UNIVERSITÉ DE MONTRÉAL

# DÉVELOPPEMENT DE STRATÉGIES DE BIO-DÉTOXIFICATION DE PRÉ-HYDROLYSATS LIGNOCELLULOSIQUES POUR FERMENTATION ABE EN BUTANOL

MARIEM THEIRI

DÉPARTEMENT DE GÉNIE CHIMIQUE

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION

DU DIPLÔME DE PHILOSOPHIAE DOCTOR

(GÉNIE CHIMIQUE)

AOÛT 2018

UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Cette thèse intitulée :

# DÉVELOPPEMENT DE STRATÉGIES DE BIO-DÉTOXIFICATION DE PRÉ-HYDROLYSATS LIGNOCELLULOSIQUES POUR FERMENTATION ABE EN BUTANOL

présentée par : THEIRI Mariem

en vue de l'obtention du diplôme de : Philosophiae Doctor

a été dûment acceptée par le jury d'examen constitué de :

M. LEGROS Robert, Ph. D., président

M. JOLICOEUR Mario, Ph. D., membre et directeur de recherche

M. CHADJAA Hassan, Ph. D., membre et codirecteur de recherche

Mme MARINOVA Mariya, Ph. D., membre et codirectrice de recherche

M. HENRY Olivier, Ph. D., membre

M. SPARLING Richard Robert M., Ph. D., membre

# DÉDICACE

*À mes parents, Abderrahmen & Aicha*

*À mes sœurs & mon petit frère*

# REMERCIEMENTS

Je tiens tout d'abord à exprimer toute ma reconnaissance à mon directeur de thèse Professeur Mario Jolicoeur pour m'avoir encadré, orienté, aidé et conseillé tout au long de ma thèse. Je le remercie infiniment pour son entière disponibilité ainsi que pour ses encouragements considérables qui m'ont grandissement motivé pour progresser dans ma thèse. Ses commentaires aux différentes étapes du projet étaient assez pertinents et précieux et m'ont aidé à la construction de mon esprit scientifique et à la meilleure gestion d'un sujet de recherche.

Je tiens à témoigner toute ma gratitude à Dr. Hassan Chadjaa, mon codirecteur de recherche et responsable scientifique du Centre National en Électrochimie et Technologies Environnementales (CNETE). Il a un rôle majeur dans le bon déroulement de ma thèse grâce à ses commentaires pertinents, ses conseils précieux, et son suivi continu de la réalisation de mon projet. Je le remercie infiniment pour sa patience, ses encouragements et ses recommandations pour devenir un excellent chercheur scientifique qui ont amélioré non pas seulement mon esprit scientifique mais également les aspects de ma vie personnelle.

J'adresse également mes sincères remerciements à Dr. Mariya Marinova, ma co-directrice de recherche, pour le temps qu'elle a consacré tout au long de ma thèse, pour m'avoir fourni son aide précieuse sachant répondre à toutes mes interrogations. Elle m'a bien guidé et conseillé au cours des différentes étapes de mon projet et elle n'a pas hésité de me communiquer son expertise et ses connaissances qui m'ont énormément aidé dans la progression de ma thèse.

Je voudrai remercier aussi Nancy Deziel, directrice du CNETE, et Nicholas Berrouard, responsable de recherche et développement au CNETE, pour m'avoir accueillie au sein de leur secteur de recherche où la partie expérimentale de la thèse était conduite. Je remercie aussi Jean-François Lemay, Patrick Quessy et l'ensemble de l'équipe de CNETE pour leur accueil et leur sympathie. Leurs conseils et expertises m'ont énormément aidé à bien conduire mon projet de recherche.

Un grand merci s'adresse à mes parents, mes sœurs Mouna, Asma et Chaima et mon petit frère Mohamed Amine pour m'avoir soutenu, encouragé incessamment et motivé malgré la distance qui nous sépare. Vous avez partagé avec moi les bons et les mauvais moments durant mes études.

Vous avez beaucoup sacrifié pour moi. Je vous remercie infiniment mes chers et j'espère vous rendre égayés par ma réussite dans la thèse.

# RÉSUMÉ

L'industrie des pâtes et papiers au Canada connait une grave crise économique depuis dix ans pour plusieurs raisons, dont entres autres une baisse de la demande en produits papetiers, une compétition internationale aigüe et une hausse des coûts de l'énergie [1, 2]. Une solution d'avenir prometteuse pour redynamiser l'industrie consiste à intégrer une bioraffinerie au sein d'usines existantes. Une bioraffinerie intégrée permet de co-produire, en plus des produits de base de l'industrie papetière, des produits à haute valeur ajoutée par valorisation des déchets et sous-produits sans grande valeur commerciale. Priorisant une conversion efficace des hémicelluloses composant la biomasse lignocellulosique avec la lignine et la cellulose, leur extraction en amont du procédé de cuisson par une étape de préhydrolyse représente une avenue prometteuse. Les hémicelluloses extraites permettent la génération de produits à haute valeur ajoutée dont le butanol et l'éthanol, par fermentation, ainsi que le furfural et diverses autres molécules carbonées d'intérêt pour l'industrie chimique. Cependant, une conversion efficace du préhydrolysat en biocarburants, par fermentation, exige la disponibilité des monomères des glucides à haut poids moléculaire présents sous formes de polymères de ces monomères. L'intégration d'une étape d'hydrolyse est ainsi indispensable pour obtenir des glucides fermentescibles (i.e. monomères ou oligomères solubles ou transportables). Les technologies courantes de préhydrolyse et d'hydrolyse des hémicelluloses sont à base de réactifs chimiques qui permettent des rendements élevés de dégradation et de mise en solution des glucides simples. L'utilisation de ces réactifs est également responsable de la génération de divers inhibiteurs de procédés de fermentation. Pour assurer une conversion efficace, l'enlèvement des produits toxiques est donc requis. Ce problème appelle à l'intégration d'un procédé de détoxification pour amoindrir les effets secondaires néfastes du procédé d'hydrolyse. Incidemment, la combinaison des processus d'hydrolyse et de détoxification des hémicelluloses via l'application d'un consortium de microorganismes, représente une option prometteuse qui pourrait contribuer à la production de biocarburants économiquement et environnementalement acceptables. En effet, les procédés impliquant des microorganismes sont généralement respectueux de l'environnement et sont caractérisés par une faible utilisation de produits chimiques et une plus faible génération de produits toxiques, par une sélection adéquate du consortium microbien.

Le but de cette thèse consiste ainsi à développer des technologies novatrices de détoxification à base de microorganismes pour une hydrolyse bactérienne performante de préhydrolysat

hémicellulosique et pour une conversion subséquente efficace en butanol par la souche *Clostridium acetobutylicum* ATCC 824.

La première partie de la thèse présente une étude proposant l'utilisation d'une culture aérobie mixte de bactéries, *Ureibacillus thermosphaericus* NCIMB 13819 et *Cupriavidus taiwanensis* DSM 17343, produisant et sécrétant les enzymes permettant la détoxification de préhydrolysat de bois. Tout d'abord, des expériences d'optimisation de la température réactionnelle de la co-culture ont été conduites sur un bouillon de soja trypsique (TSB) à titre de système modèle, et la température optimale commune permettant de favoriser les cinétiques réactionnelles spécifiques aux deux microorganismes préconisés, étudiée en mode mono- et co-cultures, a été déterminée à 41 °C. Par la suite, la performance des cultures pures et mixtes pour la détoxification de milieux synthétiques comprenant quatre classes de composés phénoliques (i.e. acide gallique, vanilline, catéchol et syringaldéhyde), le furfural, le 5-hydroxyméthyl furfural (5-HMF), l'acide acétique, les sels et la source des nutriments a été étudiée. En parallèle et dans un premier volet, une étude comparative sur l'effet d'une supplémentation de deux sources de nutriments sur la performance de dégradation de différents composés inhibiteurs par les cultures pures a été effectuée. Les cultures enrichies avec des ions métalliques comme ajout nutritionnel ont montré une performance supérieure de dégradation. Dans un deuxième volet, une étude a cherché à comparer la performance de co-cultures simultanées et séquentielles (i.e. inoculations décalées) sur la dégradation des composés phénoliques présents dans un mélange d'inhibiteurs. Des pourcentages de dégradation élevés, de l'ordre de 90% avec les co-cultures simultanées, ont été obtenus avec les différents types de co-cultures en présence de 2.8 g/L de composés phénoliques totaux. En augmentant la concentration initiale de ces composés à 8 g/L, la meilleure performance de détoxification observée a été similaire avec 87% pour une inoculation en séquence de *U. thermosphaericus* suivie de *C. taiwanensis* après 12 h d'incubation. Le suivi de détoxification en composés inhibiteurs dans les différentes cultures a permis d'identifier la séquence d'inoculation des bactéries maximisant la dégradation spécifique de chaque type de composés phénoliques à différentes concentrations initiales. On a par la suite précisé dans un troisième volet l'efficacité de détoxification par les différentes cultures microbiennes des composés phénoliques individuels. Étonnamment les performances de dégradation observées en seule présence des composés phénoliques ont été inférieures en comparaison avec les résultats obtenus en coprésence des autres inhibiteurs et des composés phénoliques. Finalement, on a évalué la performance de détoxification

d'un préhydrolysat réel par les mono- et co-cultures et les pourcentages de dégradation des composés phénoliques n'ont pas dépassé 14% avec les cultures séquentielles de *U. thermosphaericus* suivie de *C. taiwanensis*.

Dans la deuxième partie de la thèse, nous avons poursuivi le développement d'une technologie de détoxification efficace et performante en intégrant une méthode chimique de floculation à l'étape biologique (i.e. coculture de *U. thermosphaercius* avec *C. taiwanensis*) afin d'augmenter l'efficacité de détoxification. Tout d'abord, différentes combinaisons de méthodes de détoxification ont été évaluées dans le but de déterminer la stratégie favorisant une dégradation maximale des inhibiteurs. La stratégie la plus performante a consisté en la floculation de l'hydrolysat de bois avec $Fe_2(SO_4)_3$ suivie de la biodétoxification avec la co-culture (*U. thermosphaericus* suivie de *C. taiwanensis* après un délai d'inoculation de 24 h, déterminé optimal dans le préhydrolysat). Des taux d'enlèvement de 63%, 73% et 28% des composés phénoliques, de furfural et de 5-HMF ont été respectivement obtenus. Finalement, on a validé la stratégie sélectionnée par la production de butanol via fermentation de type acétone-butanol-éthanol (ABE) par *Clostridium acetobutylicum* ATCC 824. Une concentration en butanol de 8 g/L a alors été atteinte après 120 h de culture, avec un milieu de culture composé d'un hydrolysat d'hémicelluloses détoxifié, en jumelant une étape de floculation à une étape biologique puis dilué pour une concentration initiale en composés phénoliques de 0.3 g/L soit le seuil acceptable pour ces inhibiteurs, de même que l'ajout des nutriments nécessaires pour la croissance et le métabolisme de la souche solvantogénique.

La troisième et dernière partie expérimentale de cette thèse a cherché à compléter le procédé développé en intégrant une hydrolyse biologique aux étapes de floculation chimique et de détoxification biologique, par la bactérie *Paenibacillus campinasensis* DSM 21989. Une efficacité similaire de dégradation des composés phénoliques inhibiteurs (56%) a été obtenue avec les approches d'hydrolyse et de détoxification séparées ou simultanées, en co-culture des trois souches bactériennes. L'hydrolyse microbiologique comme traitement unique de préhydrolysat a également montré contribuer pour 36% de la détoxification en phénoliques. Cette approche d'hydrolyse biologique n'a pas contribué à l'hydrolyse des xylooligosaccharides en xylose, un glucide fermentescible en butanol. Il a également été intéressant qu'aucune des populations microbiennes présentes ne semble utiliser de façon préférentielle le xylose, et ce glucide est ainsi demeuré majoritairement disponible pour la fermentation ABE et la production de butanol. Une

validation finale par fermentation ABE avec *Clostridium acetobutylicum* ATCC 824 des hydrolysats détoxifiés générés par hydrolyse et détoxification simultanées puis dilués jusqu'à 0.3 g/L en composés phénoliques, a mené à 7 g/L de butanol après 116 h de culture. Cette concentration est la plus élevée obtenue en comparaison avec les résultats de fermentation d'hydrolysats biologiques non détoxifiés et d'hydrolysats obtenus par hydrolyse et détoxification séparées, travaux réalisés dans le cadre de cette thèse. De plus, il est d'intérêt de noter que la production de butanol dans les hémicelluloses hydrolysées microbiologiquement comme traitement unique de préhydrolysat a été 6 g/L, soit la meilleure performance obtenue comparativement aux essais en milieu synthétique de xylose (5.27 g/L), avec un hydrolysat acide (2.3 g/L) ou avec un hydrolysat généré par hydrolyse biologique et détoxification séparées (1.05 g/L). Nos travaux ont donc résulté en une stratégie ayant le potentiel de minimiser l'incidence environnementale de même que les coûts de bioprocédé de bioraffinage forestier pour la production de butanol.

# ABSTRACT

The Canadian pulp and paper industry is facing a great economic crisis in the last decade for several reasons (e.g. decline in the demand for paper based products, acute international competition and high cost of energy) [1, 2]. The integration of a biorefinery into existing factories is a promising future solution for revitalizing the industry. An integrated biorefinery allows the coproduction of high-value added products alongside the industrial core products by up-grading low commercial valour wastes and co-products. Emphasizing an efficient conversion of hemicelluloses, contained in lignocellulosic biomass along with lignin and cellulose, their extraction with a prehydrolysis step prior to digestion is a promising avenue for high value-added products generation as butanol and ethanol, by fermentation, as well as furfural and various other carbon molecules of interest to the chemical industry. However, an efficient conversion of the prehydrolysate to biofuels by fermentation requires the availability of monomers from high molecular weights carbohydrates present as polymers of these monomers. Hence, the integration of an hydrolysis step is required to release fermentable sugars (i.e. Soluble or transportable monomers or oligomers). The conventional methods of prehydrolysis and hydrolysis of hemicelluloses are chemical-based techniques which favor high yields of degradation and solubilize the monomeric carbohydrates. These methods contribute equally to the formation of diverse fermentation inhibitors along with the carbohydrates. A compelling need to remove toxic compounds for an efficient conversion to bioproducts is inciting the integration of a detoxification process to impede side effects of the hydrolysis step. By the way, the development of a microbial consortium for combined hydrolysis and detoxification processes would be an economic as well as an environmental avenue for sustainable biofuels production. In fact, microorganisms-based processes are generally characterized by their environmental aspect, small use of chemicals and lower contribution to toxic compounds formation, which would be achieved by a good selection of the microbial consortium.

The aim of this thesis is thus to develop novel technologies of detoxification with microorganisms for a performant biohydrolysis of hemicelluloses pre-hydrolysate and for an efficient subsequent conversion to butanol with *Clostridium acetobutylicum* ATCC 824.

