an initial population. However, this dimensionless parameter α does not give more information than μ_m and λ itself, due to the equation to a mathematical product. A redefinition of α as quotient provides more information (equation 4.2-8).

$$\beta = \mu_m / \lambda \qquad \text{with } [\beta] \equiv 1 / h^2 \qquad /4.2\text{-}8/$$

β can be interpreted as the efficiency rate of an increased maximum growth rate in respect to the limitation of a higher lag time. A higher β indicates that MOs can endure lignin more efficiently in fermentation. Figure 4.2-3 shows the dependence of growth parameters on the inoculum concentration. Due to this behaviour, it seems of interest to interpret β in the context of the cell concentration, as shown in equation 4.2-9. This procedure allows for the observation of the behaviour of β with increasing lignin concentration.

$$\gamma = \mu_m / (\lambda \cdot \Delta y \cdot y_0) \qquad \text{with } [\gamma] \equiv 1 / h^2 \qquad /4.2\text{-}9/$$

In Figure 4.2-4, the β and γ of the three strains are shown. In Figure 4.2-4A, it becomes apparent that DSM No. 2314 and DSM ID 14-301 show a raising curve of β until 0.2 g/L of lignin. After that small increase, the parameters decrease. DSM No. 2314 and DSM ID 14-301 in Figure 4.2-4B display the increase of the efficiency parameter γ until 0.2 g/L of lignin as well, but DSM No. 2314 shows the higher value of γ. DSM ID 14-298 displays a steady falling in β and γ but its descent is not as rapid as the descents of DSM No. 2314 and DSM ID 14-301. The efficiency of DSM No. 2314 and DSM ID 14-301 is lower than the efficiency of DSM ID 14-298 at an inhibitor concentration that is higher than 0.6 g/L. Furthermore, in Figure 4.2-4B, there is indication of an interception point of γ for the three strains about 0.5 g/L of lignin.

To further compare the MOs, the interception point with the x-axis of a linear interpolation of the descending part of β or γ is used (Figure 4.2-4A, Figure 4.2-4B). A higher interception point of the x-axis represents a more effective tolerance of lignin of the MOs. The interception indicates the highest possible lignin concentration in which growth is possible under the current unregulated Bioscreen conditions.

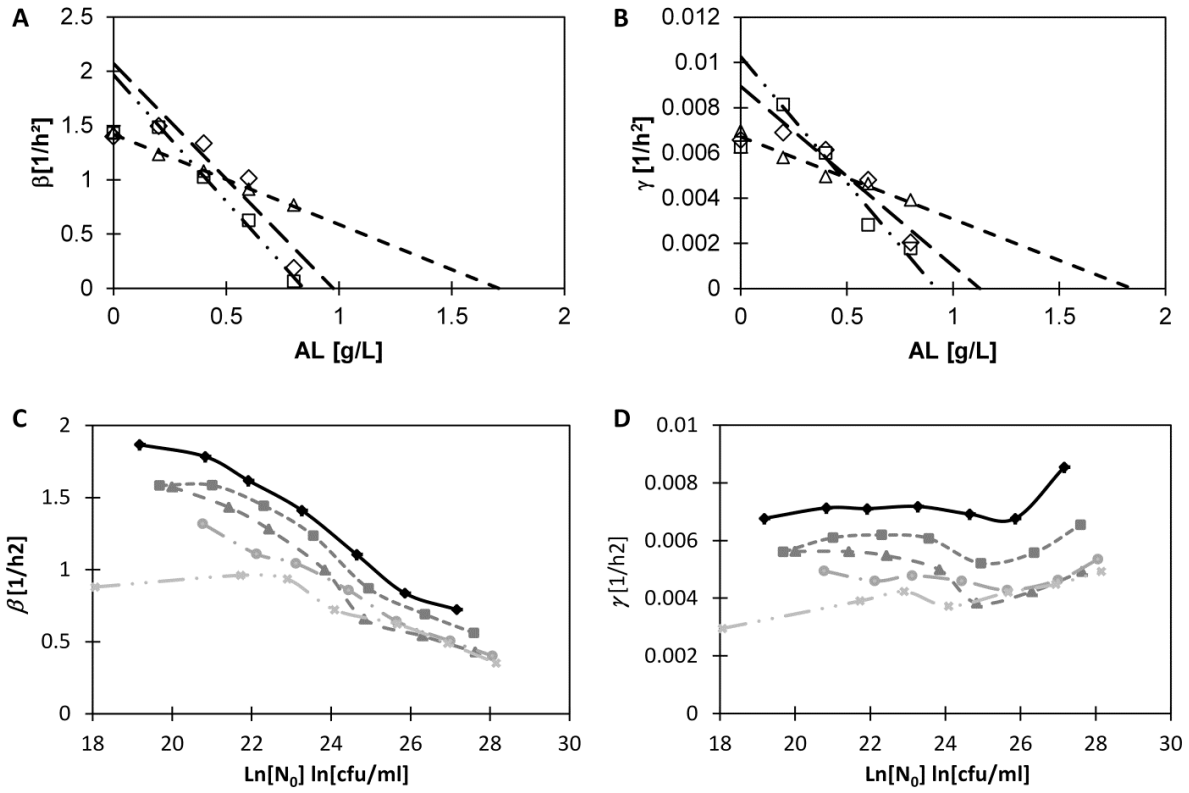

Figure 4.2-4: Progression, regression and interpolation of β and γ with increasing lignin concentration (0.0, 0.2, 0.4, 0.6, 0.8 g/L). Regression and interpolation of the descending part of β and γ with equation of y = ax+b. Increasing lignin concentration from black (0.0 g/L) up to light gray (0.8 g/L)
A: Progression and interpolation of β of the three *Bacillus coagulans* strains.
B: Progression and interpolation of γ of the three *Bacillus coagulans* strains.
C: Dependence of β towards y0 of DSM ID 14-298 (decreasing value of β).
D: Independence of γ towards y0 of DSM ID 14-298 (constant value of γ)

Regarding the dependence of the estimated parameters of the cell concentration, Figure 4.2-4C and Figure 4.2-4D show the values of β and γ of DSM ID 14-298 in respect to the inoculum concentrations. While β shows a decreasing behaviour, γ is nearly constant as the inoculum concentration increases. This circumstance indicates that γ might be more independent from the inoculum concentration and seems to be a more efficient parameter than β. For example, it can be used as a characterisation parameter prior to a scale-up of the process. Based on the interpolation results, it is to be assumed that the MO with higher interception is a better MO for a scale-up process. DSM No. 2314 and DSM ID 14-301 have nearly the same effectiveness to the phenolic compound. Theoretically, β indicates a growth of DSM No. 2314 and DSM ID 14-301 to lignin tolerance below 1 g/L (equations 4.2-10 and 4.2-13). γ indicates a growth of DSM No. 2314 until 0.9 g/L (equation 4.2-12) and a possible growth of DSM ID 14-301 up to 1.13 g/L (equation 4.2-14). The interpolation of DSM ID 14-298 shows the strongest effectivity in β and γ. The interception indicates a tolerable lignin concentration of DSM ID 14-298 at a maximum of 1.7 g/L in β (equation 4.2-12) and of 1.84 g/L in γ (equation 4.2-15) in current Bioscreen cultivation conditions.

DSM No. 2314:	β = -2.473x + 1.983	$x(\beta=0)$ = 0.80 g/L	/4.2-10/
DSM ID 14-301:	β = -2.215x + 2.070	$x(\beta=0)$ = 0.97 g/L	/4.2-11/
DSM ID 14-298:	β = -0.830x + 1.418	$x(\beta=0)$ = 1.70 g/L	/4.2-12/
DSM No. 2314:	γ = -0.0117x + 0.0105	$x(\gamma=0)$ = 0.90 g/L	/4.2-13/
DSM ID 14-301:	γ = -0.0079x + 0.0089	$x(\gamma=0)$ = 1.13 g/L	/4.2-14/
DSM ID 14-298:	γ = -0.0036x + 0.0067	$x(\gamma=0)$ = 1.84 g/L	/4.2-15/

In summary, the strains show a different behaviour in the reduced controllable environment of the well plates of the Bioscreen C screening. It is also noticeable that the lignin concentration has different effects on diverse bacterial strains. The raising of the lignin concentration influences the DSM ID 14-298 in a consistently negative way. The growth of DSM No. 2314 and DSM ID 14-301 differs from this. The use of low concentrations of lignin (for example 0.2 g/L), seems to stabilize the growth of the bacteria, which is an unexpected behaviour. Also, all strains show a different intensity of lignin inhibition. Therefore, in processes with lignin concentrations below 0.5 g/L, which refers to the interception of γ, DSM No. 2314 and DSM ID 14-301 should be used in a scale-up process. A process with higher lignin concentration should be done with DSM ID 14-298.

4.2.5. Conclusion

The procedure as described above facilitates the identification of more interesting bacteria, which can be used in other complex inhibitory environments. Using a mathematical approach, it was possible to characterize LA-producing bacteria for a lignocellulose biorefinery. It was shown that a strain, isolated from a natural lignin-containing environment, had the best growth results. It also was shown that low concentrations of lignin can stabilise the growth of two other strains. Our described mathematical approach can help to identify the amount of a substance, such as lignin, that might stabilise bacterial growth.

4.3. Model-based characterisation of growth performance and L-lactic acid production with high optical purity by thermophilic *Bacillus coagulans* in a lignin-supplemented mixed substrate medium. [3]

4.3.1. Abstract

Three *Bacillus coagulans* strains were characterised in terms of their ability to grow in lignin-containing fermentation media and to consume the lignocellulose-related sugars glucose, xylose, and arabinose. An optical-density high-throughput screening was used for pre-characterisation by means of different mathematical model parameter fitting for comparison (Logistic, Gompertz, Baranyi, Richards & Stannard, and Schnute). The growth response was characterised by the maximum growth rate and lag time. For a comparison of the screening and fermentation results, an unstructured mathematical model was proposed to characterise the lactate production, bacterial growth, and substrate consumption. The growth model was then applied to fermentation procedures using wheat straw hydrolysates. The results indicated that the unstructured growth model can be used to evaluate lactate-producing fermentation. Under the experimental fermentation conditions, one strain showed the ability to tolerate a high lignin concentration (2.5 g/L) but lacked the capacity for sufficient pentose uptake. The lactate yield of the strains that were able to consume all sugar fractions of glucose, xylose and arabinose was ~83.4%. A photometric measurement at 280 nm revealed a dynamic change in alkali-lignin concentrations during lactate-producing fermentation. A test of decolourisation of VAN, FA, and alkali-lignin samples also showed the decolourisation performance of the *B. coagulans* strains being studied.

4.3.2. Introduction

LA is a chemical widely used in different disciplines and with versatile applications, for example, in the food, textile, and leather industries. It has become a common component for chemical synthesis of polymers in the chemical, pharmaceutical, and medical industries (Andreopoulus and Theophandies, 1996; John et al., 2007; John et al., 2009 Castillo Martinez et al, 2013; Coutu et al., 2009; Gupta et al., 2007; Peter et al., 1998; Papenburg et al., 2009; Sanders et al., 2002). As a naturally occurring organic acid, LA can be produced by biotechnological fermentation of sugars obtained from renewable resources such as sugarcane. Due to the increasing demand of LA, carbon sources are currently very expensive (Okano et al., 2010). The less expensive lignocellulose raw materials, such as wheat straw and wood, are carbohydrate sources that have attracted much attention in recent studies (John et al., 2007; Adsul et al., 2007b; Carole et al., 2004; Mooney et al., 1999; Wang et al., 2015). However, a

[3] Based on: Robert Glaser and Joachim Venus, 2017. Model-based characterisation of growth performance and L-lactic acid production with high optical purity by thermophilic *Bacillus coagulans* in a lignin-supplemented mixed substrate medium. New Biotechnology 37; 180–193. http://dx.doi.org/10.1016/j.nbt.2016.12.006

pre-treatment of the lignocellulose is needed to make the carbohydrates, such as glucose, xylose, and arabinose, available as monosugars. During the pre-treatment, it is possible that soluble by-products such as furfural and hydroxymethylfurfural and the component lignin can be formed (Yamamoto et al., 2014). Lignin, as a phenolic compound, has been identified as an inhibitor of enzymatic hydrolysis and microbial fermentation (Glaser et al., 2014; Hofvendahl and Hahn-Hägerdal, 2000; Palmqvist et al., 1996; Palmqvist and Hahn-Hägerdal, 2000a, 2000b; Zeng et al., 2014). Additional pre-treatment processes are often required to remove lignin. Several methods for delignification of lignocellulose are now being discussed (Peng et al., 2012), such as alkaline pre-treatment (Phummala et al., 2014), ethanol-alkali delignification (Shatalov et al., 2002; Sun et al., 2000), and treatment with organic solvents (Dapía et al., 2002; Vázquez and Lage, 1992).
The suitability of raw materials for lignin separation pre-treatment and delignification steps varies. Residues of solvated lignin and other inhibitory compounds might still be solvated in the fermentation medium after delignification steps. For processes aimed at reducing the inhibitory compound concentrations, process time and cost increase along with the success rate. Because they less expensive economical processes, MOs that can ferment hexoses and pentoses, as well as endure higher concentrations of inhibitory compounds or detoxify lignocellulose hydrolysates, are of high industrial relevance (Priefert et al., 2001). Furthermore, bacterial lignin degradation could be used to generate aromatic chemicals from the renewable resource lignin (Sainsbury et al., 2015). In both contexts, some *Bacillus* strains show activity in terms of degradation of lignin model compounds (Gurujeyalakshmi and Mahadevan, 1987; Robinson and Crawford, 1978; Yadav and Chandra, 2015). Among them, *B. coagulans* strains have been shown to degrade the lignin-related compounds FA and VAN to vanillic acid (Karmakar et al., 2000) or have been analysed for native detoxification of furfural (Ye et al., 2014). The anaerobic degradation of phenolic or aromatic compounds requires adenosine triphosphate (ATP) as an energy source to break the strong bonds in the molecules (Boll et al., 2002; Heider and Fuchs, 1997; Heider et al., 1998; Sahar, 2006).

B. coagulans is a spore-forming thermophilic facultative anaerobic LA bacterium that grows at 50–55°C and pH 5.0 (De Clerk et al., 2004; Lucas et al., 2006). *B. coagulans* can grow facultative anaerobe, normally producing ATP by aerobic respiration and it is also capable of switching to fermentation under anaerobic conditions. It is a ubiquitous soil organism that is also able to ferment hexose and pentose sugars (that constitute plant biomass) to L-(+)-lactic acid with high titers (Ye et al., 2014; Patel et al., 2006). *B. coagulans* was shown to utilise xylose through the pentose-phosphate pathway, which enables homolactic acid fermentation. This pathway leads to a yield of 5 moles of lactic acid from 3 moles of xylose (Payot et al., 1999; Rhee et al., 2001; Su et al., 2011; Walton et al., 2010).
This study presents data on three strains of *B. coagulans*, cultured on lignin-containing substrates. The three strains of *B. coagulans* were pre-characterised by a high throughput screening

procedure using kinetic parameters to evaluate resistance to alkali-lignin (AL). Additional fermentation experiments were performed to determine the ability of the strains to consume the saccharides glucose, xylose and arabinose and to characterise the ability of the strains to withstand AL in comparison to the previous screening. To derive the necessary key performance indices, a parameter estimation was conducted to adjust a mathematical growth model. The proposed fermentation model was applied to fermentation experiments using the artificial medium (AM), wheat straw hydrolysate (WSH) and aspen wood hydrolysate (AWH). Its applicability was verified by comparing empirical and theoretical lactate production, bacterial growth, and sugar consumption.

4.3.3. Materials and methods

4.3.3.1. Bacillus coagulans strains

B. coagulans strains were isolated from different environments. To ensure that the MOs were of the species *B. coagulans*, they were characterised by the DSMZ. *B. coagulans* DSM No. 2314 was used as a reference strain, which was originally isolated from potato washing water. Strain DSM ID 14-298 was isolated from chicken food, and DSM ID 14-301 was isolated from rotten foliage.

4.3.3.2. Optical density screening

The high-throughput optical-density screening was implemented as described previously (Glaser and Venus, 2014; Glaser and Venus, 2017). The modifications are described in Glaser and Venus (2017).

4.3.3.3. Fermentation, culture conditions, biomass quantification

Cultivation was carried out in a 5 L glass bioreactor (Biostat B; B. Braun Biotech, Melsungen, Germany). For the bioreactor cultivation, 2 L of a defined medium was inoculated with 2.5% (v/V_R) of inoculum (by volume). The medium contained 50 g/L D-(+)-glucose, 20 g/L D-(+)-xylose, and 10 g/L L-(+)-arabinose (Carl Roth GmbH + Co. KG, Germany) and 15 g yeast extract. The medium was mixed with AL to achieve 0.0 g/L, 0.625 g/L, 1.25 g/L, and 2.5 g/L initial AL concentration in the medium. The sugar/lignin solution and yeast extract were autoclaved at 121°C for 20 min. The sugar/lignin solution was autoclaved separately from the yeast extract to minimize the Maillard reaction. The pH level was adjusted after autoclaving with
20% sodium hydroxide to 6.0 and was kept constant during fermentation. The process temperature was kept constant at 52°C. Samples were taken at different time points via a manual

bypass system. Blank samples were collected prior to adding the inoculum. To inactivate the samples for metabolite measurement, they were placed in a hot water bath at 92°C for 30 min, and then stored in a freezer at -8°C for further use. After thawing, the samples were subjected to centrifugation (Sigma 4k15 centrifuges) at 5000 rpm for 15 min at 4°C. The supernatant was passed through a 20 µm cellulose acetate membrane micro filter (Th. Geyer GmbH & Co. KG, Germany). The filtrate was used for detection of fermentable sugars on HPLC (Section 2.5).

For biomass quantification, each pellet was washed in 2.5 mL of demineralised water and resuspended and centrifuged (Sigma 4k15 centrifuge) at 5000 rpm, 4°C for 15 min. The supernatant was discarded. The pellets were placed in porcelain pots and dried for 2 hrs at 105°C. After that, the pots were cooled down in a desiccator in a vacuum of 40 kPa for 1 hr. The tare weight was measured using a precision scale. The pellets were resuspended in demineralised water and transferred to the porcelain pots and dried in a vacuum for 24 hrs at 105°C. After cooling to room temperature, the pots were weighed. After the first weighing, the pots were dried again for 1 hr at 105°C. If the second weighing showed a lower weight than the first, additional drying was needed, and the procedure was repeated. Blank samples were used to subtract the effects of medium composition. As reference, see also DIN EN 12880.

4.3.3.4. Detection of sugars and lactate

The measurement of sugar and lactate concentrations was done by HPLC using a Dionex ICS 3000 (Thermo Fisher Scientific Inc., USA) equipped with a Eurokat H column (300 × 8 mm, 10 µm, eluent: 0.01 N H_2SO_4, end capped, including precolumn, Knauer GmbH, Germany) with an operation pressure of 65 bar. For detection, a Refractive Index Detector RI-101 (SHODEX, Showa Denko Europe GmbH) was used at wavelength 254 nm. The column was used at a constant temperature of 35°C at a flow rate of 0.8 mL/min of the mobile phase. The injection volume was 10 µL. The operation pressure was set to 65 bar. For the previously mentioned fermentation conditions, LA was in the form of a salt and therefore was reported as lactate.

4.3.3.5. Detection of lignin

The amount of soluble lignin in the artificial medium as well as the lignin amount in the WS and AW normalised hydrolysates was estimated using UV spectrometry at 280 nm. Regression curves were constructed with AL (for further information, see Foster et al., 2010; Fukushima and Hatfield, 2004; Lee et al., 2013; Lupoi et al., 2015).

4.3.3.6. Decolourisation by lignin, ferulic acid, and vanillin uptake

The decolourisation tests of FA and VAN are modified tests based on the assays for FA decarboxylase activity and VAN dehydrogenase activity and methods described by Kaur et al. (2013a, 2013b) and Karmakar et al. (2000). The modifications are described in detail in Glaser and Venus (2017).

4.3.4. Theory and calculation

Predictive modelling is a special field of food microbiology in which mathematical equations are used to describe the behaviour of MOs under different environmental conditions, physical or chemical, such as temperature, pH, or activity and resistance to growth inhibitors. Building these mathematical models requires measurement of the bacterial growth. Bacterial growth often shows different phases. The typically named phases are 1) the initial lag phase in which the MO adapts to a new environment, resulting in a lag time (λ); 2) the growth phase, in which the specific growth rate of the MO (μ) starts at a value of zero and increases to a maximal value (μ_{max}); and 3) the final phase, where the growth rate decreases to zero, so that the curve ends reaching an asymptote (A) that results in the shape of a sigmoidal function (Zwietering et al., 1990).

4.3.4.1. Screening of growth models

Several sigmoidal functions, a logistic function, the Gompertz model, and the Richards and Stannard models were tested in a modified and reparametrized form, as proposed by Zwietering et al. (1990), and the parameters were compared to describe bacterial growth. The Baranyi equation was used as a six- and four-parameter model (Glaser and Venus, 2014; Baranyi et al., 1993b; Baranyi and Roberts, 1994; Baranyi, 1997; Buchanan et al., 1997; Grijspeerdt and Vanrolleghem, 1999) for parameter estimation. Ignoring the consumption of a substrate or formation of products, these models describe only the concentration of MOs normalised, in contrast to a model based on the Monod equation. It is also possible to define a Monod type model (MTM) using only the concentration of MOs. In the screening test, the substrate concentration is not of interest, and it can be assumed that there is enough substrate available.

In the following equations, $y(t)$ is the natural logarithm of the normed logarithmic cell concentration $y(t) = \ln(N/N_{min})$, while y_0 is the natural logarithm of the normed minimum cell concentration, and y_{max} is the natural logarithm of the normed maximum asymptotic cell concentration (Zwietering et al., 1990).

Baranyi: 6-parameter model

$$y(t) = y_0 + \mu_{max} \cdot t + \frac{1}{\mu_{max}} ln\left(\exp\left(-\nu \cdot t\right) + exp(-h_0) - exp(-\upsilon \cdot t - h_0)\right) - \frac{1}{m} \cdot ln\left(1 + \frac{exp(m \cdot \mu_{max} \cdot t) \cdot \frac{1}{\mu_{max}} ln\left(\exp\left(-\nu \cdot t\right) + exp(-h_0) - exp(-\upsilon \cdot t - h_0)\right)}{exp\left(m \cdot (y_{max} - y_0)\right)}\right) \quad /4.3\text{-}1/$$

Baranyi: 4-parameter model

$$y(t) = y_0 + \mu_{max} \cdot t + \frac{1}{\mu_{max}} ln\left(\exp\left(-\mu_{max} \cdot t\right) + exp(-h_0) - exp(-\mu_{max} \cdot t - h_0)\right) - ln\left(1 + \frac{exp(m \cdot \mu_{max} \cdot t) \cdot \frac{1}{\mu_{max}} ln\left(\exp\left(-\mu_{max} \cdot t\right) + exp(-h_0) - exp(-\mu_{max} \cdot t - h_0)\right)}{exp(y_{max} - y_0)}\right) \quad /4.3\text{-}2/$$

Logistic model:

$$y(t) = \frac{y_{max} - y_0}{\left\{1 + \exp\left[\frac{4 \cdot \mu_{max}}{y_{max} - y_0}(\lambda - t) + 2\right]\right\}} \quad /4.3\text{-}3/$$

Gompertz model:

$$y(t) = (y_{max} - y_0) \cdot exp\left\{-exp\left[\frac{4 \cdot \mu_{max} \cdot e}{y_{max} - y_0}(\lambda - t) + 1\right]\right\} \quad /4.3\text{-}4/$$

Model by Richards and Stannard:

$$y(t) = (y_{max} - y_0) \cdot \left\{1 + \omega \cdot \exp\left(1 + \xi\right) \cdot exp\left[\frac{\mu_{max}}{y_{max} - y_0} \cdot (1 + \xi) \cdot \left(1 + \frac{1}{\xi}\right) \cdot (\lambda - t)\right]\right\}^{\left(-\frac{1}{\omega}\right)} \quad /4.3\text{-}5/$$

Model proposed by Schnute:

$$y(t) = \left(\mu_{max} \frac{(1-b)}{a}\right) \cdot \left[\frac{1 - b \cdot exp(a \cdot \lambda + 1 - b - a \cdot t)}{1 - b}\right]^{1/b} \quad /4.3\text{-}6/$$

Monod-type model:

$$\frac{dC_{BM}(t)}{dt} = C_{BM}(t) \cdot \mu_{max} \cdot \left(\frac{q_0}{q_0 + exp(-\mu_{max} \cdot t)}\right) \cdot \left(1 - \frac{C_{BM}(t)}{C_{BM,max}}\right) \quad /4.3\text{-}7/$$

The components of model equation 4.3-7 are described in the section below (fermentation growth model). To characterise MOs via kinetic growth parameters derived from the characterisation of growth during the optical density screening, the curve fitting is not the best tool. For a better approach, Glaser and Venus (2014) have proposed two parameters according to the equations, with $\beta = \mu_{max}/\lambda$ and $\gamma = \mu_{max}/(\lambda*(y_{max}-y_0)*y_0)$ used to characterise the physiological ability of the normalised population to adjust to alternating environmental growth conditions. The parameter γ cannot be used in this work, as this work uses a different experimental setup. Glaser and Venus (2014) have used various inoculum concentrations to examine the change of the initial lag phase and maximum growth rate as a result of the alternation of inhibitor concentration. In the present examination, only one inoculum concentration was used to examine the effect of different inhibitor concentrations in the growth medium containing different sugars. Here, γ could not provide enough significant information and therefore γ was rearranged and provided in the following form as δ

$$\delta = \frac{\mu_{max}}{\lambda} \cdot (y_{max} - y_0) \quad /4.3\text{-}8/$$

4.3.4.2. Fermentation growth model

In the following section, a mathematical model is proposed. The purpose of this model is to provide a basis for better comparison and understanding of the *B. coagulans* strains that are discussed in this study. It could be used to compare data of other research later on, but due to differences in process design and the genetics of used strains of other studies, the results would be questionable.