In the first part of this thesis, we proposed the application of an aerobic co-culture, composed of *Ureibacillus thermosphaericus* NCIMB 13819 and *Cupriavidus taiwanensis* DSM 17343, which

produce and secrete enzymes responsible for the detoxification of hardwood pre-hydrolysate. First, we performed optimisation experiments of the reaction temperature in the co-culture using tryptic soy broth (TSB) as a model system, and the common optimal temperature favoring the specific reaction kinetics for both recommended bacteria studied in pure and mixed cultures, was determined to 41 °C. Secondly, the performance of mono- and co-cultures has been studied for the detoxification of synthetic media comprising four classes of phenolic compounds (i.e. gallic acid, vanillin, catechol and syringaldehyde), furfural, 5-hydroxymethyl furfural (5-HMF), acetic acid and nutrients source. In parallel and in a first section, a comparative study of two nutrients sources for their supplementation effects on the inhibitors' degradation performance with pure cultures has been developed. The cultures enriched with metal ions as a nutritional supplement showed superior degradation performance. In a second section of the study, the performances of sequential (i.e. offset inoculations) and simultaneous cultures for the removal of key phenolics present in a mixture of inhibitors were compared. At 2.8 g/L of total phenolic compounds, the different co-cultures exhibited high degradation percentages, up to 90% with an offset inoculation of *Cupriavidus taiwanensis* with respect to *Ureibacillus thermosphaericus*. By increasing the initial concentration of phenolic compounds to 8 g/L, similar performance of phenolic compounds removal up to 87% was observed with the sequential inoculation with a 12 h delay in the inoculation of *C. taiwanensis*. The optimal bacteria sequence maximising specific degradation of each type of phenolics present at different concentrations was determined from the detoxification monitoring of inhibitory compounds. Subsequently and in a third section, the degradation efficiency of individual phenolic compounds with the bacterial cultures was investigated. Surprisingly, the degradation performances observed in single presence of phenolic compounds were lower than those obtained in the co-presence of other inhibitors along with the phenolics. Finally, the detoxification performance of a real pre-hydrolysate was evaluated with mono- and co-cultures and the degradation values of phenolic compounds did not exceed 14% obtained with the sequential cultures of *U. thermosphaercius* followed by *C. taiwanensis*.

In the second part of the thesis, we continued the development of a performant and efficient detoxification technology by coupling a chemical method of flocculation to the biological method (i.e. co-culture of *U. thermosphaericus* and *C. taiwanensis*) in order to increase the performance of detoxification. First, different combinations of detoxification methods were evaluated and the strategy yielding maximum inhibitors degradation was selected. The most performant strategy

consisted in flocculation of the wood hydrolysate with $Fe_2(SO_4)_3$ followed by biodetoxification with the co-culture (sequential cultures with *U. thermosphaercius* inoculated 24 h prior to *C. taiwanensis*, as optimised in the pre-hydrolysate). Removal rates of 63%, 73% and 28% of phenolic compounds, furfural and 5-HMF were respectively obtained with this strategy (co-culture followed by flocculation). Finally, the detoxification method was validated by the production of butanol with *C. acetobutylicum* ATCC 824 via acetone-butanol-ethanol (ABE) fermentation. A butanol concentration of 8 g/L was produced after 120 h of culture in a medium consisting of the detoxified hemicelluloses hydrolysate, obtained by coupling a flocculation step with a biological one then dilution to 0.3 g/L of phenolic compounds for acceptable thresholds, and the nutrients source required to solventogenic bacteria for their growth and metabolism.

The third and last experimental part of this thesis sought to complete the process developed by coupling biological hydrolysis with *Paenibacillus campinasensis* (*P. campinasensis*) DSM 21989 to flocculation and biodetoxification steps. Similar performances of phenolic compounds degradation were obtained (56%) with separate or simultaneous, with the co-culture of three bacteria, hydrolysis and detoxification steps. Microbial hydrolysis as the sole treatment method of hemicelluloses pre-hydrolysate has also been shown to contribute to 36% of phenolics removal, while it didn't result in the hydrolysis of xylooligosaccharides to xylose which is a fermentable carbohydrate to butanol. It has also been interesting that none of the existing microbial populations seem to preferentially consume xylose, and this carbohydrate remained mostly available for ABE fermentation and butanol production. A final step of validation by ABE fermentation with *Clostridium acetobutylicum* ATCC 824 of detoxified hydrolysates obtained by simultaneous detoxification and hydrolysis then diluted to 0.3 g/L of phenolic compounds, contributed to 7 g/L of butanol production after 116 h of culture. This concentration was the highest among values from non-detoxified biohydrolysates and hydrolysates from separate hydrolysis and detoxification, work carried out in the context of this thesis. Furthermore, it is worthy to note that a butanol concentration of 6 g/L was produced from hemicelluloses obtained by hydrolysis of the pre-hydrolysate as the sole treatment method. This performance was higher than those with synthetic xylose medium (5.27 g/L), acid hydrolysate (2.3 g/L) or with hydrolysate obtained by separate hydrolysis and detoxification (1.05 g/L). As a result, our work has led to a potential strategy which mitigates environmental impact as well as costs of forest bioprocessing biorefineries for butanol production.

# TABLE DES MATIÈRES

# LISTE DES TABLEAUX

# LISTE DES FIGURES

# LISTE DES SIGLES ET ABRÉVIATIONS

| | |
|---|---|
| 1-HBT | 1-hydrobenzotriazole |
| 5-HMF | 5-hydromethylfurfural |
| ABE | Acetone-Butanol-Ethanol |
| ABTS | Acide 2,2'-azino-bis-(3-éthylbenzothiazoline-6-sulfonique) |
| ADN | Acide désoxyribonucléique |
| AFEX | Ammonium Fiber Expansion |
| ARN | Acide ribonucléique |
| ATCC | American Type Culture Collection |
| ATP | Adénosine triphosphate |
| BD | Becton, Dickinson and Company |
| *B. licheniformis* 77-2 | *Bacillus licheniformis* 77-2 |
| *C. acetobutylicum* | *Clostridium acetobutylicum* |
| *C. beijerinckii* | *Clostridium beijerinckii* |
| *C. flavigen* PR-22 | *Cellulomonas flavigena* PR-22 |
| *C. taiwanensis* | *Cupriavidus taiwanensis* |
| CMCase | Carboxyméthyle cellulase |
| cP | Centipoise |
| DSM | Deutsche Sammlung von Mikroorganismen |
| *E. coli* | *Escherchia coli* |
| FPase | Enzymes de dégradation de papier filtre |
| MIC | Concentration Minimale Inhibitrice |
| NADH | Nicotinamide adenine dinucleotide |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| NCIMB | National Collection of Industrial Food and Marine Bacteria |
| *P. campinasensis* | *Paenibacillus campinasensis* |
| *P. curlanolyticus* B-6 | *Paenibacillus curlanolyticus* B-6 |
| Q-PCR | Quantitative Polymerase Chain Reaction |
| *S. cerevisae* | *Saccharomyces cerevisae* |
| TSB | Tryptic Soy Broth |
| *U. thermosphaericus* | *Ureibacillus thermosphaericus* |
| # cat | catalog number |

# CHAPITRE 1 : INTRODUCTION

L'industrie des pâtes et papiers, qui représente une part importante de l'économie canadienne, a été confrontée, ces dernières années, à plusieurs difficultés. Entre autres, on retrouve la vive compétition mondiale, l'augmentation du coût de l'énergie et la diminution de la demande pour des produits traditionnels à base de fibres de bois. Aussi, l'intégration d'une unité de bioraffinerie au sein de ces industries, via l'extraction des hémicelluloses et leur conversion en bioproduits à haute valeur ajoutée, est une solution prometteuse qui permettrait à cette industrie de diversifier l'éventail de produits et ainsi de générer de nouvelles sources de revenus. La production de pâtes à papiers peut être réalisée par voies chimique, mécanique ou hybride. Le procédé Kraft représente la fraction majeure des procédés chimiques de mise en pâte au Canada [3] et constitue la technique dominante grâce à la possibilité de récupération de produits chimiques employés et à la haute qualité de la pâte produite. La pâte dissoute, faisant partie des produits du procédé Kraft, est caractérisée par son excellent niveau de pureté en cellulose qui excède 95% [4]. Une étape de préhydrolyse peut être intégrée au procédé de production en amont de la digestion pour permettre la séparation de la lignine des fibres de cellulose et l'extraction des hémicelluloses qui peuvent être converties en biocarburants, polymères et divers produits chimiques [5]. Le butanol, un carburant de remplacement de pétrole, peut être produit à partir des hémicelluloses via fermentation ABE. Ce biocarburant a suscité l'intérêt des communautés scientifique et industrielle grâce à ses différentes caractéristiques dont la possibilité de mélange à des pourcentages élevés avec de la gazoline, un pouvoir énergétique élevé, une faible volatilité, etc. [6]. La voie biologique de sa production utilise des microorganismes spécifiques qui assimilent les glucides simples comme source de carbone. La bactérie *Clostridium acetobutylicum* ATCC 824 est une souche reconnue pour sa capacité à assimiler à la fois les hexoses et les pentoses de la biomasse lignocellulosique pour la production de butanol [7]. L'étape de préhydrolyse permet une dépolymérisation partielle des polysaccharides contenus dans les hémicelluloses en oligo- et mono-saccharides. Pour la génération de glucides fermentescibles, une étape supplémentaire d'hydrolyse est nécessaire. Cependant, l'implantation de tels procédés à grande échelle présente certains défis. En effet, la majorité des procédés employés pour le prétraitement et l'hydrolyse permettant la conversion des hémicelluloses en glucides fermentescibles sont peu efficaces, sophistiqués, coûteux, polluants, etc. Ces procédés, employant dans la majorité des cas des conditions opératoires extrêmes (hautes températures et pression, réactifs chimiques, etc.) sont à l'origine de la formation de composés

inhibiteurs de fermentation. Des méthodes de détoxification comme la filtration nanomembranaire, la floculation, le chaulage et l'utilisation de charbon actif sont souvent utilisées pour réduire les teneurs en inhibiteurs. Mais une perte importante des glucides accompagne l'application de ces méthodes en plus de leur faible performance d'élimination d'une panoplie d'inhibiteurs. Il serait alors intéressant de trouver des alternatives novatrices limitant les coûts tout en augmentant l'efficacité de détoxification, afin de favoriser une implantation industrielle efficace. L'application de microorganismes à ces fins a récemment démontré une performance élevée de détoxification d'un grand éventail d'inhibiteurs visés (aldéhydes furaniques, composés phénoliques, acides organiques, etc.) d'une manière environnementale et économique [8]. Toutefois, les approches utilisées exigent des durées prolongées de traitement, résultent en une consommation importante de glucides, qui ne sont alors plus disponibles à la fermentation, et présentent une faible capacité de dégradation à des concentrations élevées en inhibiteurs. La souche *Amorphotheca resinae* ZN1, à titre d'exemple, requiert 4 jours pour éliminer 1 g/L de furfural [9]. La performance de dégradation de ce composé inhibiteur (i.e. furfural) par la souche *Bacillus cereus* était limitée à 50% d'une concentration initiale de 0.5 g/L [10]. Pour combler les lacunes identifiées, le développement d'un procédé de conversion des hémicelluloses qui a un faible impact sur l'environnement, économique en termes de ressources et de coûts et présentant une possible rentabilité pourrait susciter l'intérêt industriel.

Cette thèse a donc cherché à contribuer au développement d'une approche novatrice de détoxification de préhydrolysat de bois par voie biotechnologique, afin de minimiser l'incidence environnemental du processus de détoxification de même que d'en réduire les coûts, en œuvrant à l'amélioration de la performance d'hydrolyse bactérienne favorisant la production de butanol par *C. acetobutylicum* ATCC 824.

Cette thèse comprend huit chapitres. Une introduction générale précisant les motivations, la problématique ainsi que l'objectif principal de ce projet constitue le chapitre 1. Au chapitre 2, une revue de littérature détaillée est présentée. Au chapitre 3, les sous-objectifs spécifiques qui découlent de l'objectif principal de la thèse sont énumérés suivi de la méthodologie développée pour atteindre ces objectifs. Finalement, les publications issues de cette thèse sont présentées. Les chapitres 4, 5 et 6 présentent les résultats, distribués en trois manuscrits scientifiques. Au chapitre 7, une discussion générale des différents résultats obtenus est présentée, et s'en suit la conclusion générale ainsi que les recommandations (Chapitre 8).

# CHAPITRE 2 : REVUE DE LITTÉRATURE

Le chapitre de revue de littérature est divisé en quatre parties. La première partie décrit les principaux procédés de production des pâtes. La deuxième partie introduit la notion de bioraffinerie forestière industrielle. Dans la troisième partie, les produits à haute valeur ajouté, plus particulièrement les biocarburants, qui sont issus de la bioraffinerie sont décrits, puis les différents procédés de conversion des hémicelluloses en ces produits d'intérêt sont détaillés. La dernière partie résume les différents points abordés dans la revue de littérature développée et y précise les limitations des connaissances scientifiques et des développements technologiques.

## 2.1 Procédés de production des pâtes

Il existe différents procédés de production de pâtes dans l'industrie des pâtes et papiers :

### 2.1.1 Procédé mécanique de production des pâtes

Le procédé mécanique de production des pâtes représente 25% de la production des pâtes dans le monde [11]. Ce procédé a recours à des méthodes mécaniques pour la génération des fibres et les pâtes produites sont à fortes teneurs en hémicellulose et lignine en comparaison avec les procédés chimiques [12]. Il existe généralement quatre classes des technologies mécaniques pour la production des pâtes :

#### 2.1.1.1 Mise en pâte par broyage du bois

Cette méthode qui consiste à broyer la biomasse forestière contre une meule à rotation offre les avantages du rendement élevé de production des pâtes [13], faible coût, papiers volumineux, absorbants et à grande opacité [12]. Cependant, les fibres produits par ce procédé sont souvent courts et très fragiles [13].

#### 2.1.1.2 Mise en pâte par raffinage

Ce procédé, développé en 1950 [12], consiste à broyer le matériel lignocellulosique entre deux disques rainurés. Cette technique est avantageuse par rapport au procédé de broyage grâce à la génération des fibres plus forts et longues [13]. Cependant, en comparaison avec le procédé par

broyage, cette technique est plus consommatrice d'énergie et les papiers produits sont moins opaques [12].

#### 2.1.1.3 Mise en pâte par voie thermomécanique

Cette technique consiste tout d'abord à cuire les coupeaux de bois à la vapeur puis les broyer comme dans le cas du procédé par raffinage. La qualité supérieure des pâtes ainsi que les rendements élevés de production (aux alentours de 95%) s'insèrent parmi les avantages de cette technologie qui est le procédé mécanique le plus utilisé. Mais, la demande plus élevée d'énergie [13], la faible génération des fibres longues, et la faiblesse des pâtes réduisent la faisabilité de ce procédé [12].

#### 2.1.1.4 Mise en pâte par voie Chimio thermomécanique

Cette méthode intègre l'application des produits chimiques comme le sulfite de sodium en amont de l'étape de raffinage. Telle intégration améliore le nombre et la qualité des fibres (longs, rigides, résistants, etc.) produits. Cependant, une consommation élevée d'énergie accompagne cette voie de production [12, 13].

Dans le but d'élargir la production aux papiers de qualité supérieure en termes de rigidité, luminosité, résistance, etc. les procédés chimiques de production des pâtes sont majoritairement utilisés par les industriels.

### 2.1.2 Procédé chimique de production des pâtes

Les fibres de la biomasse lignocellulosique sont libérées par le recours aux réactifs chimiques. Ce procédé représente 70% des procédés de production des pâtes en Amérique du nord [11]. La consommation élevée des produits chimiques ainsi que les rendements faibles de la production des pâtes constituent les majeurs inconvénients associés à ce type de traitement [14]. Il existe deux types des procédés chimiques :

#### 2.1.2.1 Procédé à base de sulfites

Le procédé à base de sulfites utilise le dioxyde de soufre comme réactif principal pour la délignification du matériel lignocellulosique [12]. L'addition de ce réactif contribue à la formation des lignosulfonates dans une première étape. Par la suite, une étape de lavage permet d'éliminer

facilement les lignosulfonates solubles [15]. Les fibres de cellulose séparées subissent après une étape de blanchiment puis de séchage pour la production de pâte commercialisée ou acheminée aux unités de production des papiers. L'application de ce procédé est limitée par l'absence d'une unité de récupération des réactifs chimiques. De plus, la pâte produite est faiblement rigide.

#### 2.1.2.2 Procédé Kraft

Ce procédé représente 95% des procédés chimiques. Il consiste à utiliser une solution composée des réactifs chimiques $Na_2S$ et NaOH pour séparer la fraction de lignine des fibres de cellulose à haute température. Une étape de lavage en aval permet la génération de deux sortes des effluents : la pâte qui est par la suite blanchie puis finalement séchée et un effluent liquide désigné « la liqueur noire » qui est composée principalement de la lignine et des hémicelluloses. La liqueur est par la suite concentrée [11] puis brûlée dans une chaudière de récupération pour d'une part générer de l'énergie [11, 15] et d'autre part récupérer les solvants qui sont réutilisés dans la digestion des coupeaux de bois [11].

En comparaison avec le procédé à base de sulfites pour la production des pâtes, le procédé Kraft offre l'avantage d'utilisation d'une large gamme de biomasse forestière [12], de récupération des produits chimiques et leur réutilisation [11, 12, 15] pour séparer la lignine lui permettant d'acquérir un privilège économique. De plus, les pâtes générées par ce procédé sont plus blanches et sont caractérisées par leur robustesse [11]. Ces attraits ont suscité l'intérêt de plusieurs industriels. En fait, le procédé Kraft est devenu le plus dominant pour la production des pâtes dans le monde [15].

### 2.1.3 Procédé hybride de production des pâtes

Dans le but d'augmenter les rendements de mise en pâte et de rehausser la qualité de la pâte produite, les méthodes chimiques et physiques conventionnellement appliquées sont combinées. En effet, les copeaux de bois subissent initialement une étape de cuisson dans laquelle des réactifs chimiques sont utilisés. Dans une deuxième étape, la pâte issue est mécaniquement manipulée [12, 16]. Cependant, une telle combinaison augmente les coûts de production des pâtes en raison d'une consommation accrue de l'eau, de l'électricité, de l'énergie, etc. De plus, le volume élevé des effluents générés par une telle combinaison représente un obstacle majeur à la faisabilité de ce procédé de production des pâtes.

En se basant sur les caractéristiques des différentes méthodes de production des pâtes, les procédés chimiques et plus spécifiquement le procédé Kraft semble être le plus avantageux pour la production des pâtes et papiers. Cependant, la liqueur noire issue de l'étape de digestion et qui est brûlée pour la génération de l'énergie, contient, en plus de la lignine, les hémicelluloses. Ces polysaccharides sont connus par leur faible pouvoir calorifique en comparaison avec la lignine. L'énergie produite par la combustion des hémicelluloses est donc plus faible et ne représente que 25% de l'énergie totale produite par la combustion de la liqueur noire [5]. Pour augmenter la rentabilité économique du procédé de production des pâtes, l'intégration d'une étape d'extraction des hémicelluloses dans le procédé Kraft est recommandée [11, 17].

## 2.2 Développement de la bioraffinerie industrielle

La bioraffinerie industrielle était définie par « Biorefinery Euroview » comme étant une industrie intégrée qui permet la production d'une grande variété des produits à haute valeur ajoutée entre autres des produits chimiques, des biocarburants, des biomatériaux, des produits alimentaires ainsi que de l'énergie par l'intermédiaire de diverses technologies qui utilisent la biomasse comme matière première [18].

L'intégration des procédés de bioraffinerie au sein de l'industrie produisant la pâte Kraft présente plusieurs avantages entre autres l'infrastructure déjà existantes, la bonne expertise et l'efficacité énergétique au sein de l'usine réceptrice, la proximité des sources de la biomasse, etc. [4]. Pour la valorisation des hémicelluloses, plus faible pouvoir calorifique que la lignine, la bioraffinerie intègre une étape d'extraction en amont de cuisson des copeaux de bois [17] pour la conversion des hémicelluloses en une grande variété des bioproduits.

L'extraction des hémicelluloses peut être réalisée par la préhydrolyse [4]. En fait, le procédé kraft intégrant une étape de préhydrolyse a représenté 56% de la production mondiale de la pâte dissoute à la fin de 2014. Dans ce procédé, les hémicelluloses de bois ainsi qu'une fraction de la lignine sont initialement dissoutes grâce à l'autohydrolyse par la libération des groupements acides [19].

Il existe différents types de prétraitement développés pour l'extraction des hémicelluloses. La sélection entre les différents procédés dépend non pas seulement des critères exigés de la production des pâtes (rendement et qualité) mais également des types des produits finaux à partir de la conversion des hémicelluloses [4].

## 2.3 Conversion des hémicelluloses en produits à haute valeur ajoutée

La fraction des hémicelluloses représente 20-35% de la biomasse lignocellulosique [20] et est classée le deuxième polymère le plus abondant dans la nature après la cellulose [21]. Il s'agit d'un hétéropolysaccharide très ramifié qui est composé d'une grande variété des sucres dont le degré de polymérisation est plus faible en comparaison avec la cellulose [4]. Le xylane représente la fraction majeure des hémicelluloses. Il s'agit d'un polymère des monomères de xylose qui sont liés par des liaisons glucosidiques de type β-(1,4) suivant une configuration linéaire. Les chaines latérales de ce polymère peuvent être composées de glucose, galactose, mannose, arabinose, groupements acétyles, acide férulique, acide O-méthyle-4 glucuronique ou d'acide p-coumarique. La distribution des sucres dans les hémicelluloses et leur degré de polymérisation varient selon le type de la biomasse. En effet, les hémicelluloses dans le bois dur contiennent majoritairement des pentoses et le degré de polymérisation des sucres est compris entre 100 et 200 alors que les hexoses composent la majorité des sucres dans le bois tendre et le degré de polymérisation dans cette biomasse varie entre 70 et 130 [22-26]. La structure des hémicelluloses lui confère des propriétés intéressantes entre autres la solubilité, la tensioactivité, les activités physiologiques, etc. qui élargissent les domaines de ses applications entre autres l'énergie (biocarburants, etc.), l'industrie chimique (furfural), l'industrie alimentaire (xylitol), l'industrie pharmaceutique (xylooligosaccharides), etc. Les hémicelluloses sont donc considérées des polysaccharides attrayants plusieurs industriels [24, 27, 28]. La production des biocarburants renouvelables à partir des hémicelluloses de bois représente une alternative très prometteuse aux carburants fossiles.

### 2.3.1 Les biocarburants comparés aux carburants fossiles

L'utilisation excessive de ces dernières contribue au réchauffement climatique [29] par effet des gaz à effets de serre dégagés. En effet, les émanations des gaz à effet de serre issues de la combustion des carburants représentent 65% des émissions globales [30] et leur libération des usines de sables pétrolifères au Canada est prévue d'augmenter de 55 millions de tonnes en 2011 à 101 millions de tonnes en 2020 [31]. De plus, l'augmentation de la consommation de l'énergie au Canada peut induire l'épuisement des ressources en combustibles fossiles [32]. Maity et al. ont estimé la disparition des réserves de pétrole après 2050 [33]. Pour contrecarrer à ces problèmes, le développement des sources d'énergie renouvelables (i.e. Biocarburants), durables et respectueuses à l'environnement représente une meilleure alternative [30, 33]. Les biocarburants peuvent être

classés en trois groupes : Première, deuxième et troisième génération [34-36]. L'éthanol, le biodiesel et le biogaz font partie de la première génération et sont produits à partir des substrats comestibles comme l'amidon, les lipides et les sucres [34]. Mais, la compétition aigue avec l'alimentation et les terres cultivables induit une augmentation des coûts des produits alimentaires [35]. Les biocarburants de deuxième génération sont produits à partir de la biomasse lignocellulosique qui est moins coûteuse, renouvelable, abondante et pas comestible. Des résidus agricoles, industriels et forestiers et des récoltes lignocellulosiques sont des exemples de la biomasse lignocellulosique [37]. Tandis que les plantes aquatiques (i.e. algues) représentent des sources potentielles des biocarburants de troisième génération (biobutanol, bioéthanol et biogaz) grâce à leur performance élevée de production de biomasse et leur caractéristique non compétitive à l'alimentation et aux terres cultivables [34]. Mais, les étapes de récolte de la biomasse et de la production [38] et de l'extraction des hydrocarbures sont coûteuses [39]. De plus, une consommation importante d'énergie thermique est nécessaire pour le séchage des algues [38]. Les biocarburants de deuxième génération issus des biomasses abondantes et renouvelables, représentent alors une option avantageuse au niveau économique et environnemental. Le biobutanol, en particulier, a suscité l'intérêt de la communauté scientifique et industrielle comme biocarburant prometteur grâce à ses différentes caractéristiques (possibilité de mélange à des pourcentages élevés avec gazoline, pouvoir énergétique élevé, faible volatilité, etc.).

### 2.3.2 Butanol : caractéristiques et voies de production

Le butanol est un alcool à quatre atomes de carbone de formule moléculaire $C_4H_9OH$ (Figure 2-1). Il est caractérisé par sa grande miscibilité dans les solvants organiques [40]. De plus, cet alcool entre dans la formulation des liquides des freins et est appliqué dans les revêtements de surface et les laques. Il est également considéré comme un carburant liquide supérieur à l'éthanol qui permet de remplacer la gazoline [41] grâce à sa densité énergétique plus élevée de 30% [42], son degré de ressemblance élevé avec la molécule de gazoline ouvrant ainsi l'opportunité de mélanger ce biocarburant avec la gazoline jusqu'à 100% [43] comparé à un pourcentage maximal de 15% dans le cas de l'éthanol, sa faible hygroscopicité et sa faible pression de vapeur qui facilite son entreposage dans des canaux de transport [41, 44, 45].

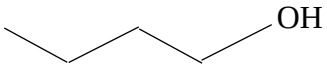



Figure 2-1 : Structure chimique de butanol.

Le butanol peut être produit par voie chimique ou biologique. Le procédé chimique courant de requiert des dérivés de pétrole comme substrat comme le procédé de synthèse oxo. Ce procédé consiste à réagir la double liaison C=C d'un propylène synthétique avec le monoxyde de carbone et l'hydrogène en présence des catalyseurs Co, Rh, ou Ru suivie d'une étape finale d'hydrogénation [46] (Figure 2-2). Mais le butanol synthétique peut également être produit à partir de l'éthanol via trois réactions chimiques. La première est une déshydrogénation de la molécule d'éthanol en acétaldéhyde. Une condensation aldolique représente la deuxième réaction et qui permet de produire un β-hydroxybutaraldéhyde suivie d'une déshydratation pour donner crotonaldéhyde qui est un aldéhyde insaturé. La dernière réaction impliquée dans la production de butanol est une hydrogénation du produit de condensation pour une solubilisation plus élevée en milieu aqueux. Les liaisons C=C sont généralement plus réactives aux réactions d'hydrogénation que les liaisons C=O pour des considérations thermodynamiques [47]. Les différentes étapes du procédé chimique de production de butanol à partir de l'éthanol sont présentées dans la Figure 2-3.

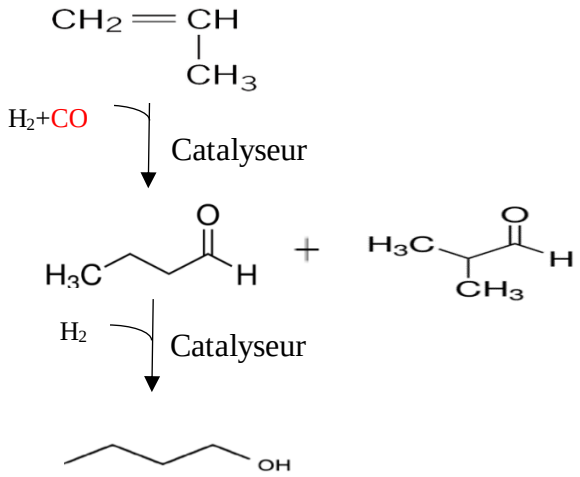



Figure 2-2 : Voie chimique de production du butanol par oxosynthèse [46].

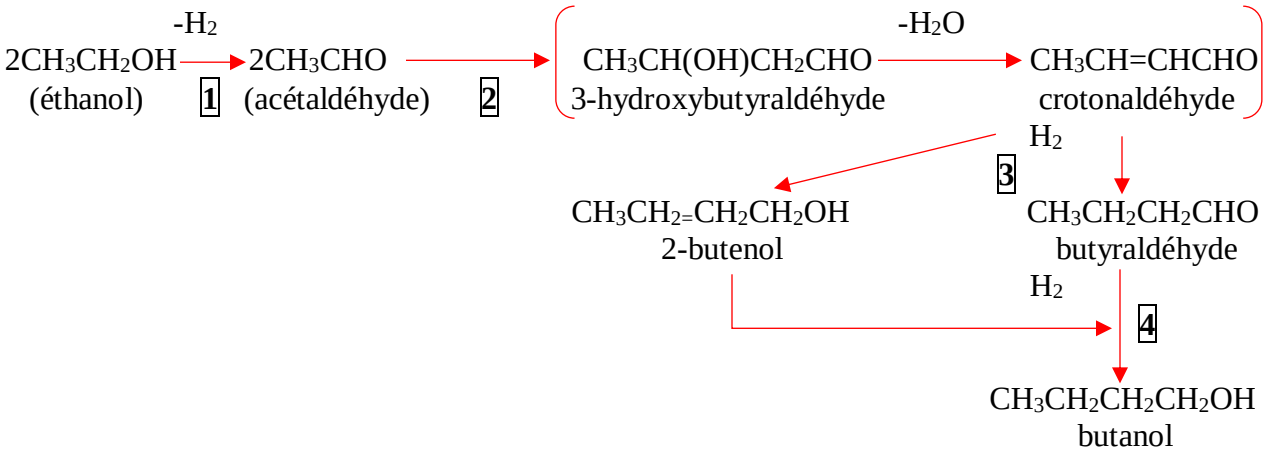



Figure 2-3 : Voie chimique de production de butanol à partir de l'éthanol.

Des fluctuations importantes des coûts du butanol synthétique limitent l'applicabilité de cette voie [45]. La production de butanol par le développement des procédés de fermentation anaérobique est une excellente alternative grâce à la disponibilité, l'abondance et l'aspect renouvelable de la biomasse lignocellulosique utilisée comme matière première. Différentes espèces de *Clostridia* solvantogéniques ont été utilisées pour la conduite de la fermentation désignée par la fermentation Acétone-Butanol-Éthanol (ABE) [42]. Cette désignation renseigne sur les trois solvants produits par les souches d'intérêt dans les proportions respectives de 3:6:1 [48]. Une fermentation ABE typique est composée de deux phases distinctes : Une phase d'acidogenèse pour la production des acides acétique et butyrique à partir des sucres consommés et une phase de solvatogenèse caractérisée par la réassimilation des acides produits pour la production des solvants organiques [49]. La Figure 2-4 suivante représente la voie métabolique de production de butanol à partir de xylose par voie biologique avec *C. acetobutylicum*.