For simulation of the batch cultivation of *B. coagulans*, a kinetic process model was derived with glucose, xylose, and arabinose as carbon sources. The model consisted of five differential equations. The kinetic of the model was based on the additive Monod type for growth with mixed substrates (Dunn et al., 2003; Zeiger et al., 2010). The nonlinear least squares algorithm performed the estimation of the parameter values, by estimation of the root mean squares (RMS), and by means of MATLAB (Mathworks, Natick, MA) optimisation tools.

In the following section, the mass balance and kinetic equations for the batch process of mixed-substrate cultivation of *B. coagulans* are presented.
As described above, bacterial growth can be classified into different phases. Therefore, the model used here for the fermentation characterisation needs different parts that consider those phases. An exponential growth phase can be described with the first part of equation /4.3-9/. After the growth phase, the following stationary phase changes into a phase in which MOs are dying due to a lack of carbohydrates in the batch fermentation medium. Therefore, the second term is introduced into equation /4.3-9/ to consider the reduction of the biomass at the end of the process in question. The parameters K_D and k_D characterise the death rate of the cells. The change in biomass is described as follows:

$$\frac{dC_{BM}}{dt} = \mu_{BM} \cdot C_{BM}(t) - k_D \cdot \left(\frac{C_{BM}(t)}{K_D - C_{BM}(t)}\right) \qquad /4.3\text{-}9/$$

The reduction in the number of MOs takes place via a visible reduction in the cell size and due to death of the MOs. The Monod model also contains a parameter describing the growth rate of the MOs. This parameter is defined as μ_{BM}. In the process to model here, the organisms are expected to grow at different rates on different substrates. Therefore, the biomass growth rate, given by the parameter μ_{BM}, should be described as a function of the growth rates of the available substrates, where each single substrate will allow a different maximum growth rate.

In this optimisation and simulation approach, it is assumed that each substrate thus allows a different maximal growth rate ($\mu_{i,max}$). The biomass growth rate μ_{BM} is assumed as specific growth rates on glucose $\mu_{Glc,}$ xylose μ_{Xyl}, and arabinose μ_{Ara} (see also equation 4.3-14). A further theoretical parameter was defined as $\mu_{max,sim,mix}$, as a quality parameter (see also Zeiger and Grammel, 2010; Dunn et al., 2003).

$$\mu_{BM} = \mu_{max,sim,mix} \cdot \left(k_1\mu_{Glc} + k_2\mu_{Xyl} + k_3\mu_{Ara}\right) \cdot \left(\frac{1}{k_1+k_2+k_3}\right) \quad /4.3\text{-}10/$$

Parameters $\mu_{max,sim,mix}$, k_1, k_2, and k_3 are theoretical parameters obtained by MATLAB simulation as well as by the specific maximum specific growth rates on glucose μ_{Glc}, xylose μ_{Xyl}, and arabinose μ_{Ara}, which are also obtained by MATLAB simulation.

It is also possible that the resulting product in the biotechnological process inhibits the growth of MOs. This situation also raises the possibility that all sugar types were consumed by the strains. Therefore, the model was extended with an additional term: $I(LA)$. This mathematical term was used to consider an effect of a critical LA concentration C_{LA} (Ben-Youssef et al., 2005; Burgos-Rubio et al, 2000; Keller and Gerhardt, 1975; Monteagudo et al., 1997) in our process.

$$I(LA) = \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right) \quad /4.3\text{-}11/$$

The model had to consider the differences in the duration of the initial lag phase. This means a second alteration was considered via an additional term described by Baranyi and Roberts (1994).

$$\alpha_i(t) = \left(\frac{q_{0,i}}{q_{0,i}+exp(-\mu_{i,max}\cdot t)}\right) \quad \text{where } i = Glc, Xyl, Ara \quad /4.3\text{-}12/$$

Adjustment function $\alpha(t)$ varies from $\alpha(t=0) = (q_0/(q_0+1))$ to $\alpha(t=\infty) = 1$. Here, q_0 represents the physiological state of the inoculum and therefore its ability to adjust to the new environment (Baranyi and Roberts, 1994). The lag time can be derived according to Baty and Delignette-Muller (2004):

$$\lambda_i = \frac{ln\left(1+\frac{1}{q_{0,i}}\right)}{\mu_{i,max}} \quad \text{where } i = Glc, Xyl, Ara \quad /4.3\text{-}13/$$

According to the approach of the description of the biomass development over time in the process, the changes in the substrate concentrations (glucose, xylose, and arabinose) were included in the model as follows:

$$\mu_i = \mu_{i,max} \cdot I(LA) \cdot \alpha_i(t) \cdot \left(\frac{C_i(t)}{K_i+C_i(t)}\right) \quad \text{where } i = Glc, Xyl, Ara \quad /4.3\text{-}14/$$

$$\frac{dC_i(t)}{dt} = -\frac{\mu_i}{Y^{BM/i}} \cdot C_{BM}(t) \quad \text{where } i = Glc, Xyl, Ara \quad /4.3\text{-}15/$$

Double- or more substrate Monod kinetics can also be written for two or more substrates as parallel reaction equation, according to the following approach (Dunne et al., 2003):

$$\mu = \mu_{max} \cdot \left(\frac{k_1C_1}{K_1+C_1} + \frac{k_2C_2}{K_2+C_2} + \frac{k_3C_3}{K_3+C_3}\right) \cdot \left(\frac{1}{k_1+k_2+k_3}\right) \quad /4.3\text{-}16/$$

This equation gives an additive, partial contribution for each substrate. Thus, for $C_1 = K_1$, $C_2 = K_2$ and $C_3 = K_3$, the result is $\mu = \mu_{max}/2$. For $C_1 = 0$, $C_2 = 0$ and a high concentration for C_3, $\mu = \mu_{max}*k_3/(k_1+k_2+k_3)$. If all three component concentrations C_1, C_2, and C_3 are high, then the growth

rate is $\mu = \mu_{max}$. Note that this kinetic equation requires twice as many kinetic parameters as the double Monod kinetics (Dunn et al., 2003). After expanding equation 4.3-10 and inserting equation 4.3-13, three terms are obtained (equation 4.3-17) that contain the parameter combination $\mu_{max,sim,mix} \cdot k_i \frac{\mu_{i,max}}{k_1+k_2+k_3}$ (equation 4.3-19). This combination can be interpreted as the maximum growth rate on the respective sugar component *i*. Herein, the sum of the individual sugar-specific growth rates is the total growth rate (Dunn et al., 2003).

$$\mu_{BM} = \mu_{max,sim,mix} \cdot \left(\left(\frac{k_1 \mu_{Glc}}{k_1+k_2+k_3} \right) + \left(\frac{k_2 \mu_{Xyl}}{k_1+k_2+k_3} \right) + \left(\frac{k_3 \mu_{Ara}}{k_1+k_2+k_3} \right) \right) = \mu_{max,sim,mix} \cdot \sum_{i=3}^{n} \left(\frac{k_i \mu_i}{k_1+k_2+k_3} \right) \quad /4.3\text{-}17/$$

$$\mu_{BM} = \mu_{max,sim,mix} \cdot \sum_{i=3}^{n} \left(\frac{k_i \cdot \mu_{i,max} \cdot \alpha_i(t) \cdot \left(\frac{C_i(t)}{K_i + C_i(t)} \right) \cdot I(LA)}{k_1+k_2+k_3} \right) \quad /4.3\text{-}18/$$

$$\mu_{BM} = \mu_{max,sim,mix} \cdot \sum_{i=3}^{n} \left\{ \left(k_i \cdot \frac{\mu_{i,max}}{k_1+k_2+k_3} \right) \cdot \alpha_i(t) \cdot \left(\frac{C_i(t)}{K_i + C_i(t)} \right) \right\} \cdot I(LA) \quad /4.3\text{-}19/$$

The relation $\mu_i / Y^{BM/i} = qi$ describes the uptake rate of a substrate. The product formation equation for lactate was based on the simplified assumption that the rate of product formation was related to the biomass formation through a production coefficient, $Y^{LA/BM}$.

$$\frac{dC_{LA}}{dt} = Y^{LA/BM} \cdot \frac{dC_{BM}}{dt} \quad /4.3\text{-}20/$$

$Y^{LA/BM}$ can be derived via the relation $Y^{LA/BM} = (Y^{LA/Sub} / Y^{BM/Sub})$. The reactor volume was assumed to be constant. The Monod model is certainly too complex to describe experimental data in a satisfactory manner and must be used with appropriate caution. The value of the substrate saturation constant K is often very small compared to the substrate concentrations. For example, the concentration C is generally much greater than K, and the term $\mu/\mu_{max} = C/(C+K)$ is in batch mode practically equal to 1 over the entire cultivation time, therefore, zero order of reaction regarding the substrate concentration. At high substrate concentrations C→∞, an asymptotically zero reaction order with respect to the substrate is achieved and μ equals μ_{max}. At low concentrations, the first reaction order for μ/μ_{max} with the increase $1/K$ applies with good approximation. With C → 0 the approach results in K = (C*μ_{max}/μ); it may be assumed that K ≈ $1/\mu$ in the small concentration range, which would be interesting for the parameter estimation. To reduce the models' degrees of freedom, parameters $\mu_{i,max}$ and K_i were defined as dependent on each other. The numerical value of the Monod parameters K was set as equal to the inverse value of the specific uptake rates, as follows: $K_{Glc} = 1/\mu_{Glc,max}$, $K_{Xyl} = 1/\mu_{Xyl,max}$, and $K_{Ara} = 1/\mu_{Ara,max}$ as well as $K_D = 1/k_D$. Note that by using this approach, the units do not fit. Only the values are used for parameter reduction to increase the repeatability and reproducibility of the parameter estimations. The values of the parameter K will be much higher than if they had been derived by laboratory experiments.

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The final model equation system can be now defined as follows:

$$\frac{dC_{BM}}{dt} = \mu_{BM} \cdot C_{BM}(t) - k_D \cdot \left(\frac{C_{BM}(t)}{K_D - C_{BM}(t)}\right) \quad /4.3\text{-}21/$$

$$\frac{dC_{Glc}(t)}{dt} = -\frac{\mu_{Glc,max} \cdot \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right) \cdot \left(\frac{q_{0,Glc}}{q_{0,Glc} + exp\left(-\mu_{Glc,max} \cdot t\right)}\right) \cdot \left(\frac{C_{Glc}(t)}{K_{Glc} + C_{Glc}(t)}\right)}{Y^{BM/Glc}} \cdot C_{BM}(t) \quad /4.3\text{-}22/$$

$$\frac{dC_{Xyl}(t)}{dt} = -\frac{\mu_{Xyl,max} \cdot \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right) \cdot \left(\frac{q_{0,Xyl}}{q_{0,Xyl} + exp\left(-\mu_{Xyl,max} \cdot t\right)}\right) \cdot \left(\frac{C_{Xyl}(t)}{K_{Xyl} + C_{Xyl}(t)}\right)}{Y^{BM/Xyl}} \cdot C_{BM}(t) \quad /4.3\text{-}23/$$

$$\frac{dC_{Ara}(t)}{dt} = -\frac{\mu_{Ara,max} \cdot \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right) \cdot \left(\frac{q_{0,Ara}}{q_{0,Ara} + exp\left(-\mu_{Ara,max} \cdot t\right)}\right) \cdot \left(\frac{C_{Ara}(t)}{K_{Ara} + C_{Ara}(t)}\right)}{Y^{BM/Ara}} \cdot C_{BM}(t) \quad /4.3\text{-}24/$$

$$\frac{dC_{LA}}{dt} = Y^{LA/BM} \cdot \frac{dC_{BM}}{dt} \quad /4.3\text{-}25/$$

4.3.5. Results and discussion

The efficient utilisation of the major sugar fractions (glucose, xylose, and arabinose) bound to the lignocellulose raw materials was a major factor for the cost-effective conversion of lignocellulose into useful chemicals such as LA. Making matters more difficult was the presence of the inhibitory phenolic compound lignin, which had to be tolerated by the microbes. For simulation of such a process, an artificial medium was used that contains the major sugar components and the inhibitory compound lignin.

4.3.5.1. Optical density screening

For the basic characterisation of *B. coagulans* strains, the strains were grown in microtiter plates. The growth medium consisting of either glucose or xylose, or a combination of both, was supplemented with various amounts of lignin. To examine the growth performance, different kinetic growth models were used to obtain the basic kinetic key parameters of the maximum growth rate μ_{max} and the lag time λ. The tested models fit well to the growth data from the screening experiments, with values of the correlation coefficient R^2 of 0.91±0.04, 0.95±0.02, and 0.91±0.05 for the strains DSM No. 2314, DSM ID 14-298, and DSM ID 14-301, respectively. A direct comparison among the different models showed visibly higher maximum growth rates in the Schnute model. In addition, the defined MTM showed partially higher values. The fitting parameters for the maximum growth rates μ_{max} and the lag time values λ, as well as for the statistical evaluation, are given in Table 4.3-1.

Table 4.3-1: Maximum growth rates and lag times for *B. coagulans* determined by high-throughput optical-density measurements.

AL	0.0 g/L		0.2 g/L		0.4 g/L		0.6 g/L		0.8 g/L	
	μ_{max}	λ	μ_{max}	λ	μ_{max}	λ	μ_{max}	λ	μ_{max}	λ
DSM No 2314										
Baranyi-3	0.8422	1.7977	0.7138	1.7911	0.4774	1.6487	0.2911	1.3213	0.3397	0.9329
Baranyi-2	0.8783	2.0885	0.7484	2.1172	0.4903	1.7353	0.2839	1.0559	0.3299	1.3006
Logistic	1.1300	3.3508	0.9006	3.6168	0.4914	3.4423	0.1329	1.0203	0.0900	1.2222
Gompertz	1.1344	3.2136	0.8914	3.4243	0.4487	2.9850	0.1369	1.0364	0.0919	1.0871
R&S	0.7703	3.0087	0.5855	3.0907	0.3227	2.8808	0.1162	1.1268	0.0815	1.0658
Schnute	2.2940	1.7553	2.1279	1.8856	1.5446	1.6181	0.6541	0.9834	0.5038	0.8835
MKM	0.8366	0.4400	0.7092	0.5029	0.6249	0.8050	0.4386	0.6246	0.4280	0.6197
Average	1.1265	2.2364	0.9538	2.3470	0.6286	2.1593	0.2934	1.0241	0.2663	1.0160
STD	0.4951	0.9610	0.4899	1.0213	0.3828	0.8796	0.1824	0.1931	0.1638	0.2113
DSM ID 14-298										
Baranyi-3	0.8738	0.5576	1.0666	0.7931	1.0072	0.7661	1:0034	0.7600	0.9659	0.7219
Baranyi-2	0.6740	0.1827	1.0464	0.6378	0.9703	0.6682	1:0304	0.7046	1.0283	0.7102
Gompertz	0.4682	0.1255	1.0200	0.7012	0.9534	0.7231	0.8415	0.6916	0.7538	0.6007
Logistic	0.4500	0.1189	1.0577	0.7004	0.9664	0.6974	0.8865	0.6937	0.7995	0.6308
R&S	0.3594	0.1791	0.7465	0.7008	0.7067	0.7280	0.6225	0.7130	0.5733	0.6196
Schnute	1.1138	0.2512	2.1622	0.6628	2.1964	0.6021	1.9741	0.6044	1.7232	0.5085
MKM	1.6363	0.0994	3.4555	0.0510	3.2289	0.0549	2.8780	0.0616	2.7003	0.0691
Average	0.7965	0.2163	1.5078	0.6067	1.4327	0.6057	1.3195	0.6048	1.2206	0.5516
STD	0.4218	0.1471	0.8937	0.2312	0.8601	0.2299	0.7504	0.2258	0.6926	0.2077
DSM ID 14-301										
Baranyi-3	1.1957	0.6734	1.3170	1.1243	1.4119	1.3392	1.3308	1.2379	1.1351	0.9851
Baranyi-2	1.1830	0.7734	1.3154	1.1276	1.4311	1.3237	1.3485	1.3246	1.1576	0.9927
Gompertz	1.3429	1.0882	1.6138	1.4344	1.8665	1.6057	1.7590	1.6747	1.4108	1.5055
Logistic	1.4125	1.1031	1.6770	1.3985	1.9214	1.5420	1.7785	1.5794	1.4630	1.4847
R&S	0.8305	1.0039	1.0662	1.3586	1.1507	1.5208	1.0803	1.5591	0.9537	1.4845
Schnute	2.0654	0.6507	2.1708	0.9124	2.8924	1.2973	2.9721	1.3446	2.5346	1.1789
MKM	1.5689	0.1464	1.4790	0.1565	1.4566	0.1700	1.5435	0.1609	0.7949	0.5208
Average	1.3713	0.7770	1.5199	1.0732	1.7329	1.2570	1.6875	1.2687	1.3499	1.1646
STD	0.3549	0.3114	0.3268	0.4122	0.5354	0.4577	0.5725	0.4755	0.5302	0.3375

Although strain DSM No. 2314 did show the expected steady decrease in the maximum growth rate, the two strains DSM ID 14-298 and DSM ID 14-301 interestingly showed a slight increase in the maximum growth rates at lower AL concentrations. The lag time is also negatively affected by an increase in its duration at lower AL concentrations. The lower determined lag time for the highest lignin concentration was a result of the small increase in the density signal, which might be caused by evaporation processes that led to inadequate determination of the lag time.

However, in the current state, the parameters μ_{max} and λ did not make it possible to characterise the MOs. The defined parameters β and δ are more suitable for rapid evaluation of the growth processes to identify interesting strains for scaled-up processes, due to the combined examination of the parameters μ_{max} and λ. Information on the maximum tolerable concentration of AL, as far as the growth of MOs in a scaled-up process should be possible, can be derived from the regression estimation, which was used for extrapolation of β and δ. Parameter β is visually displayed in Figure 4.3-1 in Glaser and Venus (2017) and the experimental data for all the strains analysed were given as well.

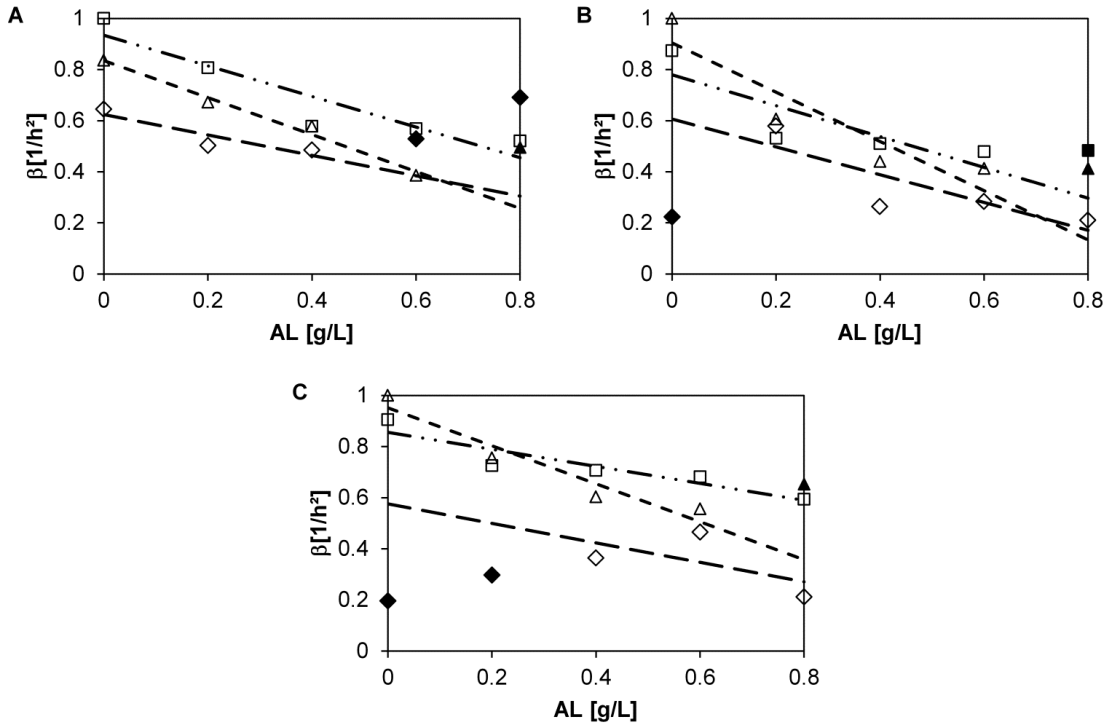


Figure 4.3-1: Growth prediction of *B. coagulans* strains based on the optical-density high-throughput screening expressed by the parameters β (□ glucose, ◊ xylose, Δ glucose+xylose). Linear regressions are indicated as lines (— ▪ ▪ glucose, — — — xylose, − − − glucose+xylose). A: β of DSM No. 2314; B: β of DSM ID 14-298; C: β of DSM ID 14-301. Black symbols were not used for regression and extrapolation.

The performance of DSM No. 2314 decreases visibly through the normed parameters δ for growth on glucose, xylose, and a mixture of the two (Figure 4.3-2A). Parameter β for DSM No. 2314 indicates growth performance on xylose, which was half as good as the growth on glucose, while the growth on the combination of glucose and xylose yielded better results. Parameter δ indicates a much worse performance on xylose because of reduced building of biomass. Both issues showed a slight increase at low AL concentrations of 0.2 g/L and 0.4 g/L. An interpolation of the decreasing part for the data points of β and δ marks a possible growth of up to an average maximum of 1.43±0.19 and 1.29±0.63 g/L AL. Disregarding the difference in biomass production, β indicates a strong possibility for a lignin concentration of up to 1.56 g/L AL in the glucose-based screening medium, while the glucose/xylose combination yielded the predicted value of 1.15 g/L. Parameter δ indicates a growth prediction for glucose fermentation of up to 0.81 g/L and of up to 0.89 g/L AL on a glucose/xylose mixed substrate. For strain DSM ID 14-298, the prediction signifies a possible growth of up to 1.11±0.14 g/L or 1.08 ± 0.14 g/L for β and δ (Figure 4.3-2B), respectively. Strain DSM ID 14-301 is marked with a possible growth performance of up to 0.8 g/L AL in the screening test. The extrapolation used for the prediction of the AL endurance indicates a mean maximum of 1.78±0.56 g/L and 1.45±0.39 g/L AL for β and δ (Figure 4.3-2C), respectively. For strain DSM ID 14-301, glucose growth was predicted for up to 2.57 g/L and to 1.98 g/L for β and δ, while xylose growth seems only possible for up to 1.28 g/L or 1.07 g/L AL.

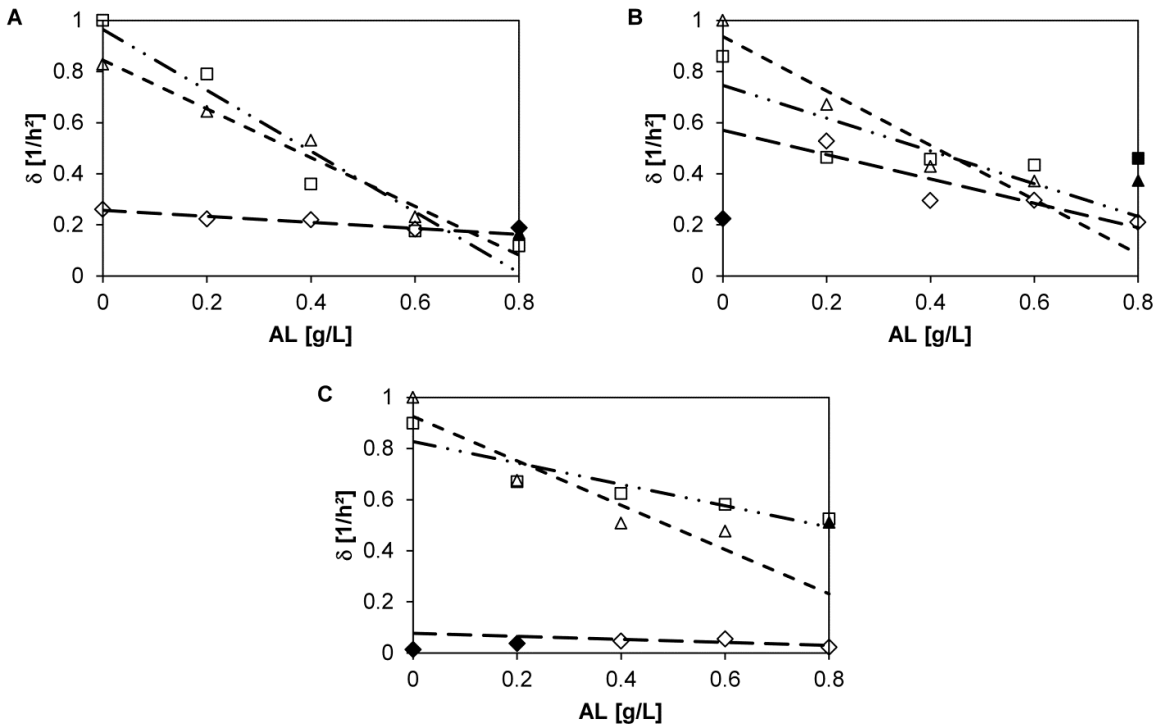


Figure 4.3-2: Growth prediction of *B. coagulans* strains based on the optical-density high-throughput screening according to parameters β and δ, which are indicated as symbols (□ glucose, ◊ xylose, Δ glucose/xylose). Linear regressions are indicated as lines (— ▪ ▪ glucose, — — — xylose, – – – glucose/xylose). A: δ of DSM No. 2314 B: δ of DSM ID 14-298; C: δ of DSM ID 14-301. Black symbols were not used for regression and extrapolation.

4.3.5.2. Fermentation performance

To schematise the major fractions of lignocellulose hydrolysate fermentation, a process in which a mixed sugar medium was supplemented with AL was used for fermentation procedures with three different strains of *B. coagulans* producing LA. To investigate the carbohydrate uptake, the fermentation medium (mixture) contained glucose 50 g/L, xylose 20 g/L, and arabinose 10 g/L, and likewise various amounts of AL: 0.0 g/L, 0.625 g/L, 1.25 g/L, or 2.5 g/L and nutrients. Thermophilic lactate production using the AM, mimicking a lignocellulose hydrolysate, resulted in different growth behaviour of the three analysed strains of *B. coagul*ans. For the basic parameters derived from the fermentation process, see Table 4.3-2 or see data in Glaser and Venus (2017).

Table 4.3-2: Parameters of growth derived from experimental data, Figs. 4.3-3 to 4.3-5.
(data published in Glaser and Venus, 2017; see Table 1)

	DSM No. 2314		**DSM ID 14-300**			**DSM ID 14-301**				
AL	**0.0**	**0.625**	**0.0**	**0.625**	**1.25**	**0.0**	**0.625**	**1.25**	**2.5**	**(g/L)**
Concentrations of biomass and sugar [a]										
$C_{BM,min}$	0.03	0.04	0.02	0.04	0.07	0.03	0.08	0.07	0.06	(g/L)
$C_{BM,max}$	6.09	7.78	4.33	5.92	5.29	5.00	4.81	5.17	5.31	(g/L)
$C_{\Delta BM}$	6.06	7.74	4.41	5.88	5.22	4.77	4.73	5.10	5.25	(g/L)
$C_{Glc,min}$	0.00	0.00	0.00	0.00	0.00	11.90	7.38	0.00	0.00	(g/L)
$C_{Glc,max}$	46.21	44.64	46.69	45.57	44.58	49.78	47.39	42.76	41.82	(g/L)
$C_{\Delta Glc}$	46.21	44.64	46.69	45.57	44.58	37.88	40.01	42.76	41.82	(g/L)
$C_{Xyl,min}$	0.00	0.00	0.00	0.00	0.00	11.27	7.09	11.55	11.16	(g/L)
$C_{Xyl,max}$	21.81	22.36	21.33	20.68	22.75	21.22	21.84	22.02	22.43	(g/L)
$C_{\Delta Xyl}$	21.81	22.36	21.33	20.68	22.75	9.95	14.75	10.47	11.27	(g/L)
$C_{Ara,min}$	0.00	0.00	0.00	0.00	0.00	8.81	7.63	8.77	7.07	(g/L)
$C_{Ara,max}$	10.44	10.76	10.58	10.66	10.95	11.70	10.77	10.58	10.55	(g/L)
$C_{\Delta Ara}$	10.44	10.76	10.58	10.66	10.95	2.89	3.14	1.18	3.48	(g/L)
$C_{\Delta Sub}$	78.46	77.76	78.6	76.91	78.28	50.72	57.90	54.41	56.57	(g/L)
$C_{LA,max}$	68.85	63.71	69.68	67.07	66.00	46.95	50.64	45.56	43.75	(g/L)
P [b]	2.648	0.885	2.488	1.397	1.375	0.978	1.055	0.949	0.912	(g/L/h)
FT	26	72	28	48	48	48	48	48	48	(h)
L-(+)-lactate purity										
L(+)-LA	98.89	98.89	99.51	99.70	99.64	99.63	98.93	98.89	98.94	(%)

[a]: Data derived by HPLC measurement described in section 4.3.3 after inoculum addition.
[b]: The productivity P was evaluated as quotient $P = (C_{LA,max} / FT)$.

The estimated model parameters are given in Table 4.3-3. Growth dynamics of the fermentation procedures and model adjustment are shown in Figures 4.3.5-3 to 4.3.5-5.

For DSM No. 2314, a clear exponential growth phase was observed during fermentation with and without a low AL (Figure 4.3-3A) amount of 0.625 g/L (Figure 4.3-3B). The growth declined visibly while the microbes were metabolising the pentoses. The reference fermentation process without AL showed complete sugar consumption within 24 hrs, with a productivity of 2.648 g/(L·h). The fermentation time (FT) for the low AL process was 72 hrs. The long FT resulted in a lowered productivity of 0.885 g/(L·h). The consumption of the pentose sugars started when the glucose concentration was reduced to the range of the initial xylose and arabinose concentrations, resulting in a simultaneous consumption of the sugars. A yield of 68.85 g/L (87.7%) LA was obtained from the mixed sugar solution in the reference process, while a yield of 63.71 g/L (81.9%) of LA was achieved in the low-AL process. The yield was reduced by 6.6%, while the obtained biomass increased from 6.09 g/L to 7.78 g/L. The AL supplementation had no effect on the L-(+)-LA purity, which was 98.89%. Bacterial growth at 1.25 g/L and 2.5 g/L AL was not detected (see data in Glaser and Venus, 2017). This finding was consistent with the previous screening, where growth was not expected for a concentration higher than 1.06 g/L AL (see section 4.1).

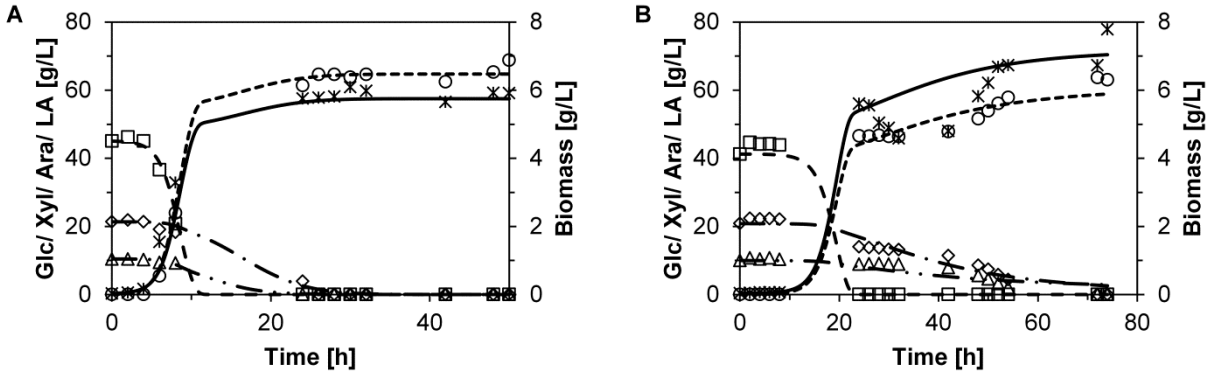


Figure 4.3-3: Fermentation dynamics of strain DSM No. 2314. Empirical results are displayed as symbols (□ glucose, ◊ xylose, Δ arabinose, ○ lactate, × biomass). Model fittings are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ arabinose, − − − lactate, ── biomass). A: Without AL. B: 0.625 g/L AL.

DSM ID 14-298 (see data in Glaser and Venus, 2017) showed stable growth until the test with 1.25 g/L AL supplementation. During the reference fermentation (Figure 4.3-4A), DSM ID 14-301 showed a high preference for consumption of glucose. Utilisation of the pentose sugars led to a slower linear growth pattern during the reference fermentation. This linear growth was not visible in the process with the low AL concentration, indicated by a faster uptake of the pentoses (Figure 4.3-4B). As a result, the FT was reduced by the increased uptake of xylose and arabinose, linked to a strong increase in biomass (36.7%). This could also be said about the LA yield, which increased from 85.9% without AL and to 89.9% at a low AL concentration. The yield of 84.3% for the process with the medium AL concentration (Figure 4.3-4C) was the same as in the reference process. The process with 2.5 g/L AL showed no MO growth. Once more, the L-(+)-LA purity of 98.92±0.03% seems unaffected by AL. The pattern of the increased growth response of DSM ID 14-298 at a low AL concentration has also be shown in a turbidimetric assay and in Glaser and Venus (2014). The screening also showed a possible growth of up to 1.25 g/L AL on the same medium.

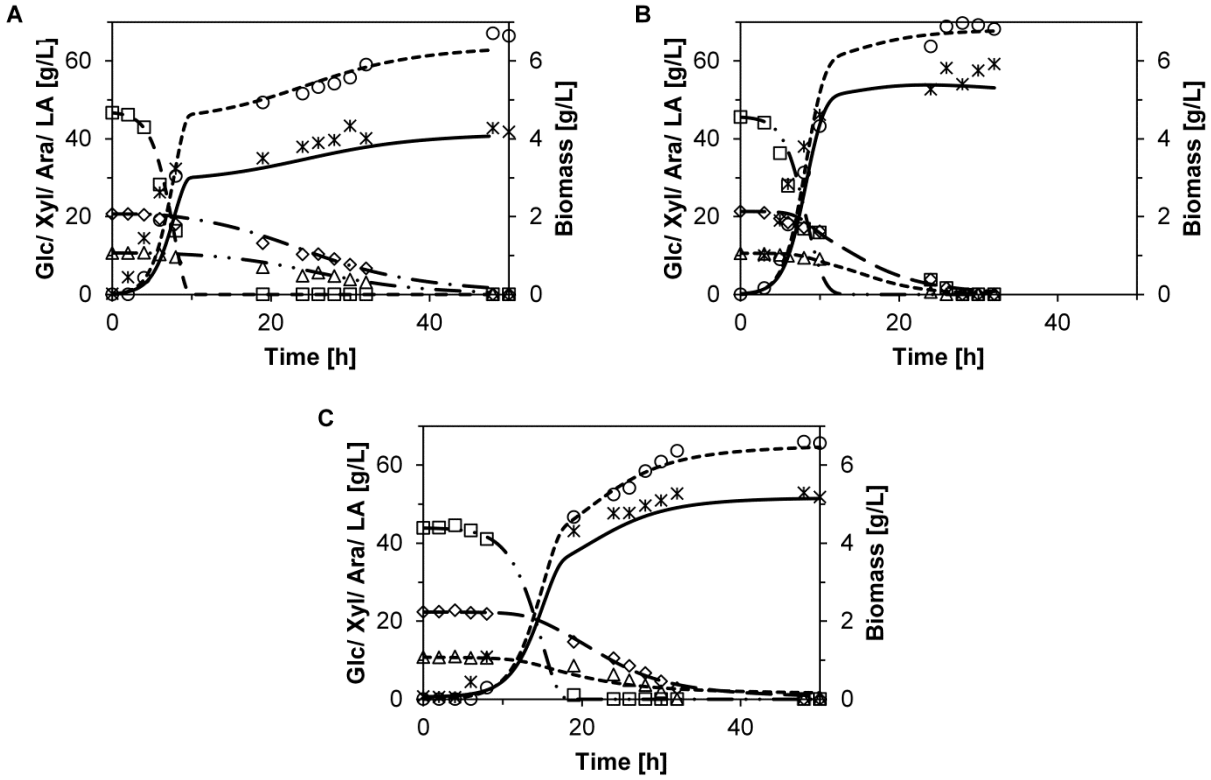


Figure 4.3-4: Fermentation dynamics of strain DSM ID 14-298. Empirical results are displayed as symbols (□ glucose, ◊ xylose, Δ arabinose, ○ lactate, × biomass). Model fittings are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ arabinose, − − − lactate, ── biomass). A: Reference fermentation process. B: 0.625 g/L AL. C: 1.25 g/L AL.

Strain DSM ID 14-301 (see data in Glaser and Venus, 2017), as shown in Figure 4.3-5, was not able to utilise all the components of the sugar mixture of the medium. However, the strain was able to utilise low amounts of xylose. It showed a mean productivity of 0.97±0.06 g/(L·h) for the 48 hrs FT, and, here too, an increased growth and production performance can be seen at low AL concentration.

DSM ID 14-301 achieved a mean LA yield of 60.4±2.9%, with a mean L-(+)-LA purity of 99.61±0.1% throughout the processes. Interestingly, DSM ID 14-301 was able to endure high AL concentrations, showing growth at 2.5 g/L AL. This result is consistent with Glaser and Venus (2014). Furthermore, with the data from the present work, a possible growth was predicted of up to 2.57 g/L AL.

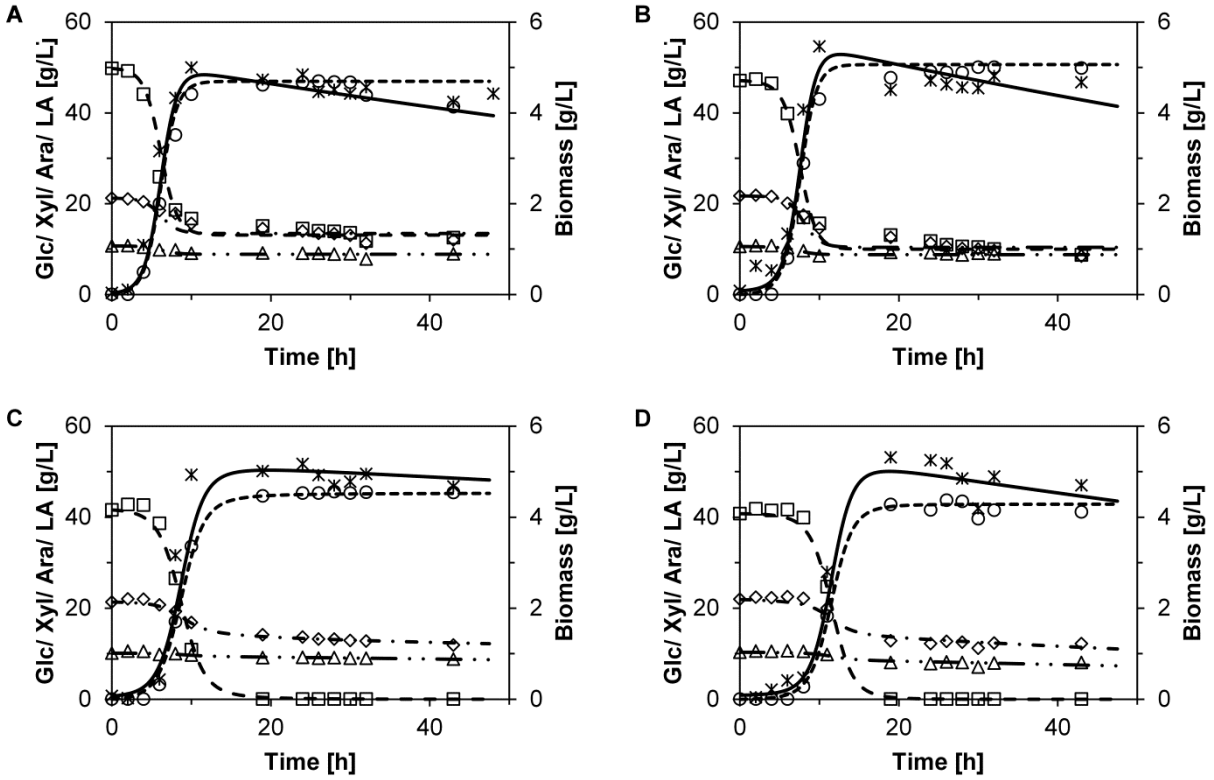


Figure 4.3-5: Fermentation dynamics of strain DSM ID 14-301. Experimental results are displayed as marks (□ glucose, ◊ xylose, Δ arabinose, ○ lactate, × biomass). Model fittings are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ arabinose, − − − lactate, ── biomass). A: Reference fermentation process. B: Fermentation process with 0.625 g/L AL. C: Fermentation process with 1.25 g/L AL and D: Fermentation process with 2.5 g/L AL.

An average LA yield of 85.9% for the two strains DSM No. 2314 and DSM ID 14-301 was demonstrated. These strains were also capable of metabolising glucose, xylose, and arabinose throughout the processes without (or while enduring) AL. Walton et al. (2010) have used *B. coagulans* MXL 9 and described its ability to fully utilise hemicellulose extracts with an LA yield of 94% on a water extract of mixed southern hard woods. They achieved an 83% yield on green liquor extract and their test fermentation with 20 g/L xylose had a yield of 90% LA. Maas et al. (2008) have reported an LA yield of 81% for *B. coagulans* DSM 2314 fermenting a hydrolysate of lime-treated wheat straw. Otto (2004) has reported a yield of 35 g/L LA from 50 g/L xylose as a sole carbon source, which gave a yield of 70% LA.

If the strains are exposed to low AL, an increase of the LA yield for DSM ID 14-289 and DSM ID 14-301 or of the biomass concentration for all strains was shown. A similar stimulatory effect on LA production has been mentioned by Ouyang et al. (2012) for *B. coagulans* NL01 when it was exposed to formic acid concentrations below 2 g/L.

In this study, the strains showed simultaneous consumption of glucose and pentoses, as the sugars were in the same concentration range. Ye et al. (2014) have also shown the simultaneous consumption of glucose and xylose at nearly equal concentrations for the fermentation of 50 g/L glucose and 53 g/L xylose by *B. coagulans* JI12. Zhang et al. (2014) have also shown data on

simultaneous consumption of glucose and xylose at equal concentrations. The glucose repression was found to be one of the major limiting factors for a cost-effective production of fuels and chemicals. Here, the tested *B. coagulans* strains provide a strong factor for efficient LA production by themselves.

For *B. coagulans* MXL 9, Bischoff et al. (2010) have reported an aldehyde tolerance at 2.5 g/L furfural and 2.5 g/L 5-hydroxymethylfurfural. It can be assumed that the inhibitory impact of lignin was at least as high as the impact of aldehydes on bacterial growth. Reducing harsh chemical conditions and thermal pre-treatments may reduce the production of furfural and hydroxymethylfurfural. In contrast, soluble lignin remained the main inhibitor of bacterial growth, particularly in simultaneous saccharification and fermentation processes.

The average value of the chiral purity of L-(+)-LA, for all fermentation procedures and strains in this study, was found to be 99.14%. This result was in the range of L-(+)-LA purity of 99.4% reported by others (Maas et al., 2008; Otto, 2004). Acetic acid as a side product could not be detected here, as confirmed for *B. coagulans* MXL 9 (Walton et al., 2010). Furthermore, an L-(+)-LA purity of over 99% for thermophilic processes has been reported (Ma et al., 2014).

The fermentation processes of the two strains DSM No. 2314 and DSM ID 14-298 consumed glucose, xylose, and arabinose. Strain DSM ID 14-298 had the best properties for use in a lignocellulose hydrolysate; it had sufficient growth performance up to an AL concentration of 1.25 g/L. The DSM ID 14-301 strain may be preferred in hydrolysates containing high glucose concentrations in combination with greater amounts of the inhibitory AL. It was able to grow at up to 2.5 g/L AL, despite lacking the ability to sufficiently metabolise pentoses. The chiral purity of L-(+)-LA was not influenced by the inhibitor.

Fermentation by the three *B. coagulans* strains showed homofermentative and thermophilic characteristics. The utilisation of pentose sugars makes these *B. coagulans* strains an ideal biocatalyst for LA production from a lignocellulosic feedstock. The strains analysed showed simultaneous consumption of the different main sugars of the cellulose and hemicellulose fractions if the concentration of the sugars reached the same level (ratio 1:1). This indicates that the glucose repression takes place only under the highly discrepant conditions of high glucose concentration and lower pentose concentration. This characteristic is a prerequisite for an effective and cost-efficient conversion of lignocellulose hydrolysates. Nonetheless, the co-fermentation of the different sugar fractions is not sufficient. The *B. coagulans* strains showed different levels of resistance to the major inhibitory compound AL. The strong resistance of strain DSM ID 14-301, and the high rate of utilisation of the main lignocellulosic sugar fractions by strain DSM ID 14-298, make a co-cultivation process possible. Using these two strains together without additional detoxification steps (or with only a reduced amount of chemicals or enzymes for detoxification of

hydrolysates) may lead to better efficiency. Avoiding detoxification steps by selecting strains of *B. coagulans* that are resistant to high concentrations of inhibitory compounds (and may be able to detoxify the growth medium by themselves) may reduce the complexity of the process and make it more effective and efficient.

4.3.5.3. Model parameters and verification

Glucose, xylose, and arabinose are the three major sugar components in lignocellulosic biomass. Efficient utilisation of these three sugars is a prerequisite for cost-efficient conversion from lignocellulose sugars to useful chemicals. In the previous section, the consumption of the three major sugar components and their reaction to different inhibitory lignin concentrations were described for *B. coagulans* using an artificial fermentation medium. A model for the description of the bacterial growth and LA production of *B. coagulans* for the parallel fermentations on glucose, xylose, and arabinose was successfully derived (see above).