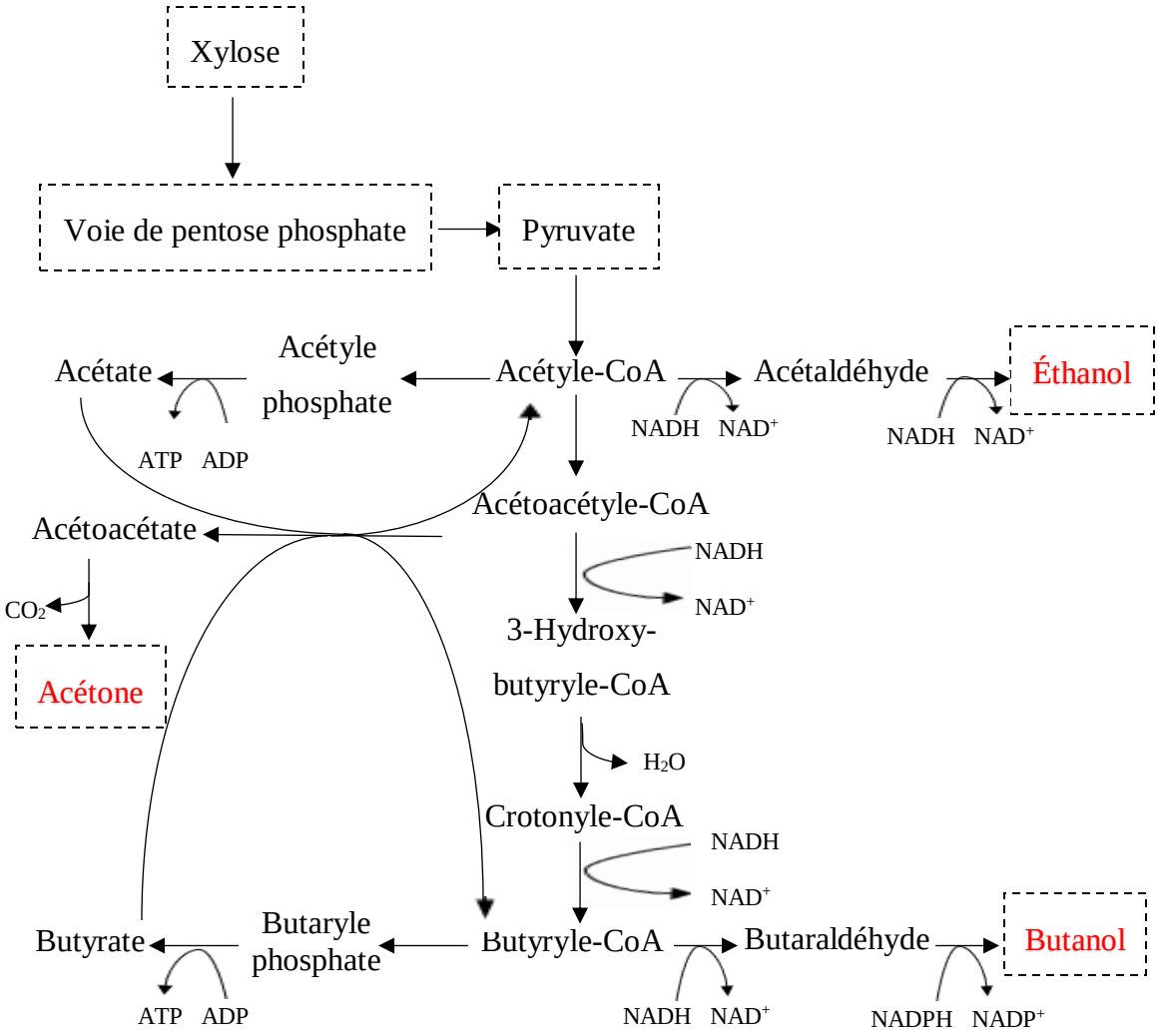



Figure 2-4 : Voie métabolique de *C. acetobutylicum* produisant acétone, butanol et éthanol à partir de xylose utilisé comme source de carbone.

### 2.3.3 Caractéristiques des souches productrices de butanol

Les souches *C. acetobutylicum* et *C. beijerinckii* sont les plus investiguées pour la conduite de la fermentation ABE [42]. Le séquençage complet de la souche *C. acetobutylicum* et son potentiel de développement de plusieurs voies de dégradation des polymères rendent de cette bactérie un meilleur modèle pour des études futures. *C. acetobutylicum* est une bactérie Gram+ isolée de sources naturelles comme les eaux usées, les racines légumineuses et le seigle [50]. Elle contient un large plasmide qui regroupe les gènes de solvantogenèse [51]. Cette souche est fortement mobile grâce à la présence de flagelle. Cette morphologie favorise non pas seulement une résistance élevée aux protéases mais également une propulsion vers les sucres et les acides améliorant ainsi la

consommation de ces derniers [52]. Une résistance aux protéases évite les risques de perte de conformation des protéines composant le flagelle et empêche alors le dysfonctionnement de cette dernière. Les propriétés de résistance aux protéases et de propulsion vers le substrat ont été attribuées selon Lyristis et al. [52] à la conformation glycosylée du flagelle. De plus, les flagelles glycosylés peuvent être plus stables dans des conditions environnementales extrêmes (e.g. acide). Une corrélation entre la mobilité de la souche et son potentiel de production des solvants a été démontrée dans des études antérieurs et une production plus élevée était observée avec un inoculum à forte motilité. Une perte de motilité de *C. acetobutylicum* était associée à une perte de production des solvants. Selon Lyristis et al. [52], la forte corrélation entre la présence de flagelle et la production des solvants a été associée à la présence des circuits génétiques de production de flagelline chez *C. acetobutylicum* qui sont corrélés à l'expression des gènes solventogénique. Le potentiel de consommation simultanée de glucose, de xylose et de cellobiose et la tolérance élevée aux inhibiteurs de la fermentation par *C. acetobutylicum* s'ajoutent aux atouts de cette bactérie [52].

La production des sucres fermentescibles issus de la biomasse lignocellulosique est abordée en trois étapes : Une étape de prétraitement qui vise à isoler et solubiliser les différents composants de la biomasse lignocellulosique [53-56]; Une étape d'hydrolyse des polysaccharides et des oligosaccharides en monomères des sucres en utilisant des acides, des enzymes ou des microorganismes spécifiques ; Une étape de détoxification pour se débarrasser des inhibiteurs de fermentation issus des étapes en amont [57].

### 2.3.4 Technologies de prétraitement des hémicelluloses

Il s'agit de dissoudre aussi bien les hémicelluloses que la lignine de la biomasse forestière par rupture des liaisons esters et des liaisons glycosidique [58]. En plus de la séparation des hémicelluloses et de la lignine, les groupements acides liés aux hémicelluloses peuvent être éliminés (groupes uroniques et acétyles, etc.). Il existe différents types de prétraitements couramment appliqués :

#### 2.3.4.1 Prétraitement au sodium

L'hydroxyde de sodium est une base forte largement utilisée dans le prétraitement [58-60] grâce aux rendements élevés de délignification favorisant une hydrolyse efficace des celluloses [59]. En

effet, cette base attaque les complexes des carbohydrates et de la lignine [59] et y rompt les liaisons éthers et esters favorisant ainsi la solubilisation des hémicelluloses et de la lignine. Elle coupe aussi les liaisons intermoléculaires de la lignine [61]. Cependant, cette technique est caractérisée par son coût élevé [59, 60].

#### 2.3.4.2 Prétraitement à l'ammoniaque

L'ammoniac présente l'avantage de récupération facile grâce à sa volatilité élevée, de plus il n'est pas corrosif. Le prétraitement à l'ammoniac favorise des rendements élevés d'élimination de la lignine allant jusqu'à 80% [61]. Les procédés à base d'ammoniac sont souvent présentés par le procédé d'expansion des fibres à l'ammoniac (AFEX) [60]. Cette technique utilise le liquide d'ammoniaque et est caractérisée par son efficacité élevée de prétraitement de la biomasse. En effet, le procédé AFEX consiste à mettre en contact l'ammoniaque avec la biomasse lignocellulosique [61] à une température entre 70 et 100 °C et une pression entre 150–400 psi [62]. Puis une décompression rapide induit l'éclatement de la biomasse permettant d'augmenter la surface accessible, de décristalliser la cellulose, de solubiliser partiellement les hémicelluloses et de dépolymériser la lignine. Bien que le procédé AFEX ne produise pas des inhibiteurs [63] et l'ammoniac est facilement récupéré [64, 65], cette technologie n'est pas efficace pour la biomasse à teneurs élevés de lignine à côté des coûts élevés l'ammoniac et la méthode de sa récupération. Il s'y ajoute le risque environnemental de dépôt de l'ammoniac [63] et la faible solubilisation des hémicelluloses qui pourraient être valorisées en des produits à haute valeur ajoutée [60].

#### 2.3.4.3 Prétraitement à l'acide dilué

Ce procédé est très largement utilisé pour des applications industrielles. Il consiste à mélanger la biomasse lignocellulosique avec l'acide sulfurique dilué (0.4%-1%) pendant quelques secondes à quelques minutes à une température entre 160 °C et 180 °C [56]. Deux fractions sont générées: a) Une fraction liquide composée des sucres, des produits de dégradation (furfural, 5-HMF et composés phénoliques) et des acides organiques (uroniques et acétique) ; b) Une fraction solide composée majoritairement de la cellulose et des fractions de la lignine [57]. Les conditions expérimentales de ce procédé favorisent une dégradation des hémicelluloses en sucres simples tout en évitant l'altération de la cellulose. Elles permettent également une déstructuration de la lignine [56, 66, 67]. Cependant, le prétraitement acide est coûteux, présente un risque de corrosion des

équipements et utilise des températures opérationnelles élevées. Tous ces facteurs engendrent des teneurs élevés des inhibiteurs, à savoir le furfural, le 5-HMF, l'acide acétique, etc. [56, 57, 67].

#### 2.3.4.4 Explosion à la vapeur

Ce procédé consiste au maintien de la vapeur à haute pression suivi d'une diminution brusque de la pression. La décompression explosive des copeaux de bois entraine la solubilisation des hémicelluloses ainsi qu'une petite quantité de la lignine. Cette technologie est caractérisée par son efficacité pour la dissolution des hémicelluloses et par sa faible demande d'énergie [57, 68]. En revanche, une destruction partielle de xylane et une disruption incomplète de la matrice du complexe lignine-carbohydrate caractérisent ce procédé [56]. De plus, l'application des températures et des pressions élevées génère une large gamme des inhibiteurs [57].

Suite à l'étape de prétraitement, des oligosaccharides sont majoritairement obtenus. Des procédés de conversion de ces glucides en des monosaccharides fermentescibles sont nécessaires.

### 2.3.5 Procédés d'hydrolyse des hémicelluloses

Les microorganismes solventogéniques qui produisent le butanol via fermentation ABE exigent des glucides simples comme source de carbone et d'énergie pour leur métabolisme. Et comme l'étape de préhydrolyse dépolymérise les polymères des glucides en des oligomères et des monomères, il est donc nécessaire d'intégrer une deuxième étape d'hydrolyse qui permet la conversion des oligomères des glucides dans le préhydrolysat en des monomères fermentescibles. Cette conversion peut être réalisée par des méthodes chimiques avec des acides ou biologiques avec des enzymes ou leurs microorganismes de production [69].

#### 2.3.5.1 Hydrolyse chimique

Elle est généralement réalisée avec l'acide sulfurique à des températures élevées (100 °C-200 °C) [4]. En effet, les acides pénètrent dans la matrice lignocellulosique et assurent la protonation de l'oxygène dans la liaison éther intermonomèrique, ce qui permet la libération des monomères des glucides [70]. Le recours à des températures élevées pour l'hydrolyse acide augmente la consommation d'énergie et entraine la génération des sous-produits toxiques (e.g. composés phénoliques, furfural, 5-HMF, acide acétique, etc.) qui exige une étape supplémentaire de purification pour achever une production subséquente des biocarburants [71]. De plus, des

problèmes de corrosion des équipements d'hydrolyse par action des réactifs acides sont fréquemment rencontrés. Il s'ajoute à ces limites la génération des volumes importants des effluents contenant l'acide et qui sont néfastes à l'environnement [71, 72]. Dans le but de minimiser les dégâts associés à l'application des acides pour l'hydrolyse des hémicelluloses de bois, le développement de nouvelles technologies d'hydrolyse biologique qui soient rentables, respectueuses à l'environnement, économiques et à moindre génération des inhibiteurs est recommandé [73].

#### 2.3.5.2 Hydrolyse biologique

##### *2.3.5.2.1 Hydrolyse enzymatique*

Pour une hydrolyse complète des hémicelluloses de bois, de structure complexe et hétérogène, en des monomères de glucides, une synergie entre différentes enzymes hydrolytiques à différentes mécanismes d'action est requise [74], à savoir endo-xylanase, exo-xylanase, β-xylosidase, arabinofuranosidase, acétyle xylène estérase, etc. [75]. Les prinipaux enzymes d'hydrolyse sont présentées dans la Figure 2-5Figure 2-7.

Les enzymes endoxylanases catalysent l'hydrolyse les liaisons xylosidiques de type β-1,4 entre les résidus xylopyranosyles [76] et génèrent des longues chaines des xylooligomères [77]. Selon la base de données CAZy database (http://www.cazy.org), les xylanases sont classifiées en plusieurs familles de glycosides hydrolases (GH) (5, 7, 8, 10, 11, 26, 30 et 43) qui différent par leurs mécanismes d'action, leurs affinités aux substrats et leurs caractéristiques physicochimiques. Les groupes GH10 et GH11 sont les plus investiguées par les chercheurs pour comprendre leurs mécanismes d'action [74]. Pour son activité, le xylanase de la famille GH10 requiert deux résidus consécutifs de xylose qui ne soient pas substitués (par arabinose, galactose, groupes acétyles, etc.). Alors que le xylanase de la famille GH11 demande trois résidus consécutifs de xylose non substitués [78]. Ce type de xylanase est considéré un vrai xylanase grâce à sa spécificité élevée au xylane. Sur le plan structural, le xylanase du groupe GH10 est caractérisée par un poids moléculaire élevé qui dépasse 30 kDa et comprend un domaine catalytique et un domaine non catalytique de liaison au glucide. Tandis que le xylanase de la famille GH11 a un poids moléculaire plus faible (<30kDa) et contient un seul domaine catalytique [74]. Les endoxylanases ont été appliquées dans plusieurs domaines industrielles, à savoir la production des biocarburants, la clarification des jus et des vins, le bioblanchiment, le traitement des déchets, etc. [76, 79].

Les exo-xylanases sont actives sur des substrats naturels à base de xylane et permettent d'hydrolyser les longues chaines des xylooligosaccharides au niveau de leurs extrémités réductrices en courtes chaines et en xylose [75, 80]. Ces enzymes appartiennent à la famille GH8 et elles sont majoritairement produites par des bactéries. Parmi les applications des exo-xylanases, l'expression de ces enzymes originaires de *Aeromonas caviae* ME1 dans *Escherichia coli*. L'hydrolyse enzymatique des xylanes de bois de hêtre avec 50 mg/L de l'exo-xylanase produite a permis de libérer 150 g/L de xylose. Et par action combinée de cette exo-xylanase avec une endo-xylanase, à partir de *Prevotella ruminicola*, introduites à des concentrations équivalentes de 50 mg/L, 330 g/L de xylose ont été obtenues démontrant le synergisme entre les différentes xylanases pour une performance améliorée d'hydrolyse en glucides simples [77].

β-xylosidases sont des exoglycosidases qui hydrolysent les xylooligosaccharides au niveau de leurs extrémités non réductrices en des courtes chaines et en xylose [81, 82]. Ces enzymes sont produites par des moisissures et des bactéries avec une production plus importante avec les moisissures et sont généralement à haut poids moléculaire qui peut dépasser 100 kDa [83]. Une des caractéristiques des β-xylosidases est leur affinité élevée au xylobiose [84]. Leur affinité aux xylooligosaccharides est plus importante avec des faibles degrés de polymérisation de ces glucides. Une autre caractéristique de ces enzymes est leur sensibilité au xylose. La gamme de pH optimum de l'activité de β-xylosidases varie entre 4 et 5 et la température optimale de l'enzyme est souvent à 60 °C [83].