The model represented the dynamic growth behaviour for the batch cultures in a clear and numerically accurate way. The overall standard mean deviations, σ, and the mean *RMS* values are shown in Table 4.3-3. The overall mean correlation coefficients R^2 were ~0.99. The results of an additional one-way ANOVA based on a 95% confidence interval had a satisfying relation of $F < F_{critical}$. With the *p* value of nearly 1.0, it could be assumed that the process data and model solution were significantly similar. The fitting parameters, as a result of the model adjustment to the fermentation data, are given in Table 4.3-2. The parameters of the maximum growth rates and the lag times of the turbidimetry screening test (Table 4.3-1), as compared to those derived through scaled-up fermentation experiments (Table 4.3-3), were slightly different, but nonetheless comparable. The values for the maximum growth rate of the model by Schnute and the MTM were in the ranges of the model parameters for the fermentation procedures. The proposed Monod model for the fermentation process could visually represent the diverse growth behaviours of the different strains. The lag times, the phase of exponential growth, the transition into the stationary, and, additionally, the phase of decreasing biomass, could successfully be shown.

Table 4.3-3: Coefficients and parameters derived by model adjustment to the fermentation growth data. (Compare Figs. 4.3-3, 4.3-4, and 4.3-5.)

	DSM No. 2314		**DSM ID 14-300**			**DSM ID 14-301**				
AL	**0.0**	**0.625**	**0.0**	**0.625**	**1.25**	**0.0**	**0.625**	**1.25**	**2.5**	**(g/L)**
Parameter derived from parameter estimations										
$\mu_{Glc,\ max}$	0.590	0.392	0.381	0.526	0.422	0.485	0.617	0.506	0.398	(1/h)
$\mu_{Xyl,\ max}$	0.285	0.087	0.147	0.309	0.197	0.163	0.374	0.125	0.112	(1/h)
$\mu_{Ara,\ max}$	0.231	0.057	0.155	0.310	0.108	0.096	0.074	0.103	0.084	(1/h)
k_D	0.006	0.024	0.005	0.069	0.025	0.090	0.103	0.050	0.087	(g/(L*h))
K_{Glc}	1.693	2.550	1.621	1.976	2.368	2.061	1.898	2.314	2.110	(g/L)
K_{Xyl}	3.505	11.46	6.796	3.236	5.088	6.130	2.671	7.978	8.893	(g/L)
K_{Ara}	4.311	17.33	6.415	3.218	9.248	10.44	13.60	9.706	11.88	(g/L)
K_D	153.3	28.45	187.0	14.35	39.69	11.09	9.671	20.15	11.51	(g/L)
$\mu_{max,sim,mix}$	1.935	2.642	2,436	2.003	2.675	2.916	2.116	2.576	3.032	(-)
k_1	1.541	1.670	3.314	2.414	1.557	1.848	2.001	0.820	2.057	(-)
k_2	0.601	1.618	2.209	0.662	2.161	0.120	0.338	0.032	0.024	(-)
k_3	0.021	0.448	1.799	0.562	0.416	0.519	0.043	0.071	0.052	(-)
$q_{0,\ Glc}$	0.435	0.036	15.30	20.66	0.052	1.094	0.159	0.371	0.045	(-)
$q_{0,\ Xyl}$	0.021	0.153	0.047	0.032	0.016	1.120	0.040	0.662	1.987	(-)
$q_{0,\ Ara}$	0.075	0.421	0.016	0.010	0.033	0.470	1.054	0.053	0.266	(-)
λ_{Glc}	2.019	8.508	0.102	0.093	5.787	1.338	3.772	3.025	6.634	(h)
λ_{Xyl}	13.61	23.09	21.07	11.24	19.74	3.913	8.684	7.347	3.626	(h)
λ_{Ara}	11.47	21.07	26.28	14.69	14.68	11.91	9.072	28.99	18.54	(h)
Fitting quality										
σ	2.060	2.611	2.434	4.619	1.476	2.739	2.484	1.438	1.275	
RMS	2.117	3.188	2.965	5.890	1.901	3.020	3.070	1.655	1.546	
R^2	0.998	0.994	0.997	0.992	0.999	0.995	0.996	0.999	0.999	
ANOVA										
F	0.001	0.023	0.002	0.001	0.001	0.002	0.015	0.007	0.002	
$F_{critical}$	3.915	3.915	3.905	3.910	3.967	3.910	3.910	3.910	3.910	
p	0.974	0.878	0.981	0.987	0.978	0.967	0.932	0.968	0.964	

Remark: The parameters K_{Glc}, K_{Xyl}, and K_{Ara}, are a result of the parameter optimisation and do not reflect the biological laboratory derived values.

Strain DSM No. 2314 had a longer FT at the low AL concentration than with the reference fermentation (Figure 4.3-3), due to a slower uptake of the pentoses and long lag time, despite the same inoculum being used. This behaviour could be reproduced by the modelling results. The uptake rates $\mu_{Glc,max}$, $\mu_{Xyl,max}$, and $\mu_{Ara,max}$ indicate a big decrease in glucose consumption (13.8%) and an even bigger decrease in pentose consumption (61.5% for xylose and 50.2% for arabinose). The model was also able to consider the significant increase in the initial lag time. Despite the significant increase in FT, as a result of the prolonged initial lag times λ, and the decrease in the uptake rates, the growth parameter showed an increase from $\mu_{max,sim,mix}$ = 2.2899 to $\mu_{max,sim,mix}$ = 3.0617, in comparison to the low-AL process.

In addition, the growth characteristics of DSM ID 14-298 could be represented by the model parameters. The increase shown in growth performance as a result of the increased consumption of pentoses was also displayed by the increased uptake rates $\mu_{Xyl,max}$ and $\mu_{Ara,max}$. Xylose concentrations showed an increased uptake (more than twofold): from 0.1471 1/h to 0.3090 1/h when compared with the fermentation with 0.625 g/L AL. The uptake of arabinose increased by slightly less than twofold, from 0.1558 1/h to 0.3107 1/h. The parameter for the process at 1.25 g/L AL revealed a decrease in the fermentation performance.

Strain DSM ID 14-301, which was able to grow at 2.5 g/L AL, showed a higher glucose uptake rate $\mu_{Glc,max}$ for AL concentrations of 0.625 g/L (30.1%) and 1.25 g/L (6.7%), in comparison to the reference process. There was also an increase for the uptake of xylose in the fermentation with

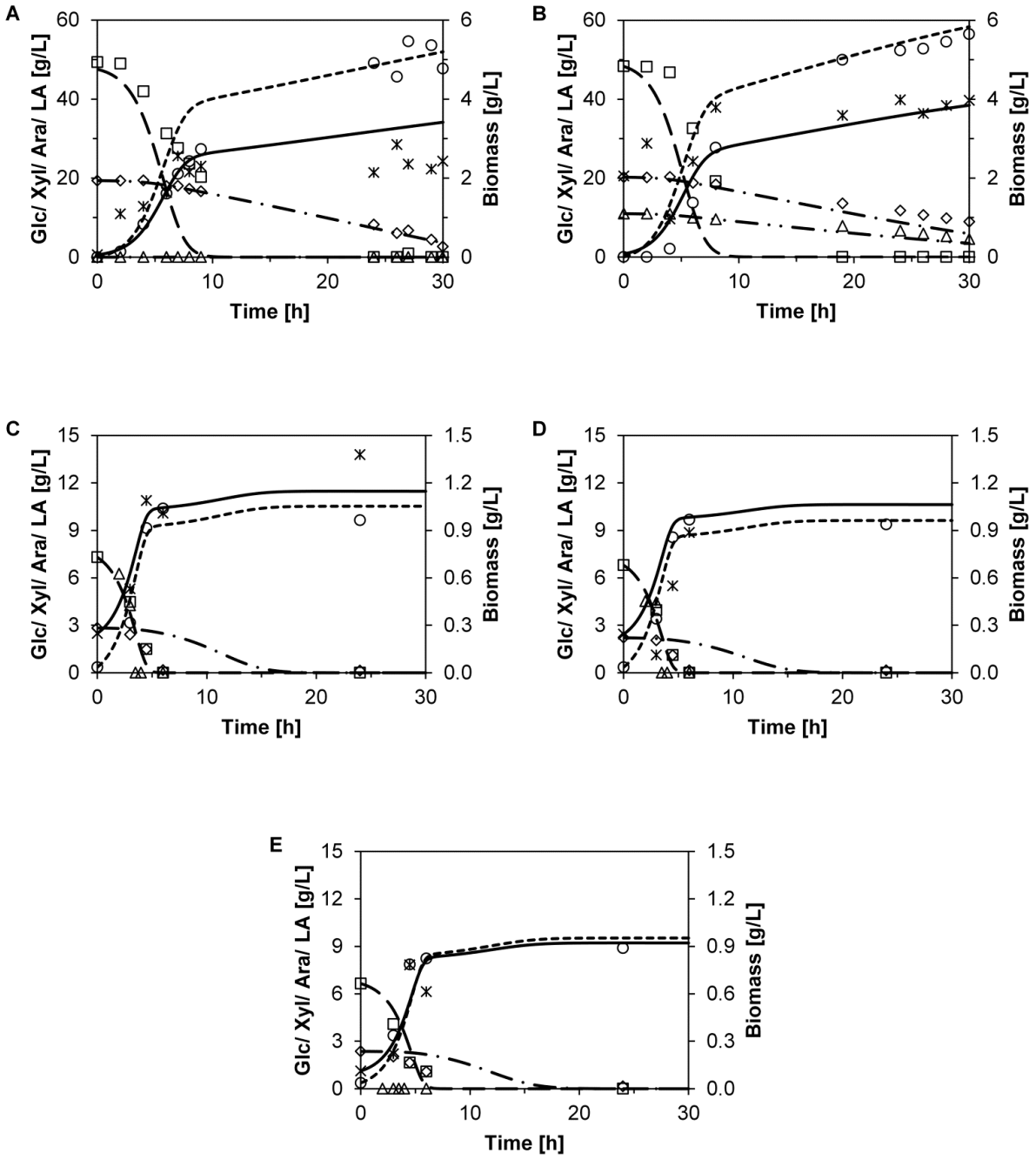
0.625 g/L AL; $\mu_{Xyl,max}$ increased from 0.1742 1/h to 0.3743 1/h. The lag times for the different sugar uptake values increase steadily with the increasing AL amount. The adjusted parameter values showed that the consumption of pentoses started after the consumption of glucose. Furthermore, the consumption of arabinose started after the uptake of xylose.

After characterising the growth of *B. coagulans* in an artificial composite fermentation medium, the MOs were then grown on different lignocellulose hydrolysates to verify the conclusions. Hydrolysis processes of the lignocellulose raw materials were performed prior to the fermentation procedure in the 2 L bioreactor, and the results of hydrolyses were described in detail in Glaser (2015a, 2015b).

To verify the fermentation Monod model, time course data was determined, and predictions were made about fermentation procedures in AM without arabinose and lignin in wheat straw hydrolysates (WSH), AWHs, and PW hydrolysates. The prediction of the AM process noticeably fits the data (Figure 4.3-6A, Figure 4.3-6B), despite the biomass being slightly overestimated; the ANOVA and the correlation coefficient confirmed this good prediction performance. The parameter selection for hydrolysis fermentation procedures was based on the lignin content of the hydrolysate.

The soluble lignin of the WSH was estimated at ~0.17 g/L, the lignin of the AWH was ~0.33 g/L and the lignin of the was PWH ~0.21 g/L. Because the fermentation was directly processed after the hydrolysis without separation from the biomass feedstocks, the bacterial biomass was calculated with regression between the biomass and the cell counts in an Abbe counting chamber of the previous fermentation procedures in AM (see also Figure 5 in Glaser and Venus, 2017).

For the WSH (Figure 4.3-6C, Figure 4.3-6D, Figure 4.3-6E) the prediction accurately tracks the assimilation of the sugars and the production of lactate and biomass. The prediction of the fermentation of the AWH (Figure 4.3-6F, Figure 4.3-6G) also yielded good results with $R^2 > 0.95$ and ANOVAs $p > 0.5$. Good agreements between the empirical and predicted results were obtained for glucose, xylose, and lactate production in all runs. Exponential growth, measured biomass concentrations, and calculated biomass concentrations are well correlated, but the model overestimated the biomass. The same results for the prediction were obtained by the fermentation results of the PWH (Figure 4.3-6H, Figure 4.3-6I, Figure 4.3-6J).


A
B
C
D
E
Glc/ Xyl/ Ara/ LA [g/L]
Biomass [g/L]
Time [h]

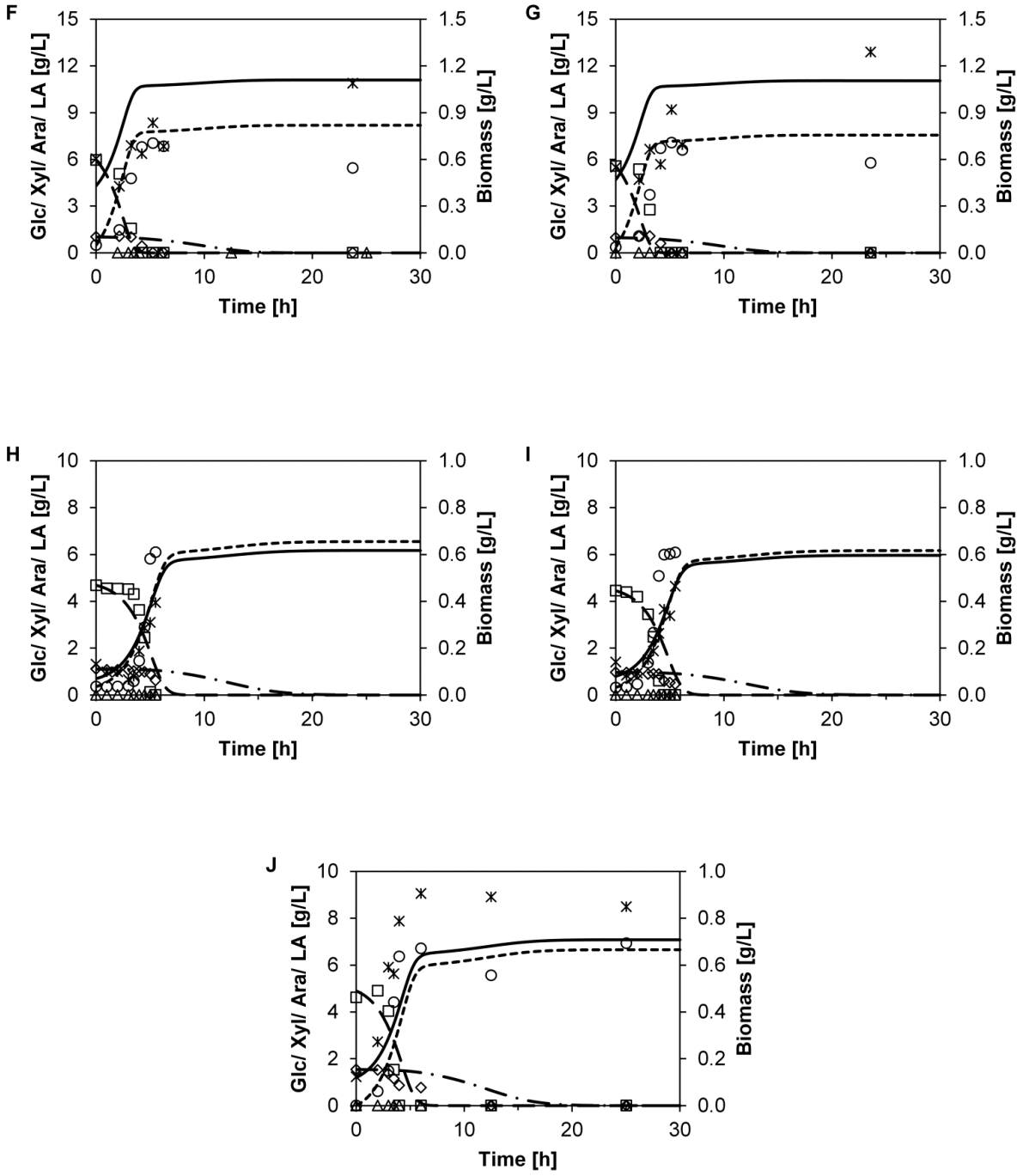


Figure 4.3-6: Fermentation time course data and model prediction. A: DSM ID 14-298 grown in AM without AL and without arabinose and in B with arabinose. C, D, E: DSM No. 2314 grown in WSH, F, G grown in AWH, and H, I, J grown in PWH. Empirical results are displayed as symbols (□ glucose, ◊ xylose, ∆ arabinose, ○ lactate, × biomass). Predictions are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ arabinose, − − − lactate, —— biomass; published in Glaser and Venus, 2017; Figure2).

The quality of the predictions is also characterised by statistical parameters: the standard mean deviations σ, coefficients of R^2 and ANOVA results, which are given in Table 4.3-4.

Table 4.3-4: Experimental data and statistical evaluations determining the prediction quality of the model equation under study (data published in Glaser and Venus, 2017; see Table 2).

	DSM ID 14 300		DSM No. 2314								
	AM		WSH			AWH		PWH			
	(-)Ara	(+)Ara									
Figure	4.3-6A	4.3-6B	4.3-6C	4.3-6D	4.3-6E	4.3-6F	4.3-6G	4.3-6H	4.3-6I	4.3-6J	
Concentrations of biomass and sugar[a]											
$C_{BM,min}$	0.06	0.04	0.25	0.24	0.11	0.43	0.47	0.13	0.14	0.12	(g/L)
$C_{BM,max}$	2.85	7.22	1.37	1.66	1.52	1.09	1.29	0.39	0.46	0.90	(g/L)
$C_{Glc,min}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	(g/L)
$C_{Glc,max}$	49.37	48.33	7.30	6.80	6.65	5.96	5.56	4.69	4.46	4.90	(g/L)
$C_{Xyl,min}$	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.51	0.47	0.00	(g/L)
$C_{Xyl,max}$	19.36	20.22	2.83	2.21	2.36	1.02	0.97	1.11	0.97	1.54	(g/L)
$C_{Ara,min}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	(g/L)
$C_{Ara,max}$	0.00	10.98	0.63	0.45	0.00	0.00	0.00	0.00	0.00	0.00	(g/L)
$C_{LA,max}$	57.52	68.81	10.38	9.68	8.88	5.44	5.77	6.10	6.09	6.94	(g/L)
Estimation quality											
σ	9.3701	9.4433	1.2630	1.0087	1.2223	1.0824	2.0104	0.8991	1.3413	2.3215	
R^2	0.9493	0.9675	0.9741	0.9828	0.9683	0.9460	0.9031	0.9403	0.8913	0.7939	
ANOVA											
F	0.0292	0.0267	0.0288	0.0132	0.0006	0.2963	0.1236	0.0313	0.0004	0.0223	
$F_{critical}$	3.9214	3.9151	4.0426	4.0426	4.0426	4.0195	4.0195	3.9777	3.9777	4.0195	
p	0.8644	0.8702	0.8657	0.9090	0.9795	0.5884	0.7264	0.8599	0.9845	0.8818	

[a]: Data derived by HPLC measurement described in section 4.3.3 after inoculum addition.

This thermophilic lactate-producing fermentation through the consumption of glucose, xylose, and arabinose was a good example that could help to verify the applicability of the proposed model for the growth of *B. coagulans* in a lignocellulose hydrolysate. The fermentation procedures in a mixed substrate medium were too complex to describe for possible growth prediction. The proposed model could reproduce differences in growth behaviour. It should help to describe the bacterial growth in complex media containing large amounts of different carbohydrates.

The quality of the fermentation results on the hydrolysates, and in particular the glucose repression, showed the same characteristics. The lignocellulose sugars are consumed almost simultaneously if the concentration of the sugars reaches the same level (ratio 1:1). Glucose is consumed first if its concentration is higher. When the concentrations of glucose reaches the same level of the pentoses, the sugars are consumed in parallel behaviour. Only a slight discrepancy between the consumption of xylose and arabinose was observed in the fermentation results of the AM and the hydrolysates. For most MOs, the simultaneous consumption of glucose and xylose has not been described; instead, the consumption of xylose starts when nearly no glucose is left in the fermentation medium (Okano et al., 2010; Taniguchi et al., 2004). The values of the consumption rate of xylose μ_{Xyl} and arabinose μ_{Ara} showed a tendency for a higher uptake rate. These results indicate (in agreement with Ye et al. [2014] and Wang et al. [2010b]) that xylose catabolism in the tested *B. coagulans* strain might be less repressed by glucose in comparison to other bacteria.

4.3.5.4. Decolourisation of the fermentation medium

Bacillus strains showed a lignin degradation activity–for example, *B. megaterium*, which has been shown to degrade ^{14}C-labeled lignin (Robinson and Crawford, 1978) and *p*-coumaric acid (Janelle et al., 2003; Wang et al., 2010b). *B. subtilis* has been shown to degrade lignin model compounds (Gurujeyalakshmi et al., 1987). Karmakar et al. (2000) have shown that a *B. coagulans* strain can degrade the lignin-related compounds FA and VAN acid. The proposed pathway of lignin metabolism in *B. coagulans* is depicted in Figure 4.3-7, which is based on the FA metabolism of *Pseudomonas* strains (Karmakar et al., 2000; Janelle and Torres, 2003; Wang et al., 2010b; Chen et al., 2012; Narbad and and Gasson, 1998; Walton et al., 2003; Campos et al., 2009).

The first step in the metabolism of FA in the proposed pathway (Chen et al., 2012) is its activation to form feruloyl-CoA. The feruloyl-CoA thioester undergoes a side chain cleavage to form VAN and acetyl-CoA. The aromatic product of the side chain cleavage of FA is VAN, which is oxidised to vanillic acid by a VAN dehydrogenase. The further degradation to vanillic acid possibly proceeds via vanillate-O-dehydrogenase (anaerobe) or a vanillate monooxygenase (aerobe) to form protocatechuate as a first common intermediate of a secondary metabolic pathway. The mentioned pathway led to the theoretical explanation of a fast degradation rate of lignin. Although sugars were available for utilisation and ATP formation for the primary pathways, fast degradation of lignin and growth at higher lignin concentrations was also possible. It also explained the stoppage of lignin degradation at process time points when utilisable sugars and the resulting ATP were absent.

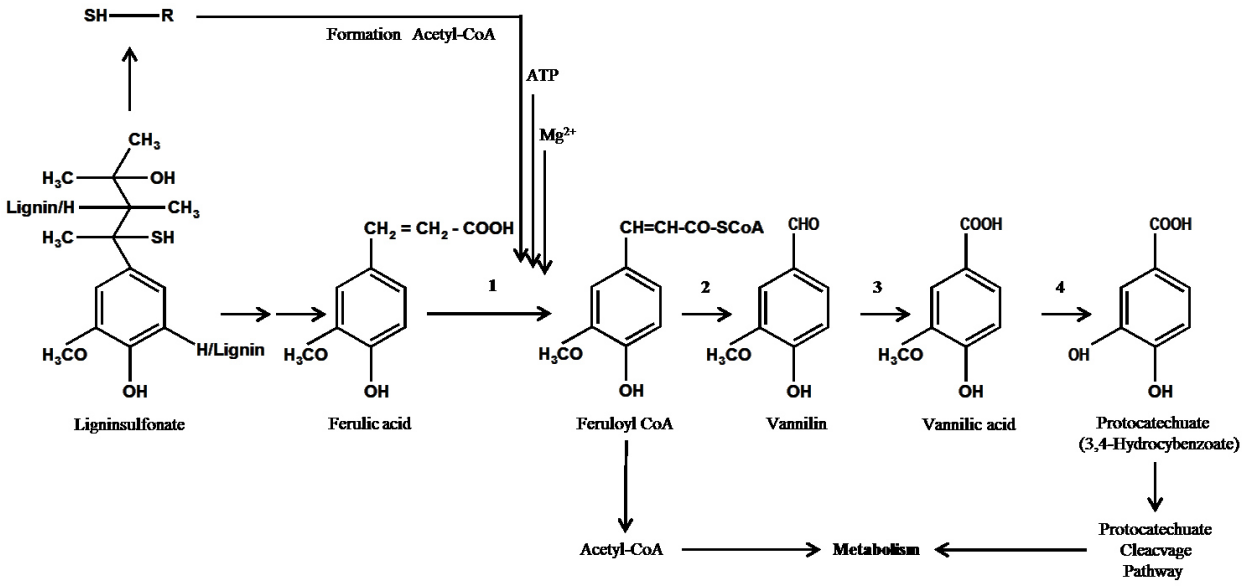


Figure 4.3-7: The proposed pathway of lignin sulfonate metabolism in *Bacillus coagulans*: (1) Ferulate-CoA ligase, (2) cleavage activity, (3) vanillin dehydrogenase, (4) vanillate-O-dehydrogenase (anaerobe), vanillate-monooxygenase (aerobe; see also Refs. Karmakar et al., 2000; Boll et al., 2002; Heider and Fuchs, 1997; Heider et al., 1998; Janelle et al., 2003;Wang et al., 2010; Chen et al., 2012).