Mais, il est important de préciser que les chaines latérales substituées sur le squelette de xylane (e.g. arabinose, acide férulique, acétyle, acide p-coumarique, etc.) peuvent limiter l'accessibilité des polymères et des oligomères des xylanes aux xylanases. L'utilisation des enzymes accessoires à côté des xylanases est donc requise pour une conversion efficace et complète des hémicelluloses [82]. Les enzymes α-L-arabinofuranosidases appartiennent à la famille GH62 et permettent de libérer les résidus L-arabinose dans le glucide. α-L-arabinofuranosidases catalysent la rupture des liaisons de types α-(1,2), α-(1,3), ou α-(1,5) qui lient les résidus α-L-arabinofuranosyles aux extrémités non réductrices des glucides [85]. À côté des résidus d'arabinose, les hémicelluloses de bois sont en majeure partie acétylées dans leurs chaines latérales rendant difficile leur solubilisation [86]. La désacétylation des glucides acétylés peut être achevée avec les enzymes acétyle estérases ce qui favorise la solubilisation des sucres [87]. Ces enzymes peuvent être produites par *Trichoderma reesei* [88], *Aspergillus niger* [87], *Pyromyces* sp. [89] favorisant par conséquent l'hydrolyse

enzymatique des glucides. De plus, la désacétylation permet de lever l'inhibition de la fermentation alcoolique par les groupes acétyles [87].

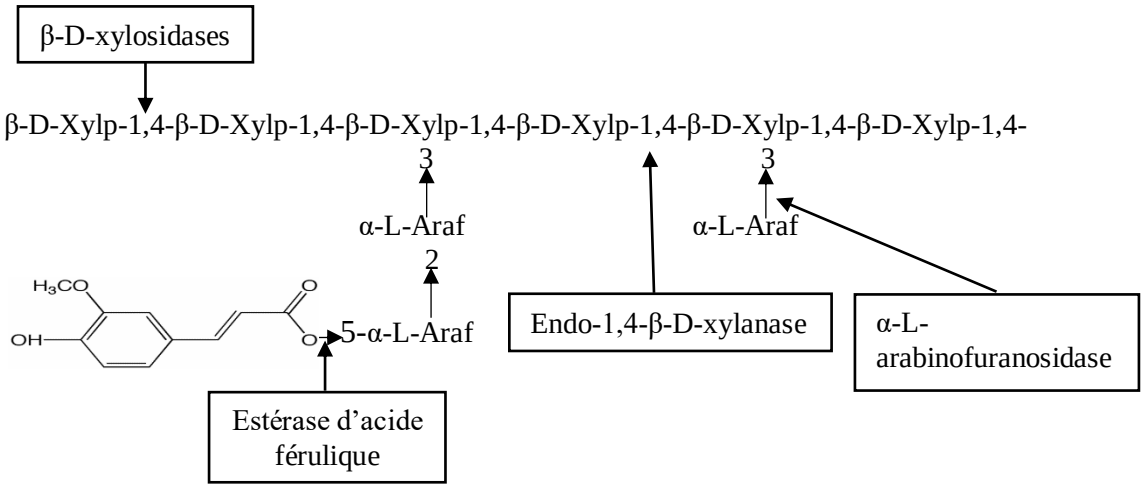



Figure 2-5 : Représentation schématique de l'hydrolyse enzymatique des hémicelluloses.

Certes, les enzymes hydrolytiques représentent une alternative très prometteuse d'hydrolyse des hémicelluloses grâce aux attributs environnementaux et économiques aux procédés de conversion et de valorisation de la biomasse lignocellulosique. Mais, les coûts élevés de production de ces enzymes, de leur extraction, de leur purification et de leur application pour l'hydrolyse du matériel hémicellulosique limitent leurs intérêts industriels [90]. Ainsi, des études d'hydrolyse de la biomasse par application directe des microorganismes productrices des enzymes hydrolytiques seraient profitables.

*2.3.5.2.2 Hydrolyse microbiologique*

Plusieurs microorganismes peuvent utiliser la biomasse lignocellulosique comme source de carbone et d'énergie afin de produire les enzymes d'hydrolyse. L'hydrolyse microbiologique permet d'éviter les étapes de purification exigées dans le cas d'hydrolyse enzymatique. De plus, les microorganismes d'hydrolyse produisent un extrait enzymatique qui peut contenir non pas seulement une variété d'enzymes de décomposition des hémicelluloses mais également des cofacteurs impliqués dans les réactions d'hydrolyse.

*Piromyces* sp. est une champignon anaérobie stricte qui est caractérisée par son potentiel élevé de dégradation de la biomasse lignocellulosique grâce à ses capacités de production de diverses enzymes comme la xylanase (13.37 mIU/mL), la carboxyméthyle cellulase (19.01 mIU/mL),

l'avicélase (5.33 mIU/mL) et l'acétyle estérase (151.5 mIU/mL) après 8 jours de culture et la protéase (4762 mIU/mL) après 16 jours d'inoculation. *Piromyces* sp. est capable d'utiliser le xylose, D-glucose, lactose, D-cellobiose, raffinose, xylane et carboxyméthyle cellulose (CMCase) comme source de carbone. Mais, le maltose, le galactose, le glycogène et l'inuline ne sont pas assimilées par *Piromyces* sp. isolée des ruminants sauvages [89]. En plus de ses capacités d'hydrolyse des glucides, *Piromyces* sp. peut dégrader les composés phénoliques jusqu'à 38% et 35% d'acide p-coumarique après 14 jours d'incubation avec des concentrations initiales respectives de 0.2 g/L et 0.8 g/L. Dans ce même travail, 34%, 67% et 52% de l'acide vanillique ont été éliminées à partir des concentrations initiales de 0.168 g/L, 0.84 g/L et 1.68 g/L respectivement [91]. *Piromyces* sp. est d'un grand intérêt dans la dégradation de la lignocellulose, mais la longue durée de traitement et le métabolisme anaérobie de ce champignon entravent sa mise en culture et sa maintenance et réduisent l'étendue de son application dans les procédés industriels. De plus, le métabolisme de la biomasse lignocellulosique avec *Piromyces* sp. peut générer entre autres l'acétate et la formate qui sont des inhibiteurs de la fermentation ABE [89].

*Trichoderma reesei* est un champignon filamenteux très largement utilisé dans les industries grâce à son potentiel de production des quantités élevées des cellulases et des hémicellulases nécessaires pour l'hydrolyse de la biomasse lignocellulosique [92]. *Trichoderma reesei* permet de produire des endoglucanases, cellobiohydrolases, xylanases, β-xylosidases, β-glucosidase, des enzymes de dégradation de papier filtre (FPase), protéases, estérases. etc. [93]. L'enzyme endo-1.4- β-xylanase 2 (Xyn2) est une xylanase d'un grand intérêt industriel qui est produite par ce champignon. Elle présente une activité optimale au pH entre 4 et 6 et à une gamme de température de 50 °C-55 °C [94]. En utilisant un milieu de mélasse de canne comme source de carbone, *Trichoderma reesei* a produit jusqu'à 36.7 U/mg de xylanase, 3.95 U/mg de β-glucosidase, 0.94 U/mg de FPase et 1.15 U/mg de CMCase [95]. L'hydrolyse par cette souche permet de libérer différents types des sucres réducteurs. En effet, Borin *et al*. [96] a utilisé la bagasse de canne à sucre comme source de carbone à une concentration initiale de 0.5% (w/v) pour le champignon filamenteux *Trichoderma reesei*. Les auteurs ont rapporté une augmentation des concentrations de glucose, xylose, arabinose et galactose dans les milieux inoculés par rapport au milieu de contrôle (milieu non inoculé) jusqu'à des concentrations maximales de 6.8 mg/L (après 12 h d'inoculation), 4.1 mg/L, 1.2 mg/L et 0.1 mg/L (après 24 h d'inoculation) respectivement [96]. Mais, les faibles activités des enzymes hydrolytiques produites et leur faible stabilité ont incité plusieurs chercheurs à procéder aux

transformations génétiques pour rehausser les activités enzymatiques et pour améliorer la thermostabilité des enzymes [92, 97-100].

Dans le travail de Kang *et al.* [101], la souche *Aspergillus niger* a été cultivée dans un support solide de paille de riz pour étudier la production des enzymes hydrolytiques (i.e. cellulases et hémicellulases) dans ce milieu de culture. Les auteurs ont rapporté des productions de xylanase (5070 IU/g), carboxyméthyle cellulase (130 IU/g), β-glucosidase (94 IU/g) et β-xylosidase (176 IU/g) après 5 à 6 jours de fermentation et FPase (19 IU/g) après 4 jours [101]. De plus, *Aspergillus niger* peut produire les pectinases si le culm est utilisé comme source de carbone. Les enzymes α-L-arabinofuranosidase et xylanase sont majoritairement produites par *Aspergillus niger*. Des concentrations maximales de glucose, xylose, arabinose et galactose de 10.4 mg/L, 10.8 mg/L, 7.2 mg/L et 1.8 mg/L respectivement ont été obtenues après 12 h d'inoculation avec le champignon filamenteux *Aspergillus niger* poussant sur 0.5% (w/v) de bagasse de canne à sucre, utilisée comme source unique de carbone [96].

Les espèces de *Paenibacillus* sont couramment isolées et identifiées du sol et des plantes [102]. *P. campinasensis* DSM 21989 est une bactérie anaérobie facultative et Gram-variable qui pousse optimalement à 45 °C et au pH 10. Les cellules épousent la forme des tiges [103]. Les espèces de *P. campinasensis* peuvent pousser sur une large gamme de sources de carbone et d'énergie (L-arabinose, cellobiose, glucose, lactose, maltose, mannose, raffinose, rhamnose, glycérol, avicel, cellulose, amidon, xylane, gélatine, caséine, 3-cyclodextrine, acide propionique, Tween 40, acide acétique, etc.). Leurs capacités hydrolytiques reviennent à la production d'une panoplie d'enzymes entres autres la catalase, la xylanase (41 kDa), 3 cellulases (42, 57 et 86 kDa), une pectinase (28 kDa) et une glucanotransférase de cyclodextrine (38 kDa), produites par *P. campinasensis* BL11 Figure 2-6. Mais elles ne produisent pas des activités ligninase, lipase, oxydase, protéase et uréase [103, 104]. L'activité spécifique maximale de xylanase produite par la souche *P. campinasensis* BL11 et qui a été obtenue après 24 h de fermentation à 37 °C et au pH 8 était de 29,39 IU/mg [105].

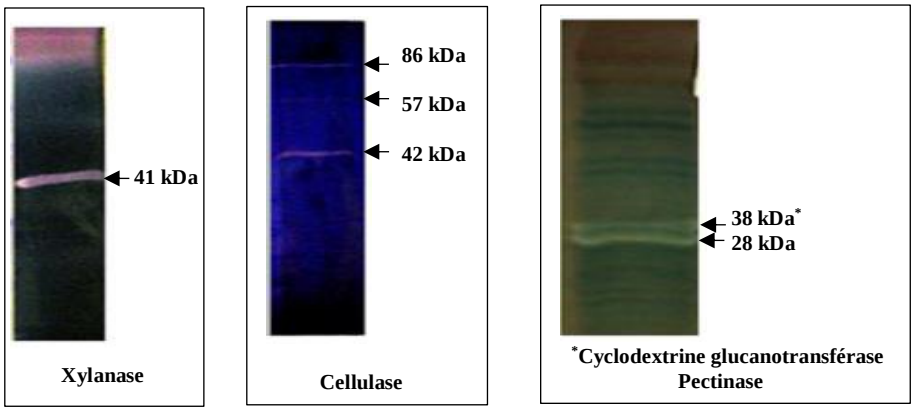



Figure 2-6 : Les hydrolases des polysaccharides produites par la bactérie *P. campinasensis* BL11 (Adaptée de [104]).

La production de xylanase par *P. campinasensis* BL11 est régie par le gène désigné XylX. Parmi ses applications, XylX a été cloné et exprimé dans *E. coli* dans le travail effectué par Ko *et al.* [102]. L'activité de xylanase recombinée purifiée était de 2392 IU/mg, une activité plus élevée que celle obtenue à partir de *Bacillus* spp. et de *Alicyclobacillus* sp. A4. La xylanase produite est caractérisée par deux régions fonctionnelles : Une région catalytique et une région de liaison au carbohydrate. Le xylotriose était le majeur produit d'hydrolyse des xylanes des glumes d'avoine avec une concentration finale de 2.34 g/L après 72 h de traitement par la xylanase. Cette dernière est caractérisée par sa stabilité sur une large gamme de pH (5-9) et de température (45 °C-70 °C) lui rendant un meilleur candidat pour la conversion de la biomasse. Sa stabilité au pH alcalin et à des températures élevées rend de la xylanase adéquate pour le prétraitement de pâte kraft avant le blanchiment [102]. Ko et *al.* [105] ont montré que le prétraitement de pâte kraft avec une activité de xylanase de 2.5 IU/g de pulpe séchée au four a permis d'augmenter la brillance de plus de 4.9% et la viscosité de plus de 0.5 cP. Ce type de prétraitement réduit l'utilisation de dioxyde de chlorine contribuant ainsi à la faisabilité environnementale et économique du procédé [105].

Plus récemment, Zainudin *et al.* [106] ont étudié le compostage de fruit de grappe exempt de l'huile de palmier avec une addition continue des boues anaérobiques des effluents de l'industrie de l'huile de palmier contenant des nutriments et des microbes indigènes. Ces microorganismes ont favorisé la dégradation du matériel lignocellulosique dans une courte durée de compostage passant de 60-90 jours à 40 jours. La rapidité dans le compostage pourrait être attribuée aux natures cellulolytique

et hémicellulolytique des 27 souches identifiées dans l'étude. Les souches étaient dominées par les genres appartenant au phylum de *Frimicutes*, y compris la bactérie *P. campinasensis* BL11 qui a été identifiée comme productrice des cellulases et des xylanases [106]. La rapidité dans la dégradation du matériel lignocellulosique par un consortium contenant la bactérie *P. campinasensis* BL11 démontre la performance de cette souche dans la conversion de la biomasse dans un court délai. De plus Ko *et al.* [105] ont démontré dans leur étude que la production de xylanase s'effectue parallèlement à la croissance cellulaire de *P. campinasensis* BL11 similairement au métabolisme de *Bacillus circulans* AB 16 et contrairement aux *B. licheniformis* 77-2 et *P. curlanolyticus* B-6 dont la production de xylanase était observée après 24 h et 36 h d'inoculation respectivement [105].

Durant les étapes de prétraitement et d'hydrolyse des hémicelluloses de bois, employant des conditions opératoires extrêmes (e.g. température et pression élevées, pH, doses des réactifs chimiques, longue durée, etc.), des inhibiteurs sont libérés entres autres des composés phénoliques, furfural, 5-HMF, acides organiques, sels, etc. [107].

### 2.3.6 Classification et mode d'action des inhibiteurs lignocellulosiques

Les composés toxiques inhibent également la fermentation ABE. En effet, ils affectent la croissance des souches solventogéniques ainsi que l'assimilation des glucides par ces microorganismes et la productivités des solvants [108]. Les différents inhibiteurs formés à travers les étapes de conversion de la biomasse lignocelllulosique sont présentés dans la Figure 2-7.

#### 2.3.6.1 Composés phénoliques

Les composés phénoliques issus principalement de la dégradation de la lignine peuvent contenir des acides (acide férulique, acide 4-hydroxybenzoïque, acide vanillique, acide p-coumarique, acide gallique, acide syringique, etc.) des alcools (e.g. catéchol, guiacol, alcool vanillique) et des aldéhydes (e.g. 4-hydroxybenzaldéhyde, vanilline, syringaldéhyde) [109]. Ces composés sont des inhibiteurs potentiels des microorganismes solvantogéniques, des microorganismes d'hydrolyse et de leurs enzymes respectifs via différents mécanismes d'inhibition. Plusieurs travaux de recherche ont démontré que les composés phénoliques sont des inhibiteurs potentiels des enzymes d'hydrolyse de la biomasse lignocellulosique. En effet, Zhai [110] a visé dans sa thèse la compréhension des mécanismes d'inhibition des cellulases avec les composés phénoliques et la

détermination des caractéristiques inhibitrices de ces composés. L'auteur a défini dans son manuscrit deux types d'interactions entre les cellulases et les composés phénoliques : Des interactions non covalentes (e.g. liaisons hydrogène et liaisons hydrophobes) qui sont à l'origine d'une inhibition réversible des enzymes et des interactions covalentes (entre les composés phénoliques oxydés et les chaines peptidiques des enzymes) responsables d'une inhibition irréversible et/ou de désactivation des cellulases [110]. Ces différentes interactions peuvent dépendre non pas seulement de la nature des composés inhibiteurs mais également de la structure des enzymes d'hydrolyse [110, 111]. Les paramètres d'inhibition par les composés phénoliques peuvent inclure le degré de polymérisation de ces composés comme un degré de polymérisation élevé peut entrainer un encombrement stérique qui empêche les interactions avec les enzymes, alors que les composés phénoliques de faible degré de polymérisation sont plus accessibles et présentent une affinité plus élevée aux protéines [110]. En plus des effets d'inhibition par la taille des composés phénoliques, les enzymes d'hydrolyse peuvent également être désactivées et inhibées par effets de la composition chimique de ces inhibiteurs. En effet, l'inhibition de ces enzymes augmente avec le nombre des groupes fonctionnels à la surface des composés phénoliques (e.g. groupes carbonyles, hydroxyles et méthoxy) [112]. La structure des protéines influe également les interactions des enzymes d'hydrolyse avec les composés phénoliques. En effet, la présence des groupes aromatiques dans la structure des acides aminés et des groupes hydrophobes à leurs surfaces agit significativement sur le degré d'affinité de ces protéines aux composés phénoliques [110]. L'hydrolyse de la bagasse de la canne à sucre à faibles teneurs en composés phénoliques (<10 mg/L) a été achevée par González-Bautista *et al.* [113] avec la carboxyméthyle cellulase et la xylanase produites par *Cellulomonas flavigena* PR-22. Malgré la faible concentration en composés phénoliques dans le substrat, 13% et 26% correspondent respectivement aux activités résiduelles des cellulases et des xylanases après 24 h de saccharification. La supplémentation de 50 mg/L des composés phénoliques dans un extrait enzymatique de *C. flavigena* PR-22 dépourvu des composés inhibiteurs a entrainé une perte de 64% de l'activité des cellulases. Alors que 100 mg/L des composés phénoliques étaient responsables de l'inhibition de 77% de l'activité des xylanases dans l'extrait enzymatique [113]. Le catéchol à 1.1 g/L et l'acide p-coumarique à 3.28 g/L ont entrainé une inhibition complète de la croissance de *Piromyces* sp., bactérie productrice des enzymes lignocellulolytiques (i.e. carboxyméthyle cellulase, xylanase, β-glucosidase et acétyle estérase). Paul *et al.* [91] ont investigué les effets des concentrations des monomères des composés

phénoliques sur la production des enzymes lignocellulolytiques par *Piromyces* sp. et ont observé une inhibition des différentes enzymes à partir des faibles concentrations de catéchol, 0.1 g/L. Une concentration de l'acide p-coumarique de 0.2 g/L était suffisante pour inhiber la production de xylanase, acétyle estérase et carboxyméthyle cellulase et une inhibition complète des activités de xylanase et de cellulase a été observée en présence de 1.64 g/L de l'acide p-coumarique. Les enzymes carboxyméthyle cellulase, xylanase, β-glucosidase et acétyle estérase n'étaient pas détectables en présence 3.88 g/L de l'acide férulique dans la culture de la souche lignocellulolytique *Piromyces sp.* FNG5 [91]. Les composés phénoliques sont des inhibiteurs potentiels des microorganismes solvantogéniques même à des faibles concentrations. La toxicité de ces composés est associée à leur hydrophobicité. En effet, à pouvoir hydrophobe élevé, les composés phénoliques ciblent entre autres la membrane cellulaire et affectent la perméabilité des cellules ainsi que le ratio lipides par protéines ce qui augmente la fluidité cellulaire [107, 108]. Une destruction des membranes par conséquent entraine la fuite des constituants cellulaires [4, 114] entres autres les protéines, les ions, les ARNs et l'ATP et altère les systèmes de transport des nutriments et des protons. La membrane perd ainsi sa caractéristique de barrière sélective. Les composés phénoliques contribuent également à la formation d'espèces réactives de l'oxygène qui dénaturent les protéines (e.g. enzymes), mutent l'ADN génomique et provoquent l'apoptose [108, 115].

Des similitudes des effets toxiques des inhibiteurs sur la fermentation éthanolique et la fermentation solvantogénique ont été démontrées dans des études antérieures. Des concentrations de 0.2 g/L de l'acide férulique et de seulement 0.02 g/L de l'aldéhyde coniférique étaient inhibitrices de la souche *Saccharomyces cerevisae*. Cette levure a été complètement inhibée en présence d'une concentration de 0.02 g/L de benzoquinone qui était également responsable de l'inhibition de la production de l'éthanol [116]. La fermentation ABE avec les espèces de *Clostridium* est également affectée par les composés phénoliques. En effet, les effets de six types des composés phénoliques (i.e. acide p-coumarique, acide vanillique, acide férulique, acide 4-hydroxybenzoique, vanilline et syringaldéhyde) sur la fermentation avec *Clostridium beijerinckii* ont été testés. À une concentration initiale de 1 g/L de ces composés phénoliques, la croissance cellulaire était inhibée de 64% à 74% et la production de butanol était complètement inhibée. Les acides férulique et p-coumarique ont été démontrés les plus inhibiteurs de la bactérie et une incapacité de production des solvants était observée à des faibles concentrations de ces acides de

0.3 g/L et 0.5 g/L respectivement. Des concentrations des composés phénoliques totaux au-delà de 2.1 g/L ont été potentiellement inhibitrices de la production des solvants par *C. beijerinckii*. Les composés phénoliques, même à des faibles concentrations, affectent également la consommation des sucres par les souches solventogéniques. En effet, une concentration initiale des phénoliques de seulement 0.74 g/L a contribué à la réduction de l'assimilation de glucose par *C. beijerinckii* de 100% dans un milieu de contrôle contenant le glucose à 92.7% [108].

#### 2.3.6.2 Aldéhydes furaniques (furfural et 5-HMF)

Le furfural et le 5-HMF proviennent des réactions de déshydratation des pentoses et des hexoses respectivement qui interviennent aux conditions opératoires sévères lors des étapes de prétraitement et d'hydrolyse chimique. Des acides formique et lévulinique sont formés par dégradation supplémentaire des aldéhydes furaniques [117]. Ces aldéhydes furaniques font des dégâts mineurs sur les enzymes d'hydrolyse [111]. Le furfural et le 5-HMF peuvent avoir des effets stimulateurs ou inhibiteurs de la fermentation ABE. En effet, la croissance de *C. beijerinckii* BA101 sur deux milieux contenant séparément 2 g/L de HMF et de furfural a montré une augmentation de la croissance cellulaire de 14% et de 7% respectivement par rapport à la croissance sur un milieu de contrôle composé de glucose comme source unique de carbone. Dans le travail de Ezeji *et al.* [107], la concentration des solvants ABE dans un milieu contenant 0.5 g/L à 1 g/L de HMF a augmenté de 19% par rapport au contrôle à base de glucose jusqu'à une concentration finale de 21.2 g/L. Une augmentation des concentrations initiales de furfural et de HMF à 2 g/L dans des cultures séparées de *C. beijerinckii* BA101 a permis une hausse dans la production des solvants de 6% et 15% respectivement par rapport au milieu de contrôle [107]. Mais en présence des concentrations élevées de ces aldéhydes, le NAD(P)H et le NADH, des cofacteurs indispensables pour la production des solvants, interviennent dans la réduction des aldéhydes en des alcools qui sont moins toxiques [118]. La transition de la phase acidogénique à la phase solvantogénique est ainsi inhibée. Le furfural et 5-HMF affectent également l'inhibition de la réplication cellulaire [108]. Les aldéhydes furaniques peuvent également inhiber des enzymes glycolytiques et fermentatives nécessaires pour le métabolisme cellulaire comme l'aldéhyde déshydrogénase, la pyruvate déshydrogénase, etc. Des interactions entre le furfural et l'ADN génomique des bactéries et des levures ont été démontrées et des cassures de la double brin de l'ADN en simples brins

étaient observées [117]. La toxicité de furfural et de 5-HMF est plus importante en présence des autres inhibiteurs dans le milieu par effets de synergisme [107].

#### 2.3.6.3 Acide acétique

L'acide acétique provient de l'hydrolyse des hémicelluloses au niveau des groupes acétyles [119]. Sa concentration dans l'hydrolysat de bois peut dépasser 10 g/L et dépend de la biomasse lignocellulosique d'origine ainsi que des types de prétraitement et d'hydrolyse employés. Ils font des dommages mineurs sur les enzymes d'hydrolyse [111]. L'acide acétique inhibe l'accroissement de la masse cellulaire et des concentrations inférieures à 0.5 g/L ont été observées inhibitrices de la croissance de *E. coli* par 50% [117]. L'inhibition par l'acide acétique est initiée par la pénétration de sa forme indissociée dans le cytoplasme pour se dissocier à l'intérieur de la cellule bactérienne en ses protons et ses anions correspondants sans synthèse de l'ATP [108, 117]. Par conséquent, les besoins énergétiques nécessaires pour le maintien du potentiel membranaire sont augmentés [120]. La diminution consécutive du pH inhibe la croissance cellulaire et entravent la transition du métabolisme des souches de la phase acidogène à la phase solvantogène. Les acides faibles participent également dans la réduction de l'accumulation de certains acides aminés dans le cytoplasme [117].

#### 2.3.6.4 Sels inorganiques

Les sels inorganiques contenus dans les hydrolysats hémicellulosiques peuvent provenir de la biomasse lignocellulosique, des réactifs chimiques dans les procédés de prétraitement et d'hydrolyse ainsi que dans les étapes d'ajustement de pH et des réactions de corrosion des équipements de prétraitement et des d'hydrolyse [121]. Les sels peuvent inhiber les xylanases durant l'hydrolyse. En effet, dans le travail de Zheng *et al.* [122], les ions $Fe^{3+}$, $Fe^{2+}$ et $Zn^{2+}$ étaient observés des inhibiteurs potentiels de xylanase secrétée par *Bacillus megaterium* MS941. $Hg^{2+}$, $Pb^{2+}$ and $Cu^{2+}$ sont des inhibiteurs de xylanases produites par *Bacillus halodurans* TSEV1 [123]. Une concentration de 1 g/L de $Hg^{2+}$ a provoqué une inhibition totale de l'activité de xylanase de *P. campinasensis* BL11 exprimée dans *E. coli*. Le mécanisme d'inhibition par ces ions métalliques consiste à leurs interactions avec les acides aminés qui composent le site actif des enzymes [102]. Les nutriments inorganiques sont également toxiques pour les souches solvantogéniques et peuvent affecter la croissance cellulaire, les rendements en solvants, la consommation des sucres, etc. Ils

sont transportés à l'intérieur de la cellule par des mécanismes passifs ou actifs [121] et à fortes concentrations dans le milieu, ils peuvent induire une pression osmotique sévère. Les ions $Na^+$ inhibent les enzymes responsables de la conversion de l'acétate et de butyrate en acétate CoA et butyrate CoA respectivement [124]. Des concentrations de NaCl au-delà de 10 g/L étaient inhibitrices de la culture *C. beijerinckii* BA101 [125]. Et une concentration de 10 g/L de NaCl était responsable de la réduction du taux de croissance de *C. acetobutylicum* de 40%. Mais en présence de la même concentration (10 g/L) en $NH_4Cl$, pas d'inhibition de la croissance cellulaire était observée. En plus de leur potentiel d'inhibition, il est important de noter que les sels minéraux peuvent également participer dans la détoxification du matériel lignocellulosique grâce à leurs implications dans les réactions d'oxydoréduction et dans la formation des radicaux phénoxyles ce qui favorise la dégradation de la lignine [124]. La présence des concentrations élevées des inhibiteurs de la fermentation ABE dans les hydrolysats de bois, utilisés comme source unique de carbone, exige une étape de détoxification dans le but de maximiser la conversion de la biomasse lignocellulosique en butanol par *C. acetobutylicum* ATCC824 [126]. Les composés inhibiteurs doivent être efficacement enlevés avec le minimum de perte des sucres fermentescibles dans le milieu par différents procédés de détoxification [127].

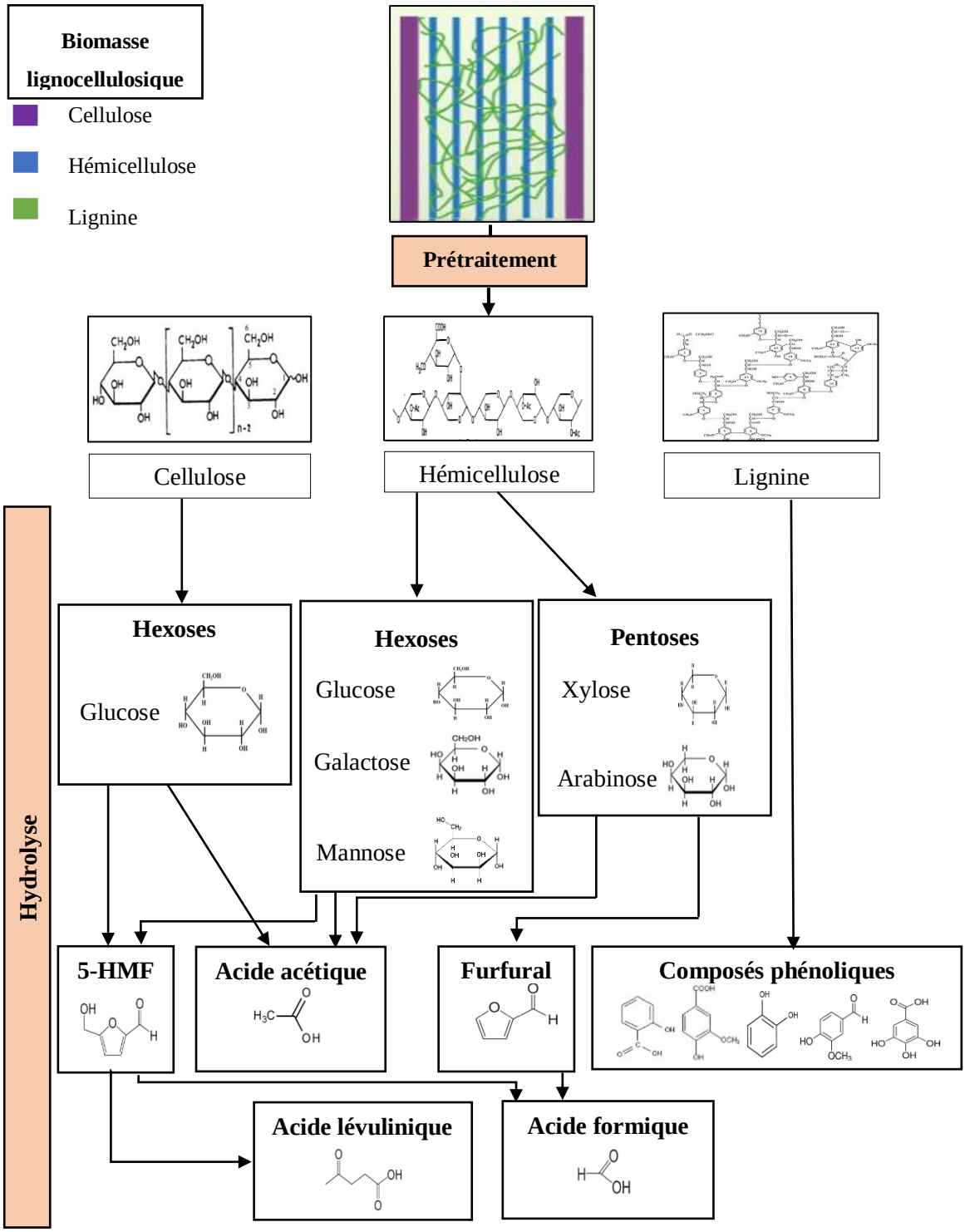



Figure 2-7 : Origine des différents composés inhibiteurs durant la conversion de la biomasse lignocellulosique.