UV measurements at 280 nm were carried out on the fermentation samples to determine changes in the AL concentration (Fig 4.3-8). During the fermentation procedures, a noticeable decrease in the absorbance rate could be detected for two of the three strains (for the data presentation see also Glaser and Venus, 2017). A decrease in the photometric signal might be due to the uptake of protein from the yeast extract by the MOs or due to alkaline dilution for the constant pH value of the fermentation broth. Comparing the reference fermentation procedures without AL, the absorbance signal shows no noticeable decrease. It is possible that for fermentation procedures at different concentrations of AL, the change in the absorbance signal was a result of a change in the AL matrix. However, a theoretical dilution behaviour of the AL signal at 280 nm is also given (see also Glaser and Venus, 2017). Here, the decreased absorbance of AL in the fermentation processes occurred earlier and stronger absorbance as compared to the dilution by the alkaline feed for the constant pH value.

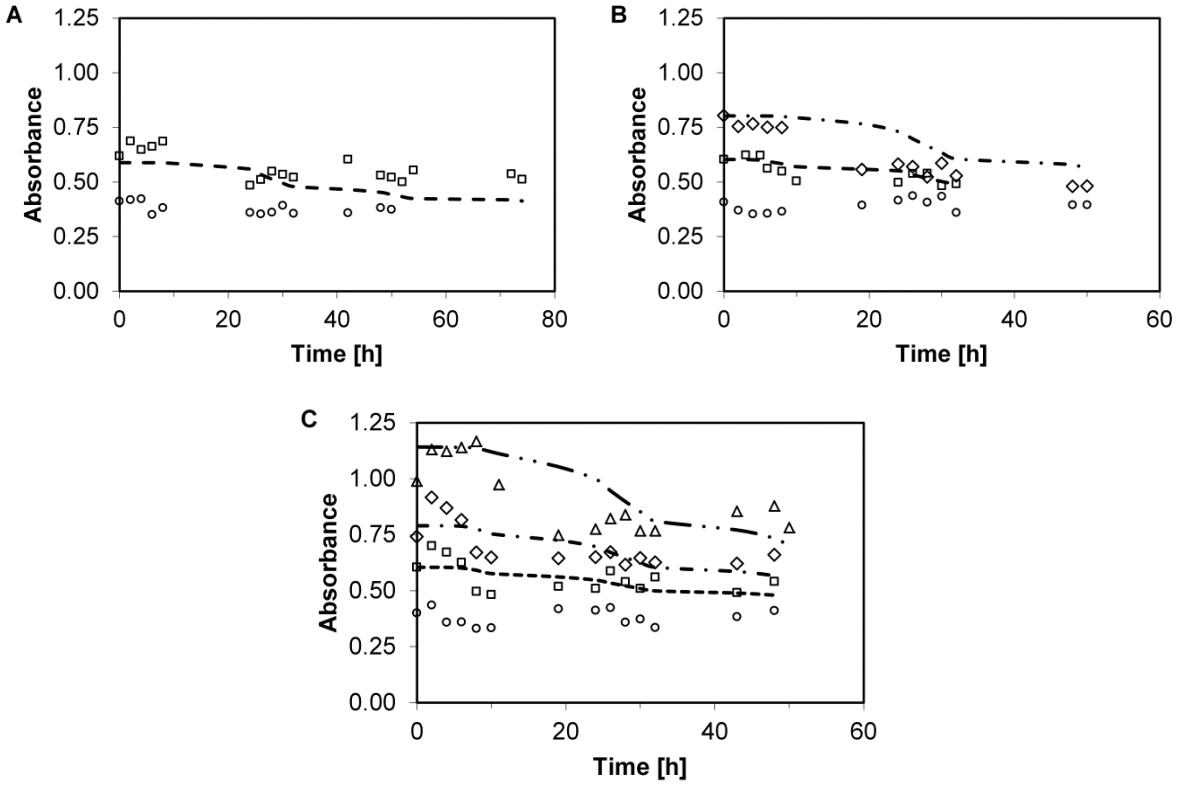


Figure 4.3-8: Decolourisation performance in fermentation measured at 280 nm. A: Strain DSM No. 2314, B: DSM ID 14-298, C: DSM ID 14-301. UV measurement results are displayed as symbols (○ blank fermentation without AL, □ fermentation with 0.625 g/L AL, ◊ fermentation with 1.25 g/L AL, Δ fermentation with 2.5 g/L AL). Theoretically expected decolourisation data due to alkaline dilution are shown as lines (— — — fermentation with 0.625 g/L AL, — ▪ — ▪ fermentation with 1.25 g/L AL, — ▪ ▪ fermentation with 2.5 g/L AL).

Strain DSM No. 2314 showed only a slight decrease in the absorbance signal at 280 nm, but this change was in the expected dilution range. The absorbance for strain DSM ID 14-298 tended to decrease more than the theoretical absorbance. A decrease in the absorbance is noticeable during the glucose fermentation period. Only a small decrease was detected during the pentose fermentation. Strain DSM ID 14-301 showed a bigger decrease in absorbance. A substantial drop in the absorbance signal was also observed during the glucose fermentation (see also Glaser and Venus, 2017).
After a phase of decrease in the absorbance signal, an increase could also be detected for a brief period. This strongly matched the time point when the MOs started to consume the pentose sugars at the end of the fermentation. At both process points, the absorbance can be influenced by proteins, which were segregated during the death of the MOs.

To determine whether the decolourisation of the fermentation broth was an artefact of the dilution by sodium hydroxide, decolourisation experiments in 5 mL tubes were conducted. Therefore, the MOs were grown in a sodium potassium buffer at pH 7 with ATP and yeast extract supplemented with AL, FA, or VAN. The spectrometric absorbance was measured between 250 nm and 400 nm (see Materials and Methods). The results clearly showed a decolourisation of the test media. While the AL medium showed a decrease in the complete range of the analysed wavelengths between 250 nm and 400 nm (Figure 4.3-9A; Figure 4.3-9B; Figure 4.3-9C), the results for FA (Figure 4.3-9D; Figure 4.3-9E; Figure 4.3-9F) and VA (Figure 4.3-9G; Figure 4.3-9H; Figure 4.3-9I) showed a decrease in absorbance.

AL shows a peaked between 250 nm and 260 nm. That peak was reduced in the first 2.5 hrs to 5 hrs of the process. The signal peak at 280 nm was also reduced, followed by a decrease in the overall signal. A measurement at 10 g/L ATP and 1 g/L yeast extract over 48 hrs also showed a decrease in the absorbance signal. However, increasing the ATP concentration did not reveal a measurable increase in decolourisation (data not shown; see also Karmakar et al., 2000).

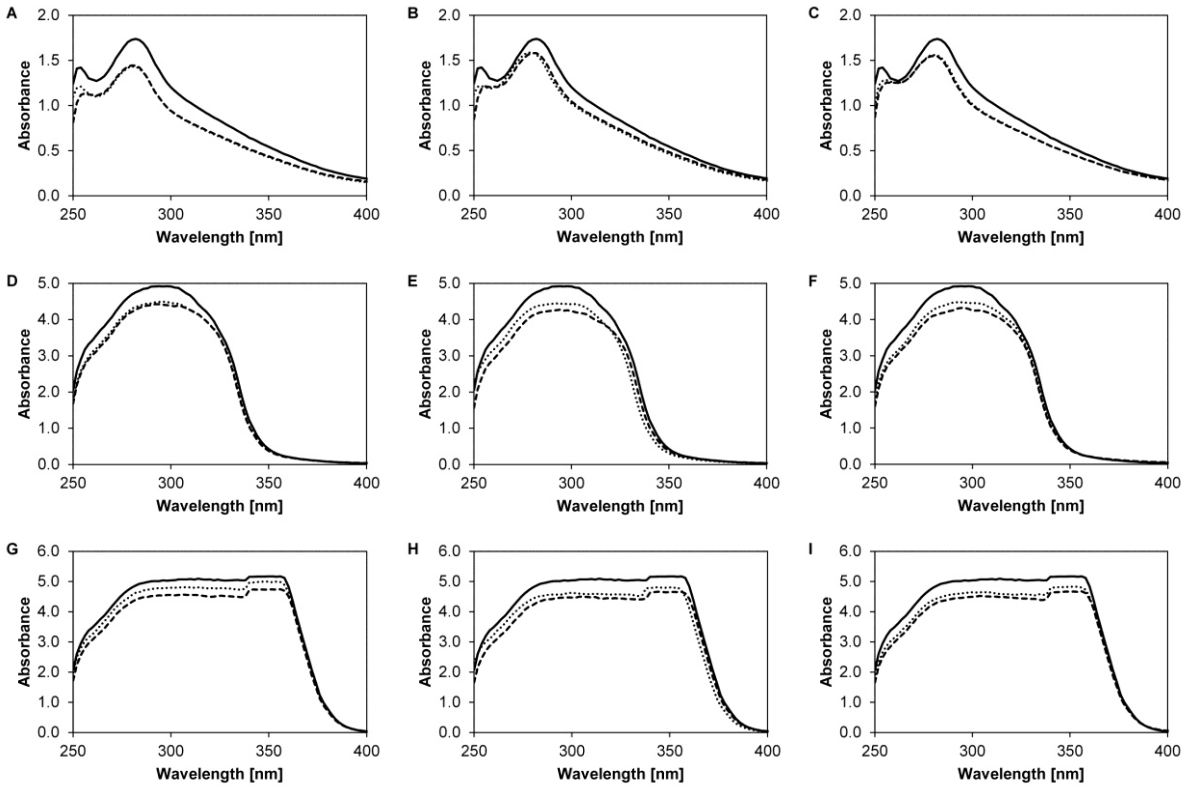


Figure 4.3-9: UV absorbance plots of the three selected lactic acid strains (A, D, G: DSM No. 2314; B, E, H: DSM ID 14-298; C, F, G: DSM ID 14-301) for the decolourisation of A, B, C: AL solution, D, E, F: ferulic acid solution, and G, H, I: vanillin solution. (─ blank solution, ▪ ▪ ▪ 2.5 h sample, - - - 5 h sample). (published in Glaser and Venus, 2017; see Figure 3)

Although strain DSM No. 2314 showed no additional decrease in the absorbance signal for FA from 2.5 hrs to 5 hrs, the strains DSM ID 14-298 and DSM ID 14-301 showed a further decrease. This was also detected in the measurement of the VAN decolourisation. Uptake of AL by the analysed strains of *B. coagulans*, and as a result the decolourisation of the fermentation medium, could also be verified by the decolourisation of FA and VAN.

The comparison of the LA yields showed a slight increase at an initial AL concentration of 0.625 g/L for strain DSM No. 2314 and strain DSM ID 14-301. A comparable pattern of an increased growth response at low inhibitor concentrations was described for wine LA bacteria *Oenococcus oeni* VF and *Lactobacillus hilgardii* 5 (Campos et al., 2009) and under the influence of *p*-coumaric acid (Rozes et al., 2003). It has been mentioned that the increased acetate production of *O. oeni* VF and *L. hilgardii* 5 is a result of increased ATP production to overcome the chemical stress. It has also been hypothesised that the increased production of lactic and acetic acid was a result of other carbohydrates present in the medium.

The slight increase in the LA yield during fermentation procedures with strain DSM No. 2314 and strain DSM ID 14-301 could be theoretically explained by the increased formation of acetyl-CoA by the sulfonated side chain of AL. The increased growth could also be seen in the model parameters for the uptake rates of glucose, xylose, and arabinose. It has been explained

elsewhere that the anaerobic degradation of aromatic compounds could depend on the reduction of ATP to ADP and Pi (Heider and Fuchs, 1997), and it seems that *B. coagulans* may be able to degrade lignin in a satisfactory manner when the central degradation pathways of the sugars provide ATP. In order to investigate the degradability of AL, the decolourisation test was conducted, and the results showed that uptake of AL was possible in the fermentation process. The degradation of AL can result subsequently in metabolisation by the intermediates: AL via FA and VAL seems possible for the tested strains.

Considering the inhibitors of growth that can be found in various waste products – for example, lignocellulosic hydrolysates for acid- or alkali-dependent hydrolysis or organosolvent-processed materials – *Bacillus* strains, especially *B. coagulans* strains, showed significant and interesting behaviours towards those inhibitors. Initially, they showed an increased growth response while exposed to lower concentrations of phenolic inhibitors such as AL, followed by their detoxification in possible inhibitory media, and then their utilisation of different kinds of carbohydrates such as glucose, xylose, and arabinose for the homo-fermentative production of LA (Karmakar et a., 2000; Ye et al., 2014; Kaur et al., 2013a).

The behaviour described may reduce the need for additional delignification processes, which can result in a more cost-efficient process. The results emphasise the need for further characterisation of metabolic possibilities, such as the production of ligninolytic enzymes by *B. coagulans* strains.

4.3.6. Conclusion

In this section, a mathematical model was derived to describe the process characteristics as based on fermentation procedures involving each of the three *B. coagulans* strains in a complex mixed substrate medium supplemented with AL. The fermentation medium contained three predominant carbohydrates–glucose, xylose and arabinose–that were fermented to lactate. The model was able to reproduce the effects of the prolonged initial lag time cause by the inhibitory AL. The intermediate lag periods that occur due to the change of metabolism from glucose to pentoses were also shown successfully. Using the model-based results, the fermentation processes were characterised, and two strains were identified as being capable of consuming glucose, xylose, and arabinose. The tested strains showed a capacity for degradation of AL in decolourisation tests of AL, VAN, and FA. Due to the resistance to lignin and the ability to utilise pentose sugars, strain DSM ID 14-298 has the potential to serve as a biocatalyst for the conversion of agricultural by-products into valuable chemicals.

4.4. Co-fermentation of the main sugar types from a beechwood organosolv hydrolysate by several strains of *Bacillus coagulans* results in effective lactic acid production.[4]

4.4.1. Abstract

Bacillus coagulans is an interesting facultative anaerobic MO for biotechnological production of LA. To determine the efficiency of biotechnological production of LA from lignocellulosic feedstock hydrolysates, five *Bacillus coagulans* strains were grown in lignocellulose organosolv hydrolysate from ethanol-water-pulped beechwood. Parameter estimation, based on a Monod-type model, was used to derive the basic key parameters for a performance evaluation of the batch process. Three of the *Bacillus coagulans* strains, including DSM No. 2314, were able to produce lactate, primarily via uptake of glucose and xylose. Two other strains were identified as being able to utilise cellobiose to a high degree, but they also had a lower affinity to xylose. The lactate yield concentration varied from 79.4±2.1 g/L to 93.7±1.4 g/L (85.4±4.7% of consumed carbohydrates) from the diluted organosolv hydrolysate.

4.4.2. Introduction

Renewable biomass is suitable for the biotechnological production of biofuels and basic chemicals. Such biomass can include starchy biomass, sugar-based feedstocks, and lignocellulosic biomass (Laopaiboon et al., 2010). The lignocellulosic feedstocks include agricultural residues, forest residues and wastes, fast-growing woods, such as hybrid poplar and willow, and herbaceous crops (Peters, 2007; Sreenath et al., 2001; Taherzadeh and Karimi, 2007). Lignocellulosic biomass requires a conversion process to make it suitable for further biotechnological processing (Hirth et al., 2012a, 2012b). Several pre-treatment methods exist to hydrolyse the bound carbohydrates–such as glucose, xylose, arabinose, galactose, and mannose–for fermentation. The pre-treatment methods that can be used to break down the structure of lignocellulosic biomass are categorised into five groups: physical treatment (e.g., mechanical disruption), chemical treatment (e.g., alkali, dilute acid, organosolv), thermal treatment (e.g., steam explosion), physicochemical treatment (e.g., ammonia fibre explosion, AFEX), and biological treatment (e.g., degradation by enzymes; Monlau et al., 2013; Ntaikou et al., 2010; Saratale et al., 2008; Taherzadeh and Karimi, 2008). Most of the existing pre-treatment processes for lignocellulosic biomass lead to the formation of undesirable by-products such as furfural, 5-hydroxymethylfurfural, and soluble lignin, which reduce the fermentation ability of hydrolysates, creating a major

[4] Based on: Robert Glaser and Joachim Venus, 2018. Co-fermentation of the main sugar types from a beechwood organosolv hydrolysate by several strains of Bacillus coagulans results in effective lactic acid production. Biotechnol. Rep. 18; e00245. https://doi.org/10.1016/j.btre.2018.e00245

hindrance to product formation (Klinke et al., 2004; Mussatto and Roberto, 2004; Mills et al., 2009; Ren et al., 2009; Chang and Holtzapple 2000; Saritha et al., 2012).

Among the chemical degradation methods, the ethanol organosolv process is a promising technology for the biorefining of lignocelluloses (Alvira et al., 2010). The organosolv process allows for the fractionation of lignocellulose fractions of cellulose, hemicellulose-derived mono-sugars, and lignin (Becker and Leschinsky, 2014; Kumar et al., 2009; Laure et al., 2014). Ethanol-water-based organosolv pre-treatment uses ethanol and water at elevated temperatures for a partial extraction of lignin and hemicellulose. The remaining cellulose fraction can be used to produce mono-sugars by an enzymatic hydrolysis step, as the majority of lignin, the major hindrance to enzymatic hydrolysis, is removed (Hu et al., 2011). Although the pre-treatment allows for a relatively clear fractionation of the major wood components, the conditions of the organosolv pre-treatment reaction might cause the formation of complex compounds, such as furans and solvated phenolic components, residues of lignin, and organic acids. These residues are present in the different fractions of cellulose- and hemicellulose-derived sugars.
Methods to remove inhibitory compounds include the addition of activated charcoal, extraction with organic solvents, ion exchange or ion exclusion, molecular sieves, and treatment with laccases (de Vrije et al., 2002; Mussatto and Roberto, 2004; Ren et al., 2009; Palmqvist and Hahn-Hägerdal, 2000a, 2000b; Chandel et al., 2011; Liu and Blaschek, 2010; Ludwig et al., 2013). Nonetheless, MOs that can endure inhibitory compounds in the fermentation medium are of industrial relevance. Here, the reduction of process costs can be achieved by lowering the necessity to achieve the lowest possible inhibitor concentration (Janelle et al., 2003; Lopez et al., 2004). Such a reduction may be achievable using *Bacillus coagulans*.

B. coagulans, a spore-forming thermophilic facultative anaerobe and LA producing bacterium, has the ability to grow at low pH (De Clerk et al., 2004) and is able to ferment hexose and pentose sugars to L-(+)-lactic acid with high titres (Ou et al., 2011; Glaser and Venus, 2014; Karmakar et al., 2000*).*

LA, long used in the food industry, has also become a common building block for chemical synthesis of the biodegradable polymer polylactic acid (PLA; Garlotta, 2001). With its versatile usability and the increasing demand for renewable bio-based plastics, there have been various attempts to produce LA efficiently in biorefineries using lignocellulosic feedstocks (Bischoff et al., 2010; Garde et al., 2002).

The scope of this study is to evaluate the performance of five *B. coagulans* strains on organosolv hydrolysate. The organosolv hydrolysate contains–besides potentially inhibitory compounds–glucose, xylose, and cellobiose as the predominant sugars that are fermented to lactate under thermophilic anaerobic process conditions. To derive the basic key performance parameters,

model-based parameter estimation is performed using a Monod-type model. The model gives the opportunity to evaluate the bacterial growth according to key performance parameters such as maximum growth rates on the different carbohydrates and alkaline feed, as well as the impact of lactate on product inhibition of growth.

4.4.3. Materials and methods

4.4.3.1. Microorganisms

Five strains of *B. coagulans* were used for fermentation in organosolv hydrolysate, including *Bacillus coagulans* DSM No. 2314 and four other strains that were checked and identified as *B. coagulans* by the DSMZ. These strains are named DSM ID 10-395, DSM ID 14-298, DSM ID 14-300, and DSM ID 14-301, and they are not purchasable through the DSMZ. The MOs were stored in cryogenic vials (VWR International GmbH, Germany) at -70°C and reactivated on MRS broth (Merck KGaA, Germany) at 52°C for 24 hrs. After full reactivation, the MOs were cultivated on slant culture tubes with MRS agar (Merck KGaA, Germany). Until use, the MOs were stored at 4°C. The inoculum was cultivated on 60 mL MRS medium (Merck KGaA, Germany) in 250 mL shaking flasks (52°C, 100 rpm, 15 hrs).

4.4.3.2. Growth medium

As a growth medium, hydrolysate from the enzymatic hydrolysis of the cellulose fraction of organosolv pre-treated beechwood was used (charge number Kk002H1E1). The Fraunhofer Centre for Chemical-Biotechnological Processes CBP (Leuna, Germany) provided the organosolv hydrolysate. Further information regarding the steadily improving organosolv process can be found in Becker and Leschinsky (2014) and Laure et al. (2014). At the Fraunhofer Centre for Chemical Biotechnological Processes CBP, the lignocellulosic feedstock was pre-treated in a 460 L digester by an organosolv process using ethanol-water pulping at elevated temperatures at pilot scale. The solution used as a substrate for this work was produced after pre-treatment of beechwood chips at 170°C using a 50% (w/w) ethanol/water solution containing 0.5% sulphuric acid (based on dry wood). Enzymatic hydrolysis of the obtained pulp fraction was performed in a stirred tank reactor using 6% of Cellic® CTec2 and 0.25% Cellic® HTec2 (w/w) o.d. pulp provided by Novozymes at 50°C with a 10% solids concentration for 48 hrs. The obtained sugar solution was concentrated using a falling film evaporator. The undissolved organosolv hydrolysate contained 307.5 g/L glucose, 72.1 g/L xylose, 85.3 g/L disaccharides, 9.6 mg/L hydroxymethyl-furfural, 3.5 mg/L furfural, and 7.7 g/L acetic acid (Section 2.5). The organosolv hydrolysate was used at a 1 to 4 dilution. For nutrition, the growth medium was supplemented with 15 g/L yeast extract.

4.4.3.3. Growth conditions

Cultivations were performed in a 2 L double-walled glass bioreactor Biostat B (Sartorius, Germany) with 1 L working volume. The growth medium solution was autoclaved at 121°C for 20 min separately from the yeast extract to minimise the Maillard reaction. The pH was adjusted to 6.0 after autoclaving and controlled with a one-sided pH-control during the fermentation process using 20% NaOH. The temperature was kept constant at 52°C during fermentation. The inoculum was 2% of the targeted working volume. Samples were taken at different times with a manual bypass system, and they were inactivated for metabolite measurement in a hot water bath at 92°C for 30 min [39]. After inactivation, the samples were stored at -8°C for further use. After thawing the samples, they were centrifuged at 5,000 rpm (relative centrifugal force [RCF] of 5,338 g) for 15 min at 4°C. The supernatant was filtered with a 20 µm cellulose acetate membrane micro-filter (Th. Geyer GmbH & Co. KG, Germany). The filtrate was used for the detection of fermentable sugars using an HPLC (Section 4.4.3.4.).

4.4.3.4. Biomass determination

The sample pellet was washed in 2.5 mL demineralized water, suspended, and centrifuged again at 5,000 rpm (RCF = 5,338 g) and 4°C for 15 min. While the supernatant was rejected, the pellet was placed in porcelain pots. The porcelain pots were previously dried for two hours at 105°C. After cooling down in an exsiccator at a vacuum of 40 kPa for 1 hr, the tare weight of the porcelain pots was determined on a microbalance. The pellet was suspended in demineralized water, transferred to the porcelain pots, and dried in a vacuum for at least 24 hrs at 105°C. After cooling down to room temperature, the pots were weighed. The procedure was repeated until the weight stayed constant. As a reference, see also DIN EN 12880. The averaged value of the two-fold weighing was used.

4.4.3.5. Detection of sugars, lactate, and phenolic components

Sugar, lactate, and, acetate concentrations were determined by HPLC using a Dionex ICS 3000 (Thermo Fisher Scientific Inc., USA) equipped with an Eurokat H column (300 mm × 8 mm, 10 µm, eluent: 0.01 N H2SO4, including pre-column, Knauer GmbH, Germany) with an operation pressure of 65 bar. For detection, a refractive index detector RI-101 (SHODEX, Showa Denko Europe GmbH) was used. The column was operated at a constant temperature of 35°C with a flow rate of 0.8 mL/min. The injection volume was 10 µL. For the previously mentioned fermentation conditions, LA exists as a salt and therefore is reported as lactate (Glaser and Venus, 2017)

Detection of aldehyde components was determined by HPLC using a Dionex ICS 3000 (Thermo Fisher Scientific Inc., USA) equipped with a Eurospher II C18 column (150×4 mm, pore size 100 Å, end capped, including pre-column, Knauer GmbH, Germany) and a Dionex Series VWD UV/VIS-detector at 280 nm (Thermo Fisher Scientific Inc., USA). As eluent, ultrapure water and 50% (v/v) acetonitrile solution were used in a multistep gradient process. The multistep gradient elution was performed as follows: 7 min isocratic elution with 10% B, 6 min gradient elution to 40% B, 5 min gradient elution to 100% B, 8 min isocratic elution with 100% B, 4 min isocratic elution with 10% B. The flow rate was set at 1 mL/min. The auto sampler temperature was 15°C; the column and detector temperatures were 23°C. For evaluation of the chromatograms, the Chromeleon software version 6.80 (Thermo Fisher Scientific Inc., USA) was used.

4.4.4. Theory and calculation

To calculate the basic key performance indices, such as growth rates, substrate consumption and product building rates, a kinetic model of the additive Monod-type model for growth with mixed substrates was used, as formerly described by Dunn et al. (2003), Zeiger and Grammel, (2010) and Glaser and Venus (2017). The model was adjusted to fit the needs of this work. In particular, the maintenance metabolism was not considered, and neither were the nitrogen and phosphorous metabolism and oxygen conditions. Estimation of the model parameters was performed by minimisation of the root mean squares (RMS) between the original experimental data (biomass-, glucose-, xylose-, cellobiose-, lactate concentrations, and alkaline amount used for pH-control) and model data using a generic method of MATLAB® (The Mathworks, Natick, MA) optimization tools.

The data was further analysed through ANOVA using the null hypothesis, with the statement that all mean experimental values of the process and results of the model simulation are equal.

The dynamics in biomass concentration, C_{BM}, were calculated as a function of the maximal growth rate, μ_{BM}, and dependent on the dynamic change of the reactor volume, V_R. Dilution occurs through alkaline feed, F_{Alk}, for the control of the pH value.

$$\frac{dC_{BM}(t)}{dt} = \mu_{BM} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{BM}(t) \qquad /4.4\text{-}1/$$

The growth rate μ_{BM} was described as a function of the diverse maximum specific uptake rates of the available substrates. In the process, the organisms are expected to grow at different rates on different substrates and corresponding the assumed specific maximum growth rates of the MOs on the available substrates–on glucose μ_{Glc}, xylose μ_{Xyl}, and cellobiose μ_{CB}. A further theoretical parameter $\mu_{max,sim,mix}$ was defined (see also Zeiger and Grammel, 2010).

$$\mu_{BM} = k_0 \cdot \left(k_1\mu_{Glc} + k_2\mu_{Xyl} + k_3\mu_{CB}\right) \cdot \left(\frac{1}{k_1+k_2+k_3}\right) \qquad /4.4\text{-}2/$$

The coefficients $\mu_{max,sim,mix}$, k_1, k_2, and k_3 were described as theoretical parameters that are obtained via MATLAB simulation optimization as well as via the diverse specific maximum growth rates on the available substrates $\mu_{Glc,max}$, $\mu_{Xyl,max}$, and, $\mu_{CB,max}$ (see equation 4.4-7). The flexibility of this kinetic form requires more kinetic parameters than a simpler double Monod kinetics. To reduce the number of free variables, the values of k_1, k_2, and k_3 were set to equal the values of the specific growth rates on the substrates.

$$\mu_{BM} = \mu_{max,sim,mix} \cdot \left(\mu_{Glc}{}^2 + \mu_{Xyl}{}^2 + \mu_{CB}{}^2\right) \cdot \left(\frac{1}{\mu_{Glc}+\mu_{Xyl}+\mu_{CB}}\right) \qquad /4.4\text{-}3/$$

Due to possible product inhibition, the Monod-type model was extended with an inhibition term $I(LA)$ to consider the effect of a critical lactate concentration, $C_{LA,max}$, (Ben-Youssef et al., 2005; Burgos-Rubio et al., 2000).

$$I(LA) = \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right) \qquad /4.4\text{-}4/$$

The product inhibition term was used as generalised nonlinear inhibition term $I(LA)^n$ using a power to the n^{th} grade, referred to as 'toxic power' by Levenspiel (1980), describing how the term of inhibition $(1 - C(t)/ C_{LA,max})$ strongly affects the specific growth and lactate production rates. A further term was used to take the differences of the duration of the initial lag phase into account until the MOs were ready for the uptake of the different sugar fractions. The term was described in Baranyi and Roberts (1994) in the following form:

$$\alpha_i(t) = \left(\frac{q_{0,i}}{q_{0,i}+exp(-\mu_{i,max}\cdot t)}\right) \qquad \text{with i= Glc, Xyl, CB} \qquad /4.4\text{-}5/$$

The dynamic equation to describe the glucose consumption, C_{Glc}, was used in the following way:

$$\frac{dC_i(t)}{dt} = -\frac{\mu_i}{Y^{BM/i}} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_i(t) \qquad \text{with i= Glc, Xyl, CB} \qquad /4.4\text{-}6/$$

$$\mu_i = \mu_{i,max} \cdot \alpha_i(t) \cdot I(LA)^n \cdot \left(\frac{C_i(t)}{K_i+C_i(t)}\right) \qquad \text{with i= Glc, Xyl, CB} \qquad /4.4\text{-}7/$$

The proposed product formation rate equation, dC_{LA}/dt, was based on the simplified assumption that the rate of product formation was related to the rate of biomass formation through a production coefficient, $Y^{LA/BM}$.

$$\frac{dC_{LA}(t)}{dt} = Y^{LA/BM} \cdot \mu_{BM} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{LA}(t) \qquad /4.4\text{-}8/$$

The value $Y^{LA/BM}$ can be derived through the relation $Y^{LA/BM} = (Y^{LA/Sub} / Y^{BM/Sub})$. The reactor volume V_R was set as dependent on the alkaline flow rate. The equation was derived from the dynamics of the pH-auxostat.

$$\frac{dV_R(t)}{dt} = F_{Alk}(t) = \frac{\mu_{BM} \cdot V_R(t) \cdot C_{BM}(t)}{Y^{BM/Alk} \cdot C_{OH^-}} \quad /4.4\text{-}9/$$

For a further reduction of the models' degrees of freedom, some parameters were dependent on each other: $K_{Glc} = 1/\mu_{Glc,max}$, $K_{Xyl} = 1/\mu_{Xyl,max}$, and $K_{CB} = 1/\mu_{CB,max}$ as well as $K_D = 1/k_D$ (Glaser and Venus, 2017). By using this approach, the units do not fit. Only the values are used for parameter reduction, reducing the variance in the parameter fit but increasing the repeatability and reproducibility of the parameter estimations. The values of the parameter *K* will be much higher than one would expect had they been derived by laboratory experiments. More information on the assumptions made for the approach are given in Chapter 4.3.4.

The final model equation system was defined:

$$\frac{dC_{BM}}{dt} = \mu_{BM} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{BM}(t) \quad /4.4\text{-}10/$$

$$\frac{dC_{Glc}(t)}{dt} = -\frac{\mu_{Glc,max} \cdot \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right)^n \cdot \left(\frac{q_{0,Glc}}{q_{0,Glc} - exp\left(-\mu_{Glc,max} \cdot t\right)}\right) \cdot \left(\frac{C_{Glc}(t)}{K_{Glc} + C_{Glc}(t)}\right)}{Y^{BM/Glc}} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{Glc}(t) \quad /4.4\text{-}11/$$

$$\frac{dC_{Xyl}(t)}{dt} = -\frac{\mu_{Xyl,max} \cdot \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right)^n \cdot \left(\frac{q_{0,Xyl}}{q_{0,Xyl} - exp\left(-\mu_{Xyl,max} \cdot t\right)}\right) \cdot \left(\frac{C_{Xyl}(t)}{K_{Xyl} + C_{Xyl}(t)}\right)}{Y^{BM/Xyl}} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{Xyl}(t) \quad /4.4\text{-}12/$$

$$\frac{dC_{CB}(t)}{dt} = -\frac{\mu_{CB,max} \cdot \left(1 - \frac{C_{LA}(t)}{C_{LA,max}}\right)^n \cdot \left(\frac{q_{0,CB}}{q_{0,CB} - exp\left(-\mu_{CB,max} \cdot t\right)}\right) \cdot \left(\frac{C_{CB}(t)}{K_{CB} + C_{CB}(t)}\right)}{Y^{BM/CB}} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{CB}(t) \quad /4.4\text{-}13/$$

$$\frac{dC_{LA}(t)}{dt} = Y^{LA/BM} \cdot \mu_{BM} \cdot C_{BM}(t) - \frac{F_{Alk}(t)}{V_R(t)} C_{LA}(t) \quad /4.4\text{-}14/$$

$$\frac{dV_R(t)}{dt} = F_{Alk}(t) = \frac{\mu_{BM} \cdot V_R(t) \cdot C_{BM}(t)}{Y^{BM/Alk} \cdot C_{OH^-}} \quad /4.4\text{-}15/$$

4.4.5. Results and discussion

Batch fermentation processes were performed on lignocellulose organosolv hydrolysate. The hydrolysate was obtained from the second organosolv fractionation procedure conducted in the pilot plant by the Fraunhofer Centre for Chemical Biotechnological Processes CBP in Leuna, Germany. The organosolv hydrolysate has an extremely high content of cellobiose that is not typical for this process and can be explained by a relatively incomplete enzymatic hydrolysis after the ethanol/water solvation. However, this cellobiose content is an interesting effect that allows for the investigation of the cellobiose fermentation ability (hydrolytic activity) of the *B. coagulans* strains used in this study.

To achieve comparable growth results between the strains, a supplement of 15 g/L of yeast extract (compare to Zhou et al. [2013], who had used 20 g/L of yeast extract at 240 g/L glucose) was used to exclude a nutritional lag, although the cost of such a medium would be too high for industrial purposes. Dietz et al. (2016) have detailed a comparison study for the use of a cheap nutrition source using leguminous green juice.

The growth performance was evaluated by key performance parameters, which were determined based on the model described in Section 4.4.4. The experimental data and the model are in agreement, as the values are close. Batch growth of the different strains of *B. coagulans* and results of the model simulation are shown in Figure 4.4-1 to Figure 4.4-5. The experimental data is indicated as marks. Simulations are shown as dashed lines. The experimental derived and estimated model parameters are given in Table 4.4-1.

Table 4.4 1: Coefficients and parameters of growth derived from parameter fitting and experimental data that were used for the model simulation of bacterial growth shown in Figure 4.4 1 to Figure 4.4 5.

	DSM No. 2314		DSM ID 14-301		DSM ID 14-300		DSM ID 14-298		DSM ID 10-395		
Concentrations of biomass and sugar [a]											
$C_{Sub,max}$	131.30	133.07	139.27	137.70	138.56	144.54	136.54	139.18	142.65	139.72	(g/L)
$C_{Sub,min}$	41.52	43.81	30.76	35.79	39.09	39.63	28.17	29.48	31.84	33.17	(g/L)
$C_{\Delta Sub}$	89.77	89.26	108.51	101.91	99.47	104.91	108.38	109.7	110.81	106.55	(g/L)
$C_{BM,min}$	0.03	0.03	0.09	0.05	0.06	0.02	0.04	0.03	0.03	0.03	(g/L)
$C_{BM,max}$	7.13	7.04	6.77	5.78	7.46	5.34	5.58	4.95	7.80	7.02	(g/L)
$C_{\Delta BM}$	7.10	7.01	6.68	5.73	7.40	5.32	5.54	4.92	7.77	6.99	(g/L)
$C_{Glc,max}$	82.25	83.42	87.41	86.43	86.83	90.55	88.33	86.00	87.49	85.81	(g/L)
$C_{Glc,min}$	7.80	8.88	12.58	12.73	7.87	8.48	0.00	2.67	12.74	12.86	(g/L)
$C_{\Delta Glc}$	74.45	74.54	74.83	73.70	78.96	82.07	88.33	83.33	74.75	72.95	(g/L)
$C_{Xyl,max}$	21.81	22.05	23.04	23.15	23.75	25.53	22.45	22.44	22.70	22.18	(g/L)
$C_{Xyl,min}$	8.66	9.11	12.90	12.82	8.07	8.69	6.55	5.45	12.98	13.10	(g/L)
$C_{\Delta Xyl}$	13.15	12.94	10.14	10.33	15.89	16.84	15.9	16.99	9.72	9.08	(g/L)
$C_{CB,max}$	27.23	27.59	28.83	28.12	27.97	28.46	25.72	30.74	32.45	31.72	(g/L)
$C_{CB,min}$	21.43	21.40	5.59	6.80	23.14	22.47	21.61	21.36	5.85	5.77	(g/L)
$C_{\Delta CB}$	5.80	6.19	23.24	21.32	4.83	5.99	4.11	9.38	26.60	25.95	(g/L)
$C_{LA,max}$	80.87	77.90	92.73	94.69	88.88	88.27	88.09	84.34	91.41	89.96	(g/L)
Parameter derived from experimental data											
$Y^{BM/Sub}$	0.054	0.053	0.049	0.042	0.054	0.037	0.041	0.036	0.055	0.050	(g/g)
$Y^{\Delta BM/\Delta Sub}$	0.079	0.079	0.062	0.056	0.074	0.051	0.051	0.045	0.070	0.066	(g/g)
$Y^{BM/Glc}$	0.087	0.084	0.078	0.067	0.086	0.059	0.063	0.058	0.089	0.082	(g/g)
$Y^{\Delta BM/\Delta Glc}$	0.095	0.094	0.089	0.078	0.094	0.065	0.063	0.059	0.104	0.096	(g/g)
$Y^{BM/Xyl}$	0.327	0.319	0.294	0.250	0.314	0.209	0.249	0.221	0.344	0.317	(g/g)
$Y^{\Delta BM/\Delta Xyl}$	0.540	0.542	0.659	0.555	0.466	0.316	0.348	0.290	0.800	0.770	(g/g)
$Y^{BM/CB}$	0.262	0.255	0.235	0.206	0.267	0.188	0.217	0.161	0.240	0.221	(g/g)
$Y^{\Delta BM/\Delta CB}$	1.224	1.133	0.287	0.269	1.532	0.888	1.348	0.525	0.292	0.269	(g/g)
$Y^{BM/LA}$	0.088	0.090	0.073	0.061	0.084	0.061	0.063	0.059	0.085	0.078	(g/g)
$Y^{\Delta BM/LA}$	0.088	0.090	0.072	0.061	0.083	0.060	0.063	0.058	0.085	0.078	(g/g)
$Y^{LA/Sub}$	0.616	0.585	0.666	0.688	0.642	0.611	0.645	0.606	0.641	0.644	(g/g)
$Y^{LA/\Delta Sub}$	0.901	0.873	0.855	0.930	0.894	0.841	0.813	0.769	0.825	0.844	(g/g)
$Y^{BM/ALK}$	8.023	8.065	6.480	5.502	7.729	5.596	5.667	5.276	7.391	7.002	(g/mol)
$Y^{\Delta BM/ALK}$	7.990	8.031	6.394	5.455	7.667	5.575	5.627	5.244	7.362	6.972	(g/mol)
Parameter derived from parameter estimations											
$\mu_{max,sim,mix}$	3.502	3.702	3.981	4.043	3.443	2.930	2.681	3.104	3.514	3.667	(-)
$\mu_{Glc,max}$	0.486	0.484	0.432	0.443	0.482	0.467	0.468	0.436	0.446	0.437	(1/h)
$\mu_{Xyl,max}$	0.435	0.522	0.168	0.136	0.398	0.289	0.252	0.340	0.101	0.070	(1/h)
$\mu_{CB,max}$	0.035	0.043	0.576	0.584	0.100	0.048	0.094	0.041	0.509	0.561	(1/h)
n	1.310	1.427	1.704	2.054	1.717	1.809	1.751	1.767	1.669	1.897	(-)
$q_{0/Glc}$	6.386	13.14	0.852	0.855	0.991	5.557	4.790	4.509	4.224	7.178	(-)
$q_{0/Xyl}$	0.006	0.002	0.094	0.190	0.006	0.034	0.041	0.009	0.560	2.588	(-)
$q_{0/CB}$	4.132	2.362	0.001	0.001	0.034	0.916	0.040	1.122	0.003	0.001	(-)
Estimation quality											
σ	1.888	2.256	2.095	2.322	2.325	2.117	2.804	2.476	1.613	1.502	
RMS	2.063	2.096	2.435	2.601	2.452	2.091	4.452	3.207	2.095	2.427	
R^2	0.999	0.999	0.998	0.999	0.999	0.999	0.998	0.998	0.999	0.999	
ANOVA											
F	2 E-5	3 E-4	5 E-4	8 E-4	5 E-4	3 E-4	3 E-4	9 E-4	7 E-4	7 E-6	
$F_{critical}$	3.918	3.918	3.918	3.918	3.918	3.927	3.927	3.927	3.927	3.918	
p	0.996	0.987	0.999	0.978	0.982	0.987	0.986	0.976	0.979	0.998	

[a]: Data derived by HPLC measurement described in section 2.3 after inoculum addition.

Remark: The parameter K_{Glc}, K_{Xyl}, and K_{CB} are a result of the parameter optimization and do not reflect the biological laboratory derived values.

For *B. coagulans* strain DSM No. 2314, a very short lag phase is evident (Figure 4.4-1A, 4.4-1B). The exponential growth depends mostly on the consumption of glucose and xylose with a averaged difference in the concentration of 74.5±0.1 g/L and 13.1±0.2 g/L, respectively. Glucose and xylose were nearly metabolized in parallel. No intermediate lag phase could be observed. The cellobiose was not well-metabolized, with only a small decrease in concentration of 6.0±0.3 g/L. A averaged yield of 79.4±2.1 g/L (60.1±1.9 %) LA was produced from 132.2±1.3 g/L total substrate solution. This LA production equals 88.7±2.4 % of a yield based on the total consumed sugar amount.

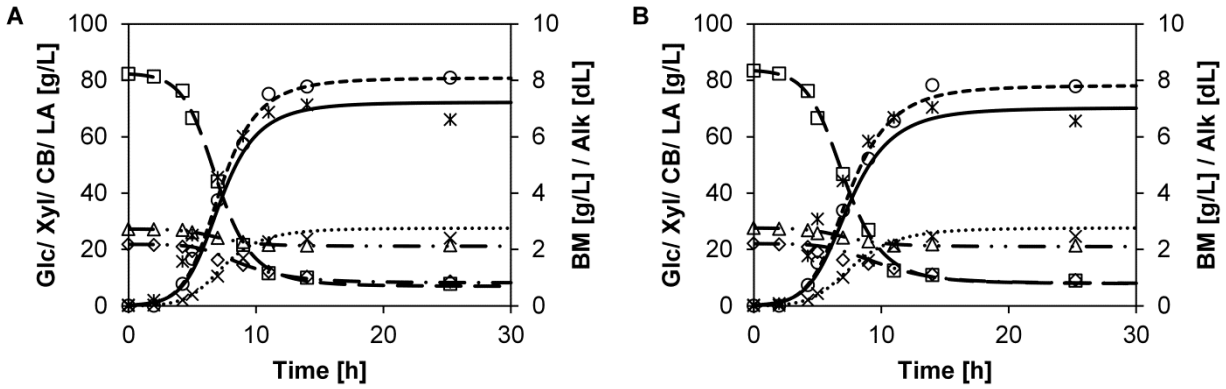


Figure 4.4-1: Fermentation dynamics of organosolv hydrolysate with strain DSM No. 2314. Experimental results are displayed as marks (□ glucose, ◊ xylose, Δ cellobiose, ○ lactate, + biomass, × alkaline). Predicted simulation results are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ cellobiose, − − − lactate, ── biomass, ▪ ▪ ▪ alkaline). Diagrams A and B refer to duplicate fermentations with the same inoculum.

Comparing the growth of DSM No. 2314 and DSM ID 14-301 (Figure 4.4-2A, 4.4-2B), a slightly longer initial lag time and a lower growth parameter, $\mu_{max,sim,mix}$ = 4.6±1.4 , was observed for the second strain. DSM ID 14-301 showed good consumption of the xylose and even higher uptake of the cellobiose fraction. The strain was able to utilize 105.2±4.7 g/L of the total available amount of carbohydrates of 138.5±1.1 g/L. The result is a difference in concentration from the process start to the end of 74.3±0.8 g/L glucose, 10.2±0.1 g/L xylose, and 22.3±1.4 g/L cellobiose. A yield concentration of 89.7±1.4 g/L of lactate could be achieved. This equals a yield of 67.7±1.3 % from the total available sugar input and 89.1±4.6 % yield of the consumed sugar amount. Although the DSM ID 14-301 strain had a slightly lower average biomass concentration (6.2±0.7 g/L) than DSM No. 2314 (7.1±0.1 g/L), only a slightly higher yield could be achieved. This is represented by the yield coefficient $Y^{BM/\Delta Subs}$, which was slightly higher than this yield coefficient of DSM No. 2314 (Table 4.4-1). That can be seen also using the average yield coefficient $Y^{LA/\Delta Sub}$ (0.9±0.1 $g_{LA}/g_{\Delta Sub}$) of DSM ID 14-301, which was not significantly higher than for DSM No. 2314 (0.9±0.1 $g_{LA}/g_{\Delta Sub}$). The molar yield coefficient of $Y^{BM/Alk}$ was lower in DSM ID 14-301 at 6.0±0.7 g_{BM}/mol_{Alk} than in DSM No. 2314 (8.0±0.3 g_{BM}/mol_{Alk}).

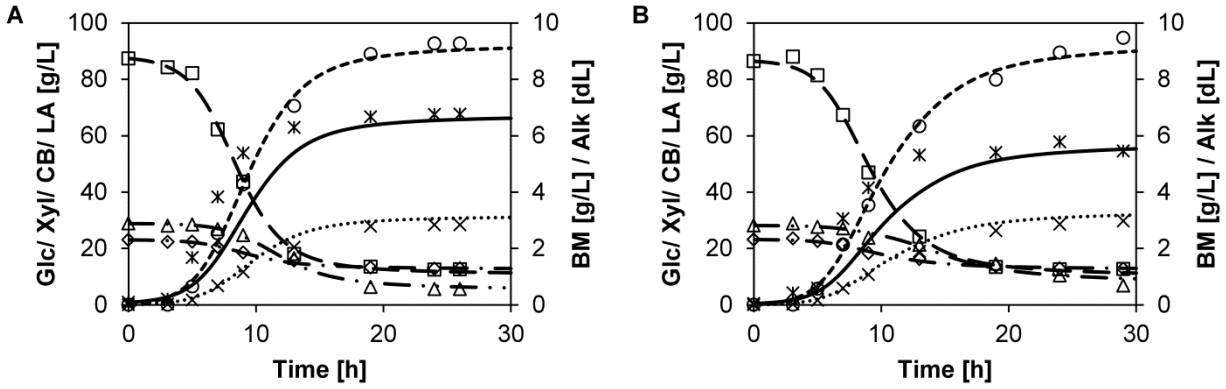


Figure 4.4-2: Fermentation dynamics of organosolv hydrolysate with strain DSM ID 14-301. Experimental results are displayed as marks (□ glucose, ◊ xylose, Δ cellobiose, ○ lactate, + biomass, × alkaline). Predicted simulation results are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ cellobiose, − − − lactate, ── biomass, ▪ ▪ ▪ alkaline). Diagrams A and B refer to duplicate fermentations with the same inoculum.