### 2.3.7 Technologies de détoxification de la biomasse lignocellulosique

Différentes méthodes de détoxification ont été utilisées pour la détoxification des hémicelluloses de bois.

#### 2.3.7.1 Le chaulage : « Overliming »

Il s'agit d'augmenter le pH du milieu à 10 par l'addition de $Ca(OH)_2$. Le gypsum, de formule chimique $CaSO_4 \cdot 2H_2O$ qui se forme suite à l'ajout de ce réactif, est enlevé par filtration et le pH est réduit par la suite à une valeur de 6. La détoxification par « overliming » permet à la fois la précipitation des composés toxiques et la déstabilisation de certains inhibiteurs et ce grâce aux variations impliquées au niveau du pH [128]. Une élimination de furfural dans les hydrolysats des déchets de bois jusqu'à 50% a été obtenue grâce à l'application de « overliming » améliorant ainsi la production de l'éthanol (12 g/L) par *Saccharomyces cerevisae* TJ1. La performance de dégradation des composés phénoliques par cette méthode de détoxification est faible ne dépassant pas 41% [129]. L'acide acétique n'est également pas ciblé par ce type de traitement [130]. De plus, une perte des sucres au-delà de 10% s'ajoute aux inconvénients de « overliming » [131]. La formation de gypsum est une contrainte majeure associée à l'utilisation de $Ca(OH)_2$ comme agent de la détoxification. En effet, les couts d'élimination de gypsum dans l'équipement en aval réduit la faisabilité économique du procédé [132].

#### 2.3.7.2 Détoxification par floculation

La floculation est un procédé qui permet l'agglomération des colloïdes. Plusieurs floculant peuvent être utilisés, à titre d'exemple le sulfate ferrique ($Fe_2(SO_4)_3.6H_2O$), l'Alum et le polyéthylenimine, pour la détoxification des différents préhydrolysats (e.g. explosion à la vapeur, prétraitement à l'ammoniaque) [133, 134]. Le mécanisme de floculation consiste en la réduction de la charge électrique suivie de la formation des agglomérats facilement éliminés. La Figure 2-8 représente le mécanisme de floculation des particules en suspension.

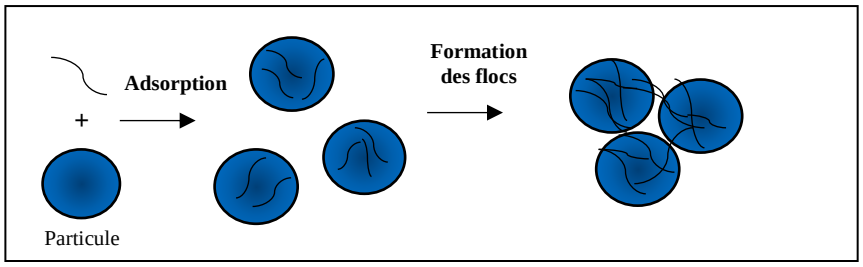



Figure 2-8 : Représentation générale du mécanisme de floculation des particules en suspension [57].

Ajao (2014) a montré dans ses études que la floculation de l'hydrolysat, prétraité par explosion à la vapeur couplée à l'eau chaude, avec le sulfate ferrique comme agent de floculation permet une meilleure dégradation des composés phénoliques jusqu'à 56% avec le minimum de pertes des sucres (<20%). Ces rendements élevés de détoxification ont permis d'améliorer la production des solvants ABE qui a passé de 0.9 g/L dans l'hydrolysat non détoxifié à 6 g/L dans l'hydrolysat floculé avec $Fe_2(SO_4)_3$ [135]. En revanche, cette méthode contribue à une faible élimination de furfural, de 5-HMF et d'acide acétique (< 20%) [4, 136]. En outre, des teneurs élevés des solides sont formés à la suite de la floculation exigeant une étape supplémentaire de filtration. Ce procédé de détoxification n'est donc pas adéquat pour l'implémentation industrielle [136].

#### 2.3.7.3 Détoxification par filtration nanomembranaire

La nanofiltration est une technologie de filtration qui s'insère entre l'osmose inverse et l'ultrafiltration. Elle favorise la rétention des molécules organiques et des protéines. En effet, ce procédé permet la concentration des sucres hémicellulosiques contenus dans les préhydrolysats de bois jusqu'à plus de 99% avec la membrane NF90 [137]. Elle favorise également une détoxification partielle par la membrane NF70 [4, 137, 138]. En effet, la concentration de furfural a été réduite de 66.2 % dans le travail de Qi *et al.* [139] par nanofiltration et aux alentours de 70% dans le travail de Ajao *et al.* [4] en utilisant la membrane NF270. Cependant, la nanofiltration présente moins de performance en termes d'élimination des composés phénoliques (les pourcentages de rétention par NF90 et NF270 varie entre 90% et 100%). Cette faible efficacité d'élimination des composés phénoliques était attribuée selon les auteurs à la formation des liaisons chimiques qui s'établissent entre la lignine et les glucides ce qui limite l'élimination de la lignine [4]. L'encastrement des membranes de filtration causé par les hydrolysats de bois à charge élevée des composés organiques s'ajoute aux limites de l'application de cette méthode [140]. Afin

d'améliorer la performance de détoxification, Ajao *et al.* [133] ont eu recours à une combinaison de la nanofiltration utilisant la membrane NF270 avec la floculation employant $Fe_2(SO_4)_3$ comme agent de floculation pour la concentration et la détoxification de préhydrolysat de bois. Cette combinaison des méthodes a permis de se débarrasser de 74% des composés phénoliques [133].

Certes, le recours à des méthodes physiques, chimiques ou physicochimiques pour la réduction des teneurs des inhibiteurs dans le préhydrolysat de bois est très efficace [127], mais ces techniques de détoxification génèrent des volumes importants d'effluents qui sont toxiques à l'environnement [104]. De plus, une perte importante des sucres caractérise ces technologies [141]. Sur le plan économique, ces méthodes sont énergivores et demandent des teneurs importantes de réactifs coûteux pour de meilleures performances de réduction des inhibiteurs. La détoxification par des microorganismes spécifiques est une alternative économiquement rentable. Elle permet de métaboliser sélectivement les composés inhibiteurs d'une manière environnementale (absence de consommation des réactifs chimiques et faible génération des effluents) [142].

#### 2.3.7.4 Détoxification enzymatique

Plusieurs enzymes produites par des bactéries, des champignons ou des plantes, sont responsables de la détoxification du matériel lignocellulosique. Les enzymes de détoxification principalement étudiées sont les laccases et les peroxidases.

##### *2.3.7.4.1 Laccase*

La laccase est une oxydase contenant du cuivre [143] qui permet l'oxydation des composés phénoliques comme l'acide férulique [143, 144]. Le mode d'action de laccase consiste à oxyder les groupements hydroxyles d'un substrat réduit en utilisant la molécule d'oxygène ($O_2$) pour produire de substrat oxydé et de l'eau. La réaction d'oxydation par laccase est catalysée par des radicaux libres [145] comme le montre la Figure 2-9. Le mode d'action de laccase peut induire la polymérisation de la lignine favorisant ainsi une élimination plus facile de la lignine [146].

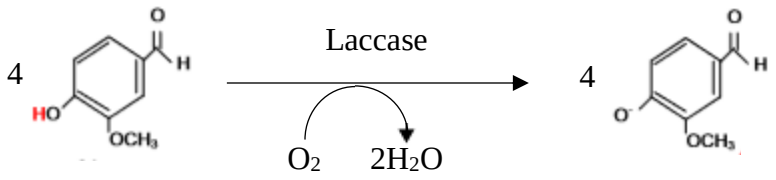



Figure 2-9 : Mode d'action de laccase sur un composé phénolique.

La laccase permet également l'oxydation des composés non phénoliques grâce à des médiateurs de laccase. Les médiateurs de faible poids moléculaire sont directement oxydés par la laccase en des radicaux organiques qui permettent l'oxydation abiotique des composés phénoliques. Le 1-hydrobenzotriazole (1-HBT) et l'acide 2,2'-azino-bis-(3-éthylbenzothiazoline-6-sulfonique) (ABTS) sont des exemples des médiateurs de laccase [147].

*2.3.7.4.2 Peroxydase*

Les peroxydases contenant le fer dans leur structure sont caractérisées par leur pouvoir réducteur élevé et exigent le peroxyde d'hydrogène pour l'oxydation de la lignine. Contrairement à la laccase, les peroxydases permettent d'oxyder directement les unités phénoliques et non phénoliques de la lignine. Ces enzymes contiennent entres autres la lignine peroxydase et la manganèse peroxydase [148]. La lignine peroxydase est caractérisée par un potentiel redox le plus élevé en comparaison avec les laccases et les autres peroxydases. Cette caractéristique favorise la dégradation des composés non phénoliques récalcitrants. Pour la manganèse peroxydase, l'enzyme permet l'oxydation des ions $Mn^{2+}$ en $Mn^{3+}$ qui permettent de déclencher les réactions d'oxydation de la lignine [149].

### 2.3.7.5 Détoxification microbienne

Plusieurs microorganismes qui dégradent les produits toxiques existent dans la nature et offrent l'avantage de détoxification directe des hémicelluloses de bois sans recours aux étapes de purification des enzymes. Ces microorganismes peuvent être des champignons ou des bactéries. Les champignons de pourriture blanche sont caractérisés par leur potentiel de dégradation de la lignine, à titre d'exemple *Stereum hirsutum* qui a éliminé jusqu'à 14% de lignine de *Pinus densiflora*. *Pycnoporus cinnabarinus* a permis une délignification de *Prosopis juliflora* jusqu'à 7.7 à 11.9%. Bien que les champignons permettent la délignification des matériaux lignocellulosique

récalcitrants, la durée de traitement qui peut aller à l'ordre de mois et la consommation élevée des glucides dans la biomasse contrecarrent l'industrialisation de la méthode de détoxification [148]. L'utilisation des bactéries pour la détoxification permet de limiter ces inconvénients, de maximiser les rendements de détoxification et d'éliminer différents types des inhibiteurs.

#### 2.3.7.6 Détoxification par la souche *Cupriavidus taiwanensis*

*C. taiwanensis* (nommée formellement *Ralstonia taiwanensis* (*R. taiwanensis*)) est une bactérie Gram- qui épouse la forme des coques et ne montre pas des spores [150]. Cette souche a été isolée des nodules racinaires de la plante de *Mimosa* spp. au sud de Taiwan [151, 152]. *R. taiwanensis* est capable d'utiliser une grande variété de sources de carbone et d'énergie comme l'acide acétique, l'acide succinique, l'acide propionique, etc. à différentes températures (i.e. 28 °C, 30 °C et 37 °C). Elle peut également consommer le phénol comme source unique de carbone à des concentrations qui dépassent 900 mg/L après 24 h de culture [150], ce qui témoigne l'intérêt biotechnologique particulier de cette souche grâce à sa capacité excellente de biodégradation des composés récalcitrants de la biomasse lignocellulosique [152]. *C. taiwanensis* peut également dégrader jusqu'à 0.2 g/L de phénol juste après 6 h [151]. Cette caractéristique de dégradation des composés phénoliques est associée à la sécrétion des catalases et des oxydases [150]. Plusieurs tentatives ont été effectuées pour améliorer la performance de dégradation des concentrations élevées de phénol par *R. taiwanensis*.

Chen *et al.* [153, 154] ont démontré que le glycérol est la source optimale de nutriments pour la stimulation de la croissance de *C. taiwanensis* et sa performance de dégradation de phénol. L'ajout de glycérol a conduit à l'augmentation du potentiel de dégradation de phénol jusqu'à plus de 900 mg/L. En ajoutant l'acide acétique ou l'extrait de levure, une croissance diauxique biphasique caractérise *R. taiwanensis*. En effet, les nutriments sont consommés en premier lieu alors que le phénol est réprimé. La dégradation de ce dernier commence uniquement après l'épuisement total des nutriments [153]. Cependant, les nutriments ajoutés dans le milieu sont coûteux et le procédé de détoxification devient alors plus onéreux.

Une autre alternative a été suggérée en 2008 par les mêmes auteurs [151]. Elle consiste en l'application du mode d'alimentation « Fed-batch » pour réduire l'accumulation des phénols dans le milieu et ainsi promouvoir la croissance cellulaire. Le recours à ce procédé a permis d'améliorer significativement la performance de biodégradation de phénol en passant d'une dégradation de 0.2

g/L par la souche après 6 h en mode batch à une dégradation 3.2 g/L après 15 h [151].

Plus récemment, Chen *et al.* [154] ont appliqué le mode continu pour contrecarrer aux problèmes d'épuisement rapide des nutriments essentiels et pour limiter la toxicité causée par l'accumulation des produits toxiques. Grâce à ce mode d'alimentation des nutriments, *C. taiwanensis* R186 était capable de dégrader jusqu'à 2.2 g/L de phénol introduites par des injections séparées pour éviter les réponses toxiques [154]. Ces souches sont également caractérisées par leur capacité de survivre dans des environnements oligotrophiques. Cette caractéristique rend de ces bactéries performantes dans les procédés de détoxification des effluents industriels [150]. La souche de *Pseudomonas sp.* UVS exige, par contre, la présence de plusieurs nutriments dans son milieu entre autres la peptone, le NaCl et l'extrait de viande pour son métabolisme [155]. En plus de son potentiel de biodégradation des composés récalcitrants, *C. taiwanensis* est caractérisée par sa tolérance élevée aux métaux lourds. En effet, cette bactérie peut tolérer jusqu'à 15 mM de Pb alors que des concentrations au-dessous de 0.2 mM étaient inhibitrices de *E. Coli* [152] (Tableau 2-1).

Tableau 2-1 : Comparaison des valeurs des concentrations minimales inhibitrices (MIC) des souches : *R. eutropha* CH34, *C. taiwanensis* TJ208 et *E. Coli* pour différents types des métaux lourds [152]

| **Souche** | **MIC (mM)** | | | | | |
|---|---|---|---|---|---|---|
| | **Pb** | **Cu** | **Cd** | **Zn** | **Co** | **Ni** |
| ***Ralstonia eutropha* CH34** | NA* | 3.0 | 2.5 | 12 | 5.0 | 2.5 |
| ***C. taiwanensis* TJ208** | 15 | 5.0 | 2.5 | 7.5 | 5.0 | 1.5 |
| ***E. coli*** | 0.2 | 1.0 | 0.5 | 1.0 | 1.0 | 1.0 |

*NA : Non disponible

*C. taiwanensis* est caractérisée par son incapacité d'assimiler les sucres entres autres D-Glucose, D-fructose et D-xylose. Ces vertus sont d'un grand intérêt biotechnologique et sont prometteurs pour une détoxification efficace des préhydrolysats de bois à l'échelle industrielle en utilisant ce bioagent. Cette caractéristique distingue *C. taiwanensis* de la majorité des souches de détoxification qui exigent la présence des sucres dans le milieu de culture pour leur métabolisme [150]. En effet, la consommation de cellulose par *Penicillium sp. strain apw-tt2* était de 28% pour dégrader 66% de la lignine [156]. Et une concentration de glucose de 1 g/L était nécessaire pour

dégrader la lignine par la souche *Pleurotus ostreatus* [157]. La souche *Tolumonas lignolytica* nécessite également la consommation de D-xylose et D-glucose pour la dégradation de la vanilline [158]. La consommation des sucres disponibles dans la biomasse lignocellulosique contribue à une insuffisance des sources de carbone et d'énergie pour les microorganismes responsables de la formation des produits à haute valeur ajoutée à titre d'exemple le butanol, l'éthanol, etc.