Strain DSM ID 14-300 (Figure 4.4-3A, 4.4-3B) gave similar results to DSM No. 2314. DSM ID 14-300 consumed glucose and xylose as the main carbohydrate sources, while cellobiose was only partially utilized. Despite the low biomass production (maximum of 6.4±1.5 g/L), a comparable lactate yield of 88.6±0.4 g/L could be achieved. The average yield for strain DSM ID 14-300 was found at 86.7±3.8 % of the fermented sugars and 62.6±3.0 % of total sugar amount.

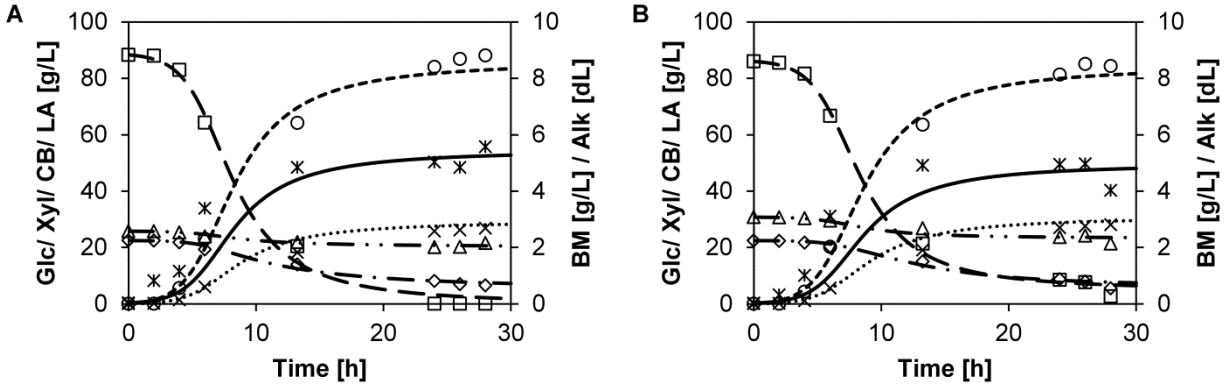


Figure 4.4-3: Fermentation dynamics of organosolv hydrolysate with strain DSM ID 14-300. Experimental results are displayed as marks (□ glucose, ◊ xylose, Δ cellobiose, ○ lactate, + biomass, × alkaline). Predicted simulation results are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ cellobiose, − − − lactate, ── biomass, ▪ ▪ ▪ alkaline). Diagrams A and B refer to duplicate fermentations with the same inoculum.

DSM ID 14-298 (Figure 4.4-4A, 4.4-4B) showed a comparable biomass yield to DSM ID 14-300 with 5.2±0.4 g/L. Nearly the entire amounts of glucose and xylose were consumed. However, this strain has a low affinity towards cellobiose as indicated by the small change in cellobiose concentration. DSM ID 14-298 had a mean yield of 67.4±1.6 % of the total available sugar amount and 79.1±3.1 % of the consumed sugars.

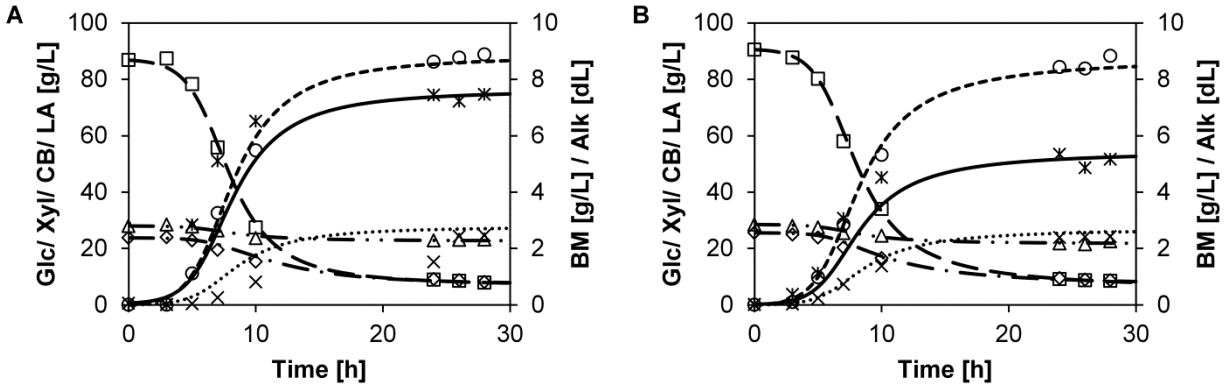


Figure 4.4-4**:** Fermentation dynamics of organosolv hydrolysate with strain DSM ID 14-298. Experimental results are displayed as marks (□ glucose, ◊ xylose, Δ cellobiose, ○ lactate, + biomass, × alkaline). Predicted simulation results are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ cellobiose, − − − lactate, ── biomass, ▪ ▪ ▪ alkaline). Diagrams A and B refer to duplicate fermentations with the same inoculum.

Strain DSM ID 10-395 (Figure 4.4-5A, 4.4-5B) performs well with cellobiose as a carbohydrate substrate but has a low affinity for xylose as shown by the small change in xylose concentration. While 73.9±1.3 g/L glucose and 26.3±0.5 g/L cellobiose were consumed, the xylose concentration only showed a decrease of 9.4±0.5 g/L. A mean biomass concentration of 7.4±0.6 g/L could be achieved. The yields were 64.2±1.6 % of the total sugar and 83.4±2.9 % of consumed sugar.

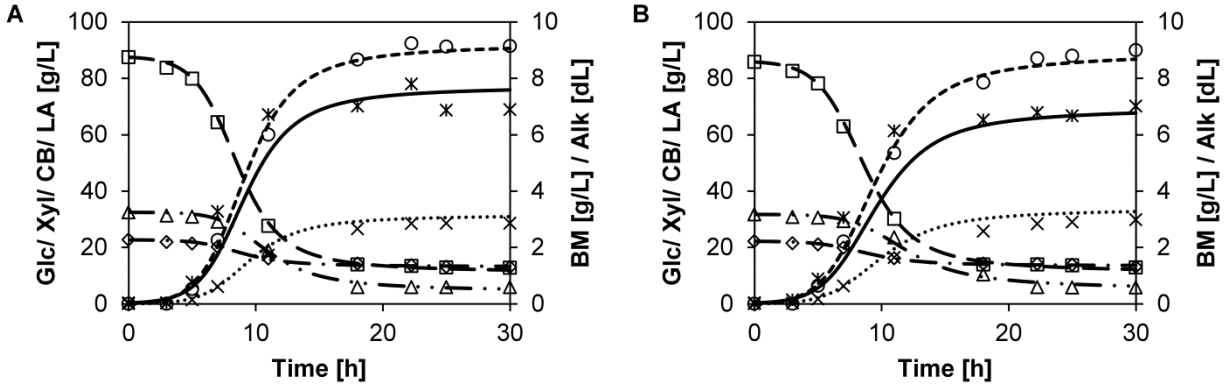


Figure 4.4-5: Fermentation dynamics of organosolv hydrolysate with strain DSM ID 10-395. Experimental results are displayed as marks (□ glucose, ◊ xylose, Δ cellobiose, ○ lactate, + biomass, × alkaline). Predicted simulation results are shown as lines (— — — glucose, — ▪ — ▪ xylose, — ▪ ▪ cellobiose, - - - lactate, ── biomass, ▪ ▪ ▪ alkaline). Diagrams A and B refer to duplicate fermentations with the same inoculum.

The parameter fitting of the proposed model to the experimental data gave good results. The overall mean standard derivation is σ^2 = 2.1±0.4 while the mean RMS values are 2.6±0.7. The overall averaged correlation coefficients are R^2 = 0.999±0.001. The results of the ANOVA based on a 95 % confidence interval have an overall satisfying relation of $F<F_{critical}$. With the *p*-value of near $p\cong 1$ with 0.987±0.008, it could be assumed that the process data and model solution are significantly equal. Reported values of the model parameters differ depending on the used strain, but the values determined here were within the range of previously reported values (Glaser and Venus, 2017).

The generalized nonlinear inhibition term considers that there is a determined concentration of product above which growth and production do not occur. In the model used here, the parameter $C_{LA,max}$ represents the lactate concentrations at which the growth and production processes are interrupted either due to the high lactate concentration itself or to a lack in nutrition. The nutrition with yeast extract lies normally in the range of 1-2 % of the carbohydrate source. The concentration $C_{LA,max}$ differs between the strains is it is expectable.

The growth model mirrors the characteristics of the strains. The maximum growth rates reveal that strain DSM ID 14-301 has the fastest growth on biomass ($\mu_{max,sim,mix}$ = 4.02±0.04) before strain DSM No. 2314 ($\mu_{max,sim,mix}$ = 3.60±0.14). Lower in maximum biomass growth, but not significantly, is strain DSM ID 10-395, with $\mu_{max,sim,mix}$ = 3.59±0.11. Significantly lower are strains DSM ID 14-300 and DSM ID 14-298, with $\mu_{max,sim,mix}$ = 3.19±0.36 and $\mu_{max,sim,mix}$ = 2.89±0.30, respectively. Comparing the glucose uptake rates, they show a good comparability (Table 4.4-1) with a low overall standard deviation. The total average value of the glucose uptake rates (Table 4.4-1) is $\mu_{Glc,max}$ = 0.46±0.02 1/h. The deviation is barely 4.7 % between the strains, so the glucose uptake rates show a good comparability between the strains with a low overall standard deviation, demonstrating comparable performance on the substrate glucose. Comparing the xylose consumption, the strains with the higher consumed xylose amount also have higher uptake rates. Here, strain DSM No 2314 has the highest value, with $\mu_{Xyl,max}$ = 0.48±0.06 1/h. Despite a higher amount of consumed xylose, strains DSM ID 14-300 and DSM ID 14-298 had uptake rates with average values of $\mu_{Xyl,max}$ = 0.34±0.08 1/h and $\mu_{Xyl,max}$ = 0.30 ±0.06 1/h, respectively. For comparison, strain DSM ID 14-301 and DSM ID 10-395 had uptake rates in the range of $\mu_{Xyl,max}$ = 0.152±0.023 1/h and $\mu_{Xyl,max}$ = 0.086±0.022 1/h. This characteristic is comparable to the behaviour of the strains with better cellobiose consumption. Here, those cellobiose-using strains have a higher uptake rate than for glucose. The strains DSM ID 14-301 and DSM ID 10-395 had uptake rates in the range of $\mu_{CB,max}$ = 0.58±0.01 1/h and $\mu_{CB,max}$ = 0.54±0.04 1/h. Among the strains with slow cellobiose consumption, DSM No 2314 had an average uptake rate of $\mu_{CB,max}$ = 0.034±0.01 1/h while the strains DSM ID 14-300 and DSM ID 14-298 had higher uptake rates of $\mu_{CB,max}$ = 0.07±0.041/h and 0.07±0.04 1/h, respectively.

With the rise in toxic power, the intensity of inhibition increases for a determined lactate concentration. With n>1, the inhibition term shows a hyperbolic behaviour. This is evident from the slow process performance, low decrease in carbohydrate concentration and lactic acid formation towards the end of the cultivation. Comparing the strains, the parameter of toxic power shows a lower impact of the lactate concentration towards strain DSM No 2314, with an average value of n = 1.37±0.08. That suggests a slightly more linear dependence on lactic acid inhibition. The other strains had higher values, with an overall total average of n = 1.80±0.13.

During the last years, thermotolerant or respectively thermophilic (50–60 °C) L-(+)-lactic acid producing MOs gained much interest, such as *B. coagulans*. Several studies concerning different Bacillus strains, isolated from nature, were published. *B. licheniformis* TY7 (Sakai and Yamanami, 2006), *B. licheniformis* BL1 (Wang et al., 2011), *B. sp.* 2–6 (Qin et al., 2009), *B. sp.* XZL9 (Wang et al., 2010b), *B. coagulans* CCM 4318 (Rosenberg et al., 2005), *B. coagulans* 36D1 and P4-102B (Patel et al., 2006), *B. coagulans* SIM-7 (Michelson et al., 2006), *B. coagulans* MXL-9 (Walton et al., 2010), and *B. coagulans* WCP10-4 (Zhou et al., 2013).
Effectivity in high LA titers, yield, and productivity are essential for a commercially cost-efficient LA production, as it reduces downstream processing costs by high substrate concentration tolerance (Zhou et al., 2013). The *B. coagulans* strain, discussed in this study, gave a total average LA yield of 87.7±5.3 g/L among all examined strains in a simple batch fermentation process. This equals to 67.4±3.7% of LA yield for the total used amount of carbohydrates and 85.4±4.7% of LA yield based on the amount of metabolised carbohydrates. This yield is at least in the same range or higher than the ethanol or LA production described by other researchers.

Otto (2004) has described the strains of *B. coagulans* DSM No. 2314, DSM No. 2319, *B. smithii* DSM No. 459, and DSM No. 460 grown in a lignocellulosic sugar containing medium. Otto (2004) has described a yield of 35 g/L (70% LA) from 50 g/L xylose as the sole carbon source. Ou et al. (2011) have described an LA production by batch fermentation using the *B. coagulans* strain 36D1 up to a yield of 60 g/L with a significant residual glucose amount. Only a yield of 40 g/L LA was achieved using xylose as a carbohydrate source (Ou et al., 2011). Lactate formation by fermentation of glucose and growth of *B. coagulans* is known to be inhibited by high substrate concentrations–for example, an initial glucose concentration higher than 100 g/L may lead to a prolonged lag time and decrease the growth rate (Michelson et al., 2006)–and higher lactate concentrations lead to unfermented carbohydrates at the process end. Higher production rates of LA in fed-batch processes are also typically suppressed by end-product inhibition, therefore batch fermentation is still the most commonly used method for industrial LA production, although it has relatively low productivity due to end-product inhibition (Michelson et al., 2006). By using $CaCO_3$ in the fermentation medium to overcome the LA inhibition, Ou et al. (2011) were able to increase the yield concentration to 110 g/L for glucose and 120 g/L for xylose. In this study, the used *B.*

coagulans strains gave a yield of about 90 g/L without any additional methods to overcome LA inhibition. However, fermentation processes using either high initial substrate concentrations, such as up to 240 g/L glucose or 200 g/L corn starch for SFF (Zhou et al., 2013), are as important as the possibility of co-fermentation of the different substrates as glucose, xylose, arabinose, and cellobiose.

Several LA-producing bacteria were reported as able to utilise lignocellulose-derived sugars. The strain *B. coagulans* MXL 9 was reported by Walton et al. (2010) for its ability to utilise a hemicellulose water extract of mixed southern hardwoods. Wang et al. (2011) have described a fermentation process for L-(+)-lactic acid produced from 195 g/L xylose using *B. coagulans* XZL4 (DSM No. 23183) and *B. coagulans* XZL9 (DSM No. 23184). Van der Pol et al. (2016) have reported about *B. coagulans* DSM No. 2314 using 72.6% glucose, 24.2% xylose, 3.2% galactose in 100 g/L Medium composition. Furthermore, Adsul et al. (2007a) have described LA-producing *Lactobacillus delbrueckii* Mutant Uc-3. Xu et al. (2013) have used the *B. coagulans* strains XZL4 (DSM No. 23183) and XZL9 (DSM No. 23184) for L-(+)-lactic acid production fermenting pentose or hexose as a carbon source. The highest amount of L-(+)-lactic acid was produced from glucose with 173 g/L, while 195 g/L L-(+)-lactic acid were produced from xylose. The yield was up to 98%. The production of L-(+)-lactic acid from reducing sugars in xylitol by-products was presented with 106 g/L. Wu et al. (2014) have presented information related to *B. coagulans* strains C106, JI12, and WCP10-4. In batch fermentations, they have shown that 66 g/L LA was produced from xylose by *B. coagulans* JI12, with a yield of 91%. *B. coagulans* C106 produced 101 g/L LA with a yield of 94%. The strain WCP10-4 produced 70 g/L of LA from 75 g/L of xylose, giving a yield of 96%. The strain *B. coagulans* MXL 9 was used in the study described by Walton et al. (2010), where its ability to fully utilise a hemicellulose water extract of mixed southern hardwoods led to an LA yield of 94%. Maas et al. (2008) have specified an LA yield of 81% for *B. coagulans* DSM No. 2314 fermenting a hydrolysate of lime-treated wheat straw.

There are a few reports available on LA-producing bacteria which are able to use cellobiose for L-(+)-lactic acid production. While Abdel-Rahman et al. (2011) have described *Enterococcus mundtii* in more detail Adsul et al. (2007a) have described *Lactobacillus delbrueckii*. Normally, these strains grow in a mesophilic temperature range of 30°C to 43°C. These temperatures do not represent the optimal conditions in the thermophilic range of 50°C to 55°C for cellulases including β-glucosidases. Processes performed in the optimum range for cellulases performance and LA by the used MOs should be more effective and economically efficient. However, little data is available on the fermentation of cellobiose by *B coagulans*. Ong et al. (2016) have found that the thermophilic *B. coagulans* WCP10-4 is able to convert cellobiose. They have reported that *B. coagulans* WCP10-4 converted 200 g/L of cellobiose to 196.3 g/L of L-lactic acid, which equals a yield of 97.8%, without supplementation of external β-glucosidases. This characteristic indicates

that the *B. coagulans* WCP10-4 strain is an efficient strain for cellobiose conversion to L-(+)-lactic acid. In this study, it was possible to present two additional *B. coagulans* strains that can utilise cellobiose to a high extent. The here presented *B. coagulans* strains have the benefit of high LA titers and productivity using glucose, xylose, and cellobiose for fermentation.

The strains with the DSM ID 14-301 and DSM ID 10-395 showed a high performance in their ability to consume an average of 9.8±0.6 g/L xylose and to utilise cellobiose to a high extent with an uptake of 24.3±2.5 g/L. However, the strains with the DSM ID 14-300 and DSM ID 14-298 are comparable to the lower performance of the DSM No. 2314. Combined, these three strains can metabolise an average total of 15.5±1.9 g/L xylose and 6.3±1.7 g/L cellobiose.

Thus, DSM ID 14-301 and DSM ID 10-395 were identified as capable of metabolising glucose, xylose, and cellobiose throughout the process, but showed a preference towards cellobiose consumption. Therefore, those strains are the most interesting for further studies characterising their hydrolytic activity on a genetic basis.

Since cellobiose is a potent inhibitor, the possibility of using strains that can directly utilise cellobiose for LA production would be beneficial to lower the cellobiose inhibition on cellulases and reduce the enzyme cost, such as in the simultaneous saccharification and fermentation processes (SSF). Often, intermediate lag phases, indicating the change in metabolism for different sugar kinds, are described, such as for *B. coagulans* MXL-9 by Walton et al. (2010). Within the used strains, intermediate lag phases were not detectable. This can be clearly seen in the online measurement of the alkaline addition.

The strains used in this study showed simultaneous consumption of glucose, xylose, and cellobiose, as these sugars were in a similar concentration range in the growth medium. Ye et al. (2014) have also shown such a simultaneous consumption of carbohydrates at nearly equal concentrations in a fermentation process using 50 g/L glucose and 53 g/L xylose for the strain *B. coagulans* JI12. Additionally, Zhang et al. (2014) have shown data for a simultaneous metabolism of glucose and xylose at equal concentrations. These results are also consistent with Glaser and Venus' study (2017), which used glucose, xylose and arabinose in an artificial fermentation medium at different concentrations. Furthermore, Maas et al. (2008) have shown that *B. coagulans* DSM 2314 has a simultaneous utilisation of glucose and xylose during LA production. The most common technical use of LA is for the synthesis of PLA. For the synthesis of PLA, only optically pure L- or D-lactic acid monomers can be used as precursors. Therefore, the production of optically pure L-lactic acid or D-lactic acid is a very important prerequisite for polymer synthesis.

The optical purity of L-(+)-lactic acid produced by the strains in this study was previously discussed by Glaser and Venus (2017) and given for strain DSM No. 2314 at 98.9%, for DSM ID 14-298 at 98.9 %, and for DSM ID 14-301 at 99.6%. Strain DSM ID 14-300 had an optical purity of 99.9% of L-(+)-lactic acid. The strain with the DSM ID 10-395 could achieve a L-(+)-lactic acid of 95.9% in a test screening (unpublished data). For comparison, Otto (2004) has described an optical purity of 96.7% to 99.7% of their L-lactic acid produced by strains of *B. coagulans* DSM No. 2314, DSM No. 2319, *B. smithii* DSM No. 459, and DSM No. 460. For the fermentation process of glucose, xylose, and xylitol using *B. coagulans* strains XZL4 (DSM No. 23183) and XZL9 (DSM No. 23184), Xu et al. (2013) have proposed an optical purity of over 99%.

Of interest, and yet rarely discussed, is a short yield comparison of LA production with the production of other chemicals, such as ethanol, butanol, and acetone. For example, Ko et al. (2009) have described a yield of 83.1% (12.7 g/L) of ethanol by *Saccharomyces cerevisiae D5A* using aqueous-ammonia pre-treated rice straw. Muñoz et al. (2015) have described an ethanol yield of 51% (35 g/L) of glucose conversion using an ethanol/water-based organosolv-treated *Eucalyptus globulus* tension wood that previously resulted in up to 69% - 77% saccharification yield. Amiri and Karimi (2011) have used an ethanol/water treatment of pine, oak, and elm wood that resulted in a yield of 73% total sugar and 11.6 g/L of combined acetone, butanol, and ethanol yield. Fukushima et al. (2004) have reported a 70% yield of D-lactic acid by *Lactobacillus delbruecki* using hydrolysed starch powder. These studies indicate the large differences in productivity of different bio-based chemicals by other organisms. This gap in productivity and the possibility of utilising several carbohydrate sources shows a further advancement in a production of the biochemical of LA beneficent.
Lignocellulose, as an abundant and renewable resource, and its conversion, has attracted much attention for its role in the production of chemicals such as LA. However, the cost-efficient pre-treatment providing fermentable sugars is still a challenge. In this context, starchy materials are reported to remain the major carbon sources for the production of LA in future (Zhou et al., 2013). Therefore, there is still a strong need for cost-effective processes for LA production from lignocellulose sugars, such as by using newly isolated *Bacillus coagulans* strains with very high LA titre, productivity and yield or by the optimisation of commercial feasible applications.
However, the tested *B. coagulans* strains provide a strong argument for efficient LA production using mixed carbohydrates from lignocellulosic sources. The results presented above show that *B. coagulans* has a rapid fermentation rate at 52°C leading to production of up to 90 g/L of L(+)-LA in a normal batch process.

The fermentation processes of the different strains used in this study showed that the strains were capable of consuming glucose, xylose, and cellobiose while enduring the presence of inhibitory compounds from an organosolv pre-treatment. Several strains also were resistant to higher lignin

concentrations and have also been shown to be able to uptake lignin from the growth medium (Glaser and Venus, 2017). These strains had the best properties for use in lignocellulose hydrolysates consisting of different carbohydrates. The production of L-(+)-lactic acid by *B. coagulans* as a bio-based chemical provides the possibility to produce LA in high yields through the effective and efficient metabolisation of available sugar components. This enables a more cost-effective production of LA than is currently available for other chemicals. The proposed model equations and parameter reduction made it easy to derive the basic kinetic key parameters for strain comparison without the need of a previous determination of additional parameters by costly screenings. The parameters derived by fitting the model to the experimental data showed a good possibility for interpretation along with the parameters derived by the fermentation process.

4.4.6. Conclusions

Using an organosolv hydrolysate fermentation process, five *Bacillus coagulans* strains were compared for their basic key performance parameters to produce LA. A proposed kinetic model used to derive the basic key performance indices was able to reflect the growth behaviour very well, despite the high degree of parameter reduction. The tested strains demonstrated good performance in fermenting the organosolv hydrolysate soluble sugars. Two strains displayed good performance in cellobiose utilisation, but the xylose consumption was lower compared to other three strains, which in turn exhibited a higher xylose uptake rate but lower consumption of cellobiose. The results indicate that it is beneficial to co-cultivate strains that are good pentose consumers with strains that perform cellobiose uptake effectively for high yield production of LA.

5. Overall discussion

In the first part of this work, an approach to increase and predict the yield of a fully cellulase-based hydrolysis of milled lignocellulose feedstock is developed. For the evaluation of the cellulase performance, different cellulase mixtures, such as CTec2 and HTec2 from Novozymes, and cellulase mixtures provided by the Moscow State University (Dr. Arkady Sinitsyn) were used to hydrolyse wheat straw, grass, PW, and AW. Sugar yields from the enzymatic hydrolyses were investigated as a function of cellulase enzyme loading, PSs, and various solid loadings. It was determined that the PS of used lignocellulosic biomass samples, characterised by the SMD, was the foundation for an increase in cellulase based hydrolysis performance prediction and yield. The lignocellulose hydrolyses performed for this work revealed that the characterisation of cellulases and hemicellulases is highly dependent on the PS distribution of the lignocellulose substrate. PS distribution as characterised by the SMD has a greater impact on the yield than the concentration of the utilised cellulase mixtures in the viewed process window.

It was shown that, by decreasing the PS distribution of lignocellulose raw materials–in this work, wheat straw–below a certain range, there was an immense improvement of lignocellulose hydrolysis with increased sugar yields. The SMDs of different PS fractions were 900 µm, 715 µm, 472.5 µm, 282.5 µm, 225 µm, 162.5 µm, 107.5 µm, 85 µm, 75.5 µm, 67 µm, and 31.5 µm. It is shown that there is significant hydrolysis performance increase at fractions with SMD < 162.5 µm. Vani et al. (2015) have declared that the PS has no significant effectiveness on hydrolyses and that hydrolysis is more influenced by biomass loading. They have examined alkali pre-treated rice straw fractions with PSs of less than 500 µm, between 500 µm and 1,000 µm, and more than 1,000 µm and mixed fractions. The results are not contractionary, due to the low resolution of the mesh size of the substrate fractions in the study of Vani et al. (2015). In this work, a significant performance increase is also not seen with SMD > 162.5 µm. Vani et al. (2015) have stated that the solid loading has a stronger impact on the yield. This behaviour is a direct result of the used PS fractions. Despite the cellulase, loading the solid loading was the only parameter change. That leads to the conclusion that for cellulase comparison studies, PS fractions with SMD < 162.5 µm should be used. For comparison reasons, a narrowly selected area of around 100 µm to 150 µm should be selected. The energy consumption for size reduction of lignocellulose biomass leads to a higher energy consumption compared to the theoretically available energy bound in the lignocellulose. The PS distribution, here characterised by the SMD, can be used as a strong quality parameter for optimising and centralising a targeted hydrolysing process.

It is shown that the sugar yield is more dependent on the PS than on the amount of enzyme used. This means that the use of a higher amount of enzyme does not necessarily result in an increased yield. Therefore, prior to a performance characterisation of cellulase mixtures, the characterisation

of the PS distribution is needed. As a key quality indicator of the lignocellulose raw materials, the SMD is proposed in this work. A further homogenisation pre-treatment to reduce the PS of the raw materials can be initiated to standardise and optimise the later hydrolysis. A substrate specific kinetic cellulase unit (wheat straw unit – WSU) was defined. The WSU was defined here as a standard approach to characterise the cellulase mixtures for glucose and xylose (WSU_{xyl}) yields. This kinetic unit is based on wheat straw as substrate and can be used for any other lignocellulose substrates, if their cellulose composition is known. An empirical mathematical approach based on the kinetic unit WSU was developed that enabled for a consistent scale-independent prediction of the glucose and xylose yields of hydrolyses. The model takes the carbohydrate composition of the used lignocellulose, the PS of the substrate, and the enzyme loading into account to predict the glucose yields. It is also possible to predict the xylose yield using a hemicellulose-based approach. The modelling approach can be easily validated for other substrates and compositions.

For a holistic approach that takes the upstream process to fermentation into account, the hydrolysates obtained from different lignocellulosic feedstocks are used for further fermentation processes. Such processes require a microbial culture that combines both substrate utilisation and product formation. LA was selected as a model product. The screening and fermentation results showed that it is possible to use LA producing bacteria from the species *B. coagulans*. As a production strain, *Bacillus coagulans* has promising characteristics; for example, many strains are regarded as safe by the U.S. Federal Drug Administration (FDA) (Keller et al., 2010; Farmer and Lefkowitz, 2004a, 2004b). The LA produced by the strains is usable for ingredients in food and cosmetics and for pharmaceutical applications. *B. coagulans* is usable to produce LA from lignocellulose biomasses (Hofvendahl and Hahn-Hägerdal, 2000; Ou et al., 2009).

By intensified bacterial screening, the *B. coagulans* strain isolates from different habitats were examined for their ability to consume glucose and xylose via high throughput screening. Mathematical models are used to derive the maximum growth rate and lag times for further analyses. Three mathematical model equations describing the bacterial growth, such as a logistic equation, Gompertz model, Baranyi model, and an equation by Richards and Stannard, are used to derive growth parameters, such as the maximum growth rate, maximum population, and the lag time.

The examined *B. coagulans* strains are shown to utilise diverse lignocellulosic pentoses, hexose, and oligosaccharides, such as glucose, xylose, arabinose, and cellobiose (Glaser and Venus 2017, 2018). It was shown that the examined strains of *B. coagulans* can co-ferment these sugars simultaneously if the carbohydrates are available in near equal concentrations. Other research groups (Zheng et al., 2018) have confirmed this simultaneous consumption of sugars.

The strains showed different abilities for hexose and pentose co-fermentation. The strains can be separated in predominantly hexose and pentose utilising strains and predominantly hexose and

cellobiose utilising strains. Aulitto et al. (2017) have observed that their *B. coagulans* strain MA-13 could not metabolise other hemicellulose-derived sugars (e.g. D-galactose, D-xylose, D-arabinose, L-arabinose) besides D-mannose. While the pentose-utilising metabolic pathway, the pentose-phosphate-pathway, is well understood, the cellobiose-utilising pathway is not as examined (Zheng et al., 2018). It is hypothesised that cellobiose utilising *B. coagulans* strains show a cellolytic activity producing active cellulases (Aulitto et al., 2017, Zheng et al., 2018).

Through intensifying the bio-screening by automated turbidimetric measurements, a mathematical approach for comparison of MOs was defined that facilitates the identification of interesting strains for use in complex inhibitory environments. This work showed that *B. coagulans* strains withstand inhibitory components, such as soluble lignin contents, or even utilise them from the growth medium. The screenings showed that low concentrations of lignin stabilise and slightly increase the growth performance–despite a longer lag time, a higher optical density was measured. The described mathematical approach was used to identify how much lignin could be included without inhibiting growth.

The screening was intensified by scaling up the high throughput turbidimetric method into lab scale fermentation processes. The *Bacillus coagulans* isolates from different habitats also show, in the small-scale fermentation processes, different abilities to ferment lignocellulose-derived hydrolyses sugars–glucose, xylose, arabinose, and cellobiose–to produce high quality L–(+)–LA. Interestingly, the strains showed a parallel consumption of the available sugars when the concentrations reached the same level. An intermediate lag-phase, which is characteristic for a diauxic growth, was not visible. This indicates that the bacteria isolates are a backbone to initialise co-fermentation processes.

Fermentation procedures were used further to characterise the ability of *B. coagulans* strains to grow in lignin-containing fermentation media while consuming the lignocellulose-related sugars. During this fermentation, the strains indicate different tolerances for lignin. While one strain (DSM ID 14-301) showed the ability to tolerate a high lignin concentration of up to 2.5 g/L, it lacked the capacity for sufficient pentose uptake. Other strains (DSM ID 10-395) also showed good cellobiose consumption. The examinations showed the best growth results in the lignin containing medium for DSM ID 14-301, isolated from a natural lignin-containing environment. To strengthen the characterisation efforts of the interesting anaerobic MO *B. coagulans,* the strains were grown in lignocellulose organosolv hydrolysate from ethanol/water-pulped beechwood. Three of the examined strains (DSM No. 2314, DSM ID 14-300, and DSM ID 14-298) produced lactate primarily via glucose and xylose uptake. Two other strains (DSM ID 14-301, DSM ID 10-395) were identified as capable of utilising cellobiose to a high degree but had a lower affinity for xylose. The fermentations of lignocellulose wheat straw hydrolysate showed that a separation step to reduce the inhibitor concentration in the hydrolysate was not needed after the enzymatic hydrolyses. It was discovered that *B. coagulans* can utilise small amounts of soluble lignin dissolved in the

growth medium, which leads to a small reduction of the inhibitor. However, soluble lignin existent in the growth medium (up to 2.5 g/L) has shown no influence on the quality of the L-(+)-lactic acid produced

A mathematical model was defined to reproduce the effects of the initial lag time caused by the effect of the inhibitory lignin concentration and changes in the availability of carbohydrates. It was possible to simulate the uptake of sugars depending on their concentration level. The models reproduced the different lag periods of the different substrates that occur until there is a change in metabolism, when the carbohydrates reach the same concentration in the growth medium. Due to the starting utilisation of sugars when they reach the same concentration in the medium, the diauxic growth was not existent in LA production. The mathematical model showed a straight production when starting utilisation of the different available sugars. Using the growth model with a parameter derived by parameter estimation on the fermentation processes with the defined medium, the equation system was used as a predictive model for fermentation using wheat straw hydrolysates. The results indicate that the growth model could be used to predict lactate-producing fermentation, bacterial growth, and substrate consumption.

The capacity for degradation of alkali-lignin in decolourisation tests of alkali-lignin was observed by measuring the dynamic change of alkali-lignin at 280 nm during lactate production of the tested strains. Decolourisation tests of the medium containing VAN, FA, and alkali-lignin that share the same metabolic pathway were conducted. The samples showed a qualitative decolourisation performance of the *B. coagulans* strains. Van der Pol et al. (2016) have shown a comparable behaviour for *Bacillus coagulans* DSM2314. Van der Pol has determined an increase in L-(+)-lactic acid productivity by a factor of 2 in a fermentation process that supplemented 1 g/L furfural to the growth medium. However, it was stated that the improved performance was not a result of the furfural consumption or conversion, but was instead connected to the elongation of *B. coagulans* cells. Van der Pol (2016) has shown that the pathways involved in the synthesis of cell wall components (such as bacillosamine, peptidoglycan, and spermidine) were upregulated. Furthermore, genes involved in stress responses in bacilli SigB, NhaX, and YsnF were upregulated in the presence of furfural.

Ye et al. (2014) have examined the strain of *Bacillus coagulans* JI12 producing L-(+)-lactic acid from both cellulose and hemicellulose. In the mixed fermentation of glucose and xylose, both sugars were simultaneously converted. Ye et al. (2014) have also stated that *B. coagulans* JI12 was tolerant of up to 4 g/L of furfural and was able to convert furfural to 2-furoic acid. In another study, Yan et al. (2018) have described an effective bioconversion of furfural to furfuryl alcohol by a furfural tolerant strain *Bacillus coagulans* NL01. The possibility of utilising the potential inhibitors enables simultaneous detoxification, saccharification, and co-fermentation processes or strain optimization approaches through artificial laboratory evolution. Owing to the tolerance for lignin and the ability to utilise pentose sugars, the tested strain DSM ID 14-298 proves to possess a high

potential for serving as a biocatalyst for the conversion of agricultural by-products into valuable chemicals.

The topic of the bioeconomy is constantly considered with the topic of economic growth. The overexploitation of natural resources in the bioeconomy will be comparable to the overexploitation of fossil resources. However, this is not a new problem, but one that began during the industrialisation process. Currently, resources have been smuggled for the wood-processing industry, such as through the trafficking of rainforest woods for furniture (www.wwf.de/fileadmin/user_upload/PDF/WWF_Holzimporte_April2008.pdf). Furthermore, there seems to be an increase of wood smuggling for heating pellet production (see also, http://www.euneighbours.eu/en/east/stay-informed/news/ukraine-all-you-need-know-about-eus-stance-wood-export-ban). Unfortunately, there is no existing law with penalties in the European Union prohibiting the import of illegally cut wood. The bioeconomy should not be focused solely on economic growth, for various reasons. One of these reasons is that the amount of resources required for constant economic growth often cannot be provided by single countries. As a result, resources from other parts of the earth, which are crucial for the development of the bioeconomy in those regions, become unavailable to the regions that produce them. The utilisation of biodegradable waste is an alternative approach in waste management.

Biodegradable wastes are predominantly found and are available in agricultural and forest materials, for example, crop residue such as straw, leaves, roots husks, sawdust, twigs, leaves, shrubs, and more. Wide ranges of the materials are used as feed for animals and other branches of the in-house circular economy of agricultural companies. Even cities create vast amounts of biodegradable waste that may contribute to environmental pollution (Sirohi et al., 2018). These materials are mainly lignocellulose based and should be recycled, which would also drive the community attention for a greener environment. A broadened recycling of lignocellulose biomass can be achieved naturally by MOs and artificially by MOs and cellulases. Cellulases are important lignocellulose-hydrolysing enzymes derived by MOs and valuable for diverse applications such as in the food and feed processing industry, pulp and paper industry, textile industry as well as cosmetic and pharmaceutical industry (Abdel-Hamid et al., 2013; Acharya and Chaudhary 2012; Jaramillo et al., 2015; Menendez et al., 2015). Cellulases also play a central role in the bioconversion of the agricultural residues to sugars and biochemicals (Sirohi et al., 2018). It is crucial to convert the lignocellulose materials effectively and efficiently to achieve economic feasibility for the following processes, and also to prevent a possible harmful impact on the environment (Hong et al., 2013).

6. Overall conclusions and outlook

The goal of this work was to develop and present methods and applications that can be used to optimise processes for the hydrolysis of lignocellulose and the production of value-added substances, such as LA. In this sense, it can support the efficient establishment of a lignocellulose biorefinery as a decentralised supporter of the economy. Besides the presentation of its varied research topics, this study provides an overview of the numerous avenues of research and applications. This is done in two ways; firstly, this work provides an approach to classifying the available biomass resources, and secondly, this work presents methods to identify areas for improvement.

A reliable quality parameter for the preparation of lignocellulose feedstock prior to an enzymatic hydrolysis process is crucially needed. For industrial applications, the standard kinetic units such as filter paper units and carboxyl-methyl-cellulase units, cellobiose units and xylose units are identified as not useful as key performance and quality parameters for a hydrolysis process. The results of this work show that a direct characterisation of the cellulase performance on industrial use substrate is more informative for the hydrolyses process performance. Methods are developed in this work to enable a better cellulase and hemicellulase characterisation on actual industrially relevant lignocellulose raw materials. In the scientific field, this work can be used as a basis or starting point to enable further characterisation strategies of lignocellulose hydrolysing enzyme development. The proposed method to characterise enzymes using non-standardised substrates, such as actual lignocellulose instead of cellulose in the form of FP, may lead to an increase in the available information. This information can be used for further optimisations and intensification for the predictive modelling approaches, as was done in this work. The results presented can have a significant impact on small- and medium-sized bioeconomies, as it would facilitate their work. Companies will be able to perform quality inspections of the raw materials and to predict the course of the process. This would allow them to rework the starting materials if quality criteria are not met. Potential applications are shown and are intended to make future amounts of renewable raw materials efficiently usable for non-centralised production of bio products. Based on an easy to determine characteristic, the SMD was defined as a quality parameter.

In the first step of a proposed lignocellulose biorefinery, precursor-containing biomasses such as milled wheat straw and wood are subjected to physical characterisation prior to enzymatic hydrolysis. For an efficient and effective lignocellulose hydrolyzation, it is crucial to have a predictive method for a possible outcome of the hydrolysis process. This is necessary to identify the need for an additional pre-treatment or to optimise the composition of the raw material or the cellulase enzyme mixture. The current set of standardised kinetic units are not usable to predict hydrolysis yields of actual lignocellulose subtracts. As discussed above, the standard kinetic units

are based on standardised optimal substrates, to characterise and achieve information on the diverse enzymes in a cellulase mixture. With a proposed mathematical model, it is now possible to simulate and predict the outcome of a hydrolysis using enzyme loading, SMD, and substrate carbohydrate properties. The developed method for lignocellulose substrate, cellulase characterisation, and model-based yield prediction can be used to optimise the effectivity and efficiency of hydrolyses processes, independent of the process scale. While for the PS distribution of the substrate, the SMD is usable as a quality parameter, for example for incoming substrate inspection, the model equation can be used to predict the yield of a possible hydrolysis process. It will become much easier to optimise and standardise hydrolysis processes for a later or parallel conversion into a product of higher value such as LA or ethanol.

As LA is a chemical usable as a building block for PLA, a LA producing bacterium should be able to ferment lignocellulose hydrolysates with its different sugar components and occurring inhibitors. The implementation of a functional high throughput screening strategy for MOs, as well as a rapid and efficient mathematical model, should make it easier and faster to identify needed optimum properties such as pH, temperature, fermentable sugars, and inhibitor stability. The discussed screening methods for screening of MOs can be also adapted to isolate wild type LA producing bacteria for production. Given the small scale fermentations, model based characterisation of the demonstrated behaviour of the *Bacillus coagulans* isolates used in this study–including good growth with low inhibitor concentrations, and utilisation of glucose, xylose, arabinose, and cellobiose–may also lead to new approaches for intensified targeted laboratory evolution for non-GMO co-cultivating processes. This could be used by small- and mid-sized companies. It was possible to identify *Bacillus coagulans* as a promising lignin resistant bacterium to produce LA from diverse hydrolysates with low risks but high efficiency. The proposed kinetic fermentation models are also useful for a first small scale process characterisation.

The results of this work, discussed and summarised above, can be used to facilitate the work of small- and mid-sized businesses. The presented results are useful to optimize processes, such as the use of the right amount of cellulases, any needed additional pre-treatment of lignocellulose raw materials, and yield prediction.

A next step should be a further optimisation of the pre-treatment. This optimisation can be achieved by combining the physical pre-treatment of milling with an easy-to-use thermal application, such as autoclaving at moderate temperatures and for a longer duration. The use of additional chemicals, such as acids or bases, should be avoided unless they are proven to be advantageous for a later fermentation process (e.g., if they provide nutrition). Efforts should be made toward the optimisation of the introduced empiric model equation that was used for the hydrolysis yield prediction. Here, different substrates and substrate mixtures with different physical properties should be used. Using this knowledge, the scale-up of the hydrolysis process can be achieved. The use of *B. coagulans* as a promising production strain should be pursued. Further

process optimisation of the production of LA could be done by further metabolic characterisation or by the genetic optimisation of promising *B. coagulans* strains. A more in-depth understanding of the degradation of lignin or aromatic acids such as FA or vanillate can be used for strain improvement. Here, it is promising to intensify the endeavour to optimise strains of *B. coagulans* by improving the naturally occurring cellulolytic activity and the substrate and product-related properties, such as inhibitor tolerance and yield. This can also be done by using recombinant methods to achieve those properties together with a heterologous cellulase and hemicellulase system. An optimised pre-treatment and fermentation process in a small scale should be followed by a scale-up approach into a pilot-scale.

The possibility of merging the three processes–the production of cellulases and hemicellulases, the hydrolysis of cellulose and hemicellulose to monomeric sugars, and the fermentation of hexose and pentose sugars as well as cellobiose–into a single system should be considered. With such a process, there is a great potential for lowering costs and increasing efficiency, due to possibly increased hydrolysis rates and reduced reactor volumes or decreased needs for additional equipment. However, more work has to be done to optimise and extend the described methods in this work. For the future optimisation and development of lignocellulosic biorefineries, the technical possibilities of using renewable raw materials have not been exhausted. Actions for the developments for economic and ecological potentials include defined research, development, and promotion strategies.

There must be a targeted promotion to establish the decentralised bioeconomy as a crucial future building block that supports family-based businesses and small industry with knowledge and easily utilised, effective, and efficient technology. To keep this in mind, there is still a need to optimise the described methods in terms of the design of the basic concepts and technical implementation options. This accompanies the improvement of cultivation and breeding adaptations of plants. Experiments and research efforts should be expanded for improving material (i.e., ingredients) as well as energy use (i.e., energy plants).

A milestone could be the direct integration of the lignocellulosic biorefinery for the production of biochemicals with the facilities for biogas that are used for the production of electric and thermal energy. This integration may lead to a better ratio of usable carbohydrates. The lignocellulose biomass can be first used for the production of biochemicals (e.g., LA) after a physical and thermal pre-treatment. The residues of the process could be further used as a feedstock in the biogas facility for the production of electricity. Free electric power can be used to start the lignocellulose pre-treatment, again establishing a production cycle inside a product life cycle.

When done in the right way, the bioeconomy can contribute to solving global problems. This includes ensuring a sustainable energy, water, and raw materials supply, and sustainable health and nutrition for an increasing world population, as well as protecting the soil, climate, and

environment. The bioeconomy is not to be understood as a new industry. Bioeconomy means getting to know the cycles of nature in terms of environmental and resource conservation. This requires bioeconomy research and development for innovation to enable sustainable production and use of biological resources and to provide products, processes and services as part of an alternative, more sustainable economic system. Political decisions are necessary to bring companies and society closer to the benefits of the necessary sustainable bio-economic management. Behavioural changes, social and political engagement are urgently needed to end the depletion of nature, to preserve the earth and the climate, and to protect it so it can remain a source for a healthy life for future generations.

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8. Theses

1. The particle size (PS) distribution of lignocellulosic biomass can be used to define the Sauter mean diameter (SMD), which was used as a quality indicator for the processing of raw materials. Decreasing the SMD of lignocellulose raw materials to below 100 µm causes up to a four-fold increase of the yield of lignocellulose hydrolysis in comparison to an increase of the cellulase concentration for the hydrolysis.
2. The SMD and cellulose or hemicellulose content of lignocellulose raw materials can be used to define a substrate specific kinetic cellulase unit. This unit was defined for the hydrolysis of milled wheat straw, hence named wheat straw unit (WSU), for diverse PSs.
3. The WSU, cellulase loading concentrations, solid lignocellulose substrate loading concentrations, and the obtained SMD of lignocellulose raw materials were used to develop a mathematical model equation to predict the sugar yield of lignocellulose hydrolysis. The model equation was validated in lab-scale and pilot-scale hydrolysis processes.
4. A method for optical high throughput screenings, in combination with a mathematical approach, is proposed that can be used to characterise MOs with respect to their growth behaviour and inhibitor tolerance on varying lignocellulose-derived carbohydrate sources. The growth of different strains of *Bacillus coagulans* was characterised regarding their growth on carbohydrates, such as glucose and xylose, with and without soluble alkaline lignin in the growth medium. The strain DSM ID 14-301 was identified as a strain with high lignin tolerance. DSM ID 14-298 had the highest pentose utilisation ability.
5. The screening method and the mathematical approach were both validated by laboratory-scale fermentation processes and by a more detailed process model. The process model enables predictions of growth behaviour, substrate utilisation, and product formation.
6. The *Bacillus coagulans* isolates from different habitats show diverse abilities to metabolise lignocellulose derived sugars to high quality L-(+)-lactic acid with high purity (99%).
7. Soluble alkaline lignin tested for concentrations of up to 2.5 g/L did not have a negative effect on the quality of the L-(+)-lactic acid production of tested *Bacillus coagulans* strains.
8. *Bacillus coagulans* isolates from different habitats show varying tolerance to soluble alkaline lignin and can decrease the lignin concentration in the growth medium. This may allow process solutions in which one of the characterised strains can be used to detoxify the growth medium from lignin and another strain can be added to ferment the available sugars.