#### 2.3.7.7 Détoxification par la souche *Ureibacillus thermosphaericus*

*U. thermosphaericus* est une bactérie thermophile Gram- [159-161] appartenant à la famille *Planococcaceae*. C'est une bactérie aérobie formant des endospores [159]. Cette souche était isolée à partir du compost de plantes aquatiques [129]. Sa gamme de température pour la croissance s'étale de 35 °C à 64 °C [162-164] avec une température optimale de 50 °C et un pH optimal de 7 [160]. Cette souche est caractérisée par son potentiel de production d'une grande variété d'enzymes, à savoir les cellulases [106], les oxydases extracellulaires, les laccases, la tyrosinase et les peroxydases [165]. Récemment, Jia *et al.* [166] ont démontré le potentiel de production de catalase par *U. thermosphaericus* FZSF03. Une production maximale de 57 630 U/mL a été obtenue après optimisation de la production. L'activité rapportée est la plus élevée parmi différentes souches sauvages. La diversité des enzymes produites par *U. thermosphaericus* permet une meilleure dégradation des composés récalcitrants couramment présents dans la biomasse lignocellulosique entres autres le furfural, le 5-HMF et les composés phénoliques [106, 167].

La diversité des enzymes produites a suscité l'intérêt de plusieurs chercheurs. En effet, cette souche a été exploitée par Okuda *et al.* [129] pour la détoxification des déchets domestiques de bois. La bactérie était directement ensemencée dans le préhydrolysat sans recours à l'addition des nutriments pour son métabolisme contrairement au *Pseudomonas sp.* SUK1 qui exige la présence de peptone, d'extrait de viande et de NaCl dans le milieu [66]. Le traitement de la biomasse lignocellulosique par *U. thermosphaericus* a favorisé une réduction significative des concentrations de furfural et HMF jusqu'à 50% et 20% respectivement. Le mécanisme de réduction des teneurs des aldéhydes furaniques par cette bactérie consiste en la dégradation de furfural en acide 2-furancarboxylique et l'oxydation de 5-HMF en acide 5-hydroxymethyl-2-furancarboxylique. Ces produits sont moins inhibiteurs de la croissance cellulaire et de la production de l'éthanol par *S. cerevisae* que les aldéhydes d'origine. En effet, la concentration finale de l'éthanol obtenue suite au traitement par *U. thermosphaericus* pendant 24 h était de 13 g/L, une concentration proche de

celle obtenue par « overliming » [129]. Plus récemment, une étude de la performance de la bactérie *U. thermosphaericus* A1 pour la détoxification de préhydrolysat Cedar était effectuée par Asada *et al.* [160]. Ces derniers ont procédé à une saccharification, une fermentation et une détoxification simultanées de la biomasse et ont réalisé une amélioration du rendement théorique de l'éthanol de 70% (une concentration finale de l'éthanol était de 7.45 g/L après 24 h de fermentation de 50 g/L de substrat). Une concentration de substrat de 200 g/L était démontrée toxique pour la souche suite au dédoublement des teneurs des inhibiteurs. Le recours au procédé « Fed-batch » par ces auteurs a contribué à une augmentation significative de la concentration finale de l'éthanol par *S. cerevisae* à 26.5 g/L après 60 h de fermentation [160].

La rapidité dans le traitement de la lignocellulose avec *U. thermosphaericus* s'ajoute aux atouts de cette bactérie [168]. En effet, une élimination de 50% de furfural était observée après 24 h de culture dans le travail de Okuda *et al.* [129]. Cette bactérie a été également explorée pour améliorer le procédé de compostage. Dans cet axe, une étude a été réalisée par Vargas-Garcı *et al.* [169] pour évaluer la capacité d'une communauté des souches microbiennes composée de *Bacillus shackletonni*, *Streptomyces thermovulgaris* et *U. thermosphaericus* dans la décomposition de la matière lignocellulosique au cours du compostage. Ce travail a montré que *U. thermosphaericus* est le microorganisme le plus efficace pour l'amélioration de compostage avec des réductions des teneurs élevés de la lignine entre 17.23% et 24.34% [169]. En 2013, des conclusions similaires ont été tirées par Zainudin *et al.* [106] qui ont développé une coculture y compris *U. thermosphaericus* pour le compostage de grappe de fruits exemptes d'huile de palmier. Ce traitement a favorisé la réduction à moitié de la durée du compostage par rapport au procédé conventionnel (passant de 90 jours à 40 jours) [106]. À côté de son application pour la décomposition de la biomasse lignocellulosique, la bactérie *U. thermosphaericus* était testée pour la biosynthèse des nanoparticules d'argent [170] et pour la production de déshydrogénase meso-diaminopimelate [171]. En plus des rendements élevés de détoxification aux courts délais, *U. thermosphaericus* est incapable d'assimiler les glucides dans le milieu ce qui favorise une détoxification économiquement fiable à grande échelle [162, 164]. En effet, la perte des sucres dans les hydrolysats des déchets domestiques du bois ne dépasse pas 5% qui est moins élevée que celle obtenue par le chaulage (10%) [129]. *U. thermosphaericus* est également caractérisée par sa tolérance aux sels de mercure et au chlorure de sodium jusqu'à 0.08 g/L et 50 g/L respectivement [164, 165]. Cependant, cette souche est peu efficace pour l'élimination des acides organiques à

l’exception de l’acide formique dont la concentration est réduite à 40% dans les hydrolysats des déchets du bois [129].

Les principales technologies de détoxification appliquées dans la littérature sont résumées dans le Tableau 2-2 suivant.

Tableau 2-2 : Liste des méthodes de détoxification fréquemment appliquées dans la littérature

| Méthodes de détoxification | Avantages | Inconvénients | Méthodes de prétraitement correspondantes |
|---|---|---|---|
| **Coagulation-Floculation** | - Meilleure dégradation des composés phénoliques (56%) [4].<br>- Faible perte des glucides (<20%) [136].<br>- Amélioration de la production d'ABE de 0.9 g/L à 6 g/L [135]. | - Élimination modérée de furfural, de 5-HMF et d'acide acétique <20% [4].<br>- Teneurs élevés des résidus solides [136]. | - Explosion à la vapeur [133]<br>- Prétraitement à l'ammoniaque dilué |
| **Filtration nanomembranaire** | - Concentration des glucides (jusqu'à 99%) [137].<br>- Réduction des concentrations de furfural, de 5-HMF et d'acide acétique à 70% [4, 139]. | - Faible élimination des composés phénoliques (0%-10%) [4].<br>- Risque de colmatage des membranes.<br>- Procédé coûteux [140]. | - Explosion à la vapeur [133]<br>- Prétraitement à l'acide dilué [135] |
| **Chaulage** | - Efficacité élevée d'élimination de furfural (~50%).<br>- Augmentation de la production de l'éthanol de 2.5 g/L à 12 g/L [129]. | - Formation de gypsum [114, 132].<br>- Faible efficacité d'élimination des composés phénoliques (41%) et de l'acide acétique [129].<br>- Perte élevée des sucres allant jusqu'à 47% de glucose dans l'hydrolysat d'épinette [114].<br>- Procédé coûteux [131]. | - Prétraitement avec l'acide sulfurique dilué [129] |

Tableau 2-2 : Liste des méthodes de détoxification fréquemment appliquées dans la littérature (suite)

| Méthodes de détoxification | Avantages | Inconvénients | Méthodes de prétraitement correspondantes |
|---|---|---|---|
| **Charbon actif** | - Adsorption jusqu'à 85% des composés phénoliques [139].<br>- Élimination importante de 5-HMF jusqu'à 38%-52%.<br>- Augmentation de la production de butanol (de 1.8 g/L à 7.3 g/L) [135]. | - Adsorption des glucides parallèlement à l'adsorption des inhibiteurs (>30%) [4, 172].<br>- Faible affinité de l'acide acétique (14%) [139].<br>- Inefficacité d'adsorption des composés phénoliques hydrophiles<br>- Saturation du charbon actif [4, 172]. | - Prétraitement à l'acide [173]<br>- Explosion à la vapeur [133]<br>- Autohydrolyse [174] |
| **Détoxification avec l'ammonium ($NH_4OH$)** | - Faible dégradation des glucides (1.2%-10%) [132].<br>- Formation du précipité due à la réaction entre les aldéhydes de furanes et l'ammonium.<br>- Effet tampon de $NH_4OH$.<br>- Élimination extensive des aldéhydes furaniques (56% de furfural et 43% de HMF).<br>- Pas de formation des sels solubles [175]. | - Faible dégradation des composés phénoliques.<br>- Pas de dégradation de l'acide acétique [176].<br>- Toxicité élevée de $NH_4OH$ aux microorganismes de fermentation [132]. | - Prétraitement avec l'acide dilué [176] |

Tableau 2-2 : Liste des méthodes de détoxification fréquemment appliquées dans la littérature (suite)

| Méthodes de détoxification | Avantages | Inconvénients | Méthodes de prétraitement correspondantes |
|---|---|---|---|
| **Détoxification électrochimique** | - Dégradation élevée des composés phénoliques (concentration initiale : 0.7 g/L) dans l'hydrolysat de paille de riz jusqu'à 71% après 10 h de traitement.<br>- Courte durée de traitement [114]. | - Consommation élevée d'énergie [114].<br>- Électrolyse et évaporation de l'eau durant la détoxification contribuant à une diminution de 14% du volume réactionnel [114].<br>- Différents voltages optimaux caractéristiques pour chaque composé toxique [177]. | - Prétraitement acide [114] |
| **Détoxification avec laccase** | - Élimination jusqu'à 75% des composés phénoliques.<br>- Pas d'attaque de la fraction des glucides.<br>- Amélioration de la concentration de l'éthanol produite jusqu'à 60% en utilisant le son de blé immergé dans l'eau comme substrat [178].<br>- Courtes durées de traitement.<br>- Faibles coûts énergétiques [179]. | - Élimination sélective des composés phénoliques sans attaque des autres inhibiteurs [178].<br>- Possibilité de formation de la lignine à faible poids moléculaire, toxique aux enzymes d'hydrolyse, par polymérisation des radicaux des composés phénoliques générés par action de laccase [180].<br>- Des médiateurs requis pour la dégradation de la fraction non phénolique de la lignine avec la laccase [148].<br>- Faible stabilité [181].<br>- Coûts de production élevés [179]. | - Prétraitement acide [179]<br>- Explosion à la vapeur [182] |
| **Détoxification avec *Ganoderma lucidum*** | - Activités maximales de laccase (141.1±0.2 U/mL) produites après 6 jours de culture sur le son de blé et la poudre d'arachide [179].<br>- Dégradation complète des composés phénoliques à une concentration initiale de 380 mg/L après 7 jours [183]. | - Longue durée d'incubation (de l'ordre des jours).<br>- Taux de consommation élevé de xylose (consommation complète après 9 jours d'incubation).<br>- Pas de production de lignine peroxydase [183]. | - Procédé Organosolv [183] |

## 2.4 Synthèse de revue de littérature

De nombreux facteurs ont contribué à la fermeture de plusieurs industries de pâtes et papiers canadiennes à la suite des crises économiques et technologiques, ainsi qu'environnementales. Le développement de bioraffineries intégrées constitue une meilleure alternative et un excellent remède aux problèmes des industries papetières grâce à la diversification de leur production. Pour la conversion de la biomasse lignocellulosique en biocarburants, différents procédés de prétraitement, d'hydrolyse et de détoxification ont été développés avant la conversion finale des sucres fermentescibles obtenus en biocarburants. Toutefois, les méthodes physiques, chimiques (e.g. floculation (Figure 2-8)) et enzymatiques (Figure 2-5 et Figure 2-9) sont fréquemment appliquées dans plusieurs travaux grâce à leur rapidité. Cependant, ces méthodes sont caractérisées par leur coût élevé, leur faible rendement d'hydrolyse et par la génération d'inhibiteurs (Figure 2-7) suite au recours à des réactifs chimiques et à des conditions opératoires sévères. De plus, des étapes de séparation supplémentaires sont nécessaires pour retirer les précipités formés suite à l'action des agents chimiques employés (Tableau 2-2). Le recours à des microorganismes pour l'hydrolyse et la détoxification de la biomasse lignocellulosique a été rarement étudié bien que ces bioagents représentent un potentiel d'avantages économique et environnemental. Peu de travaux de recherches proposant l'utilisation des microorganismes pour la conversion de la biomasse ont été publiés à ce jour de part de faibles rendements observés pour les souches étudiées, la consommation de glucides par les microorganismes de détoxification au détriment de la fermentation ABE, la durée prolongée du procédé de production par l'ajout d'un traitement microbien, etc. (Tableau 2-2). Or, la littérature a également identifié une série de souches microbiennes pour leur utilité en bioraffinage. Entre autres, la souche *P. campinasensis* s'est révélée d'intérêt pour l'hydrolyse d'hémicelluloses et les souches *U. thermosphaericus* et *C. taiwanensis* ont montré une certaine capacité pour la détoxification du matériel lignocellulosique. Les souches de détoxification *U. thermosphaericus* et *C. taiwanensis* ont été sélectionnées comme leurs génomes respectifs sont connus. Ainsi leur arsenal d'enzymes pour la détoxification de milieu est recensé, entres autres des laccases, des catalases et des oxydases qui permettent l'oxydation des composés phénoliques et des autres inhibiteurs comme les aldéhydes furaniques. De plus, *C. taiwanensis* est connue par sa tolérance élevée aux sels dans les milieux de culture. Ces propriétés pourraient relever certaines des contraintes citées ci-dessus. Toutefois, la faible tolérance aux

concentrations élevées en inhibiteurs de types phénoliques, furfural, HMF et sels (Tableau 2-1), entres autres, peut réduire significativement la performance de détoxification de ces souches microbiennes. Quelques tentatives d'amélioration de cette tolérance ont consisté à l'addition de divers nutriments ou à l'adoption des modes d'opération en bioréacteur de types « feed-batch » ou continu qui permettent de limiter l'accumulation de produits toxiques et l'alimentation continue en nutriments nécessaires. En revanche, ces techniques ne sont pas nécessairement adaptées au contexte du bioraffinage qui favorise normalement un procédé de type cuvée ou « batch ». Ces pratiques peuvent également être coûteuses et présentent l'inconvénient d'augmenter le risque de contamination microbienne. Fait important, aucune publication portant sur l'étude de co-cultures de ces souches microbiennes d'intérêt, dans le but de maximiser les rendements de conversion d'une manière plus simple, moins coûteuse et plus rapide, n'a pu être trouvée.

# CHAPITRE 3 : OBJECTIFS, MÉTHODOLOGIE ET ORGANISATION DU DOCUMENT

## 3.1 Objectifs

### 3.1.1 Objectif principal

Développer des technologies novatrices de détoxification à base de microorganismes pour une hydrolyse bactérienne performante de préhydrolysat hémicellulosique et pour une conversion subséquente efficace en butanol par la souche *Clostridium acetobutylicum* ATCC 824.

### 3.1.2 Objectifs spécifiques

Trois sous-objectifs découlent de l'objectif principal et sont répartis comme suit :

- Étudier la performance de détoxification de milieux synthétiques par des cultures pures et des co-cultures de deux bactéries, visant à évaluer le rendement de réduction en inhibiteurs de types phénoliques;

- Étudier l'effet de la biodétoxification des hydrolysats de bois, couplée ou non à une étape préalable de floculation et/ou de nanofiltration sur la performance de détoxification et de fermentation ABE;

- Déterminer l'efficacité d'hydrolyse avec *Paenibacillus campinasensis* DSM 21989, et étudier les effets d'un couplage simultané ou séquentiel d'une biohydrolyse à la biodétoxification, sur les performances d'hydrolyse, de détoxification et de fermentation ABE.

## 3.2 Méthodologie et organisation du document

La méthodologie générale est présentée à la Figure 3-1 et a été développée de manière à atteindre l'objectif principal et les sous-objectifs spécifiques de la thèse.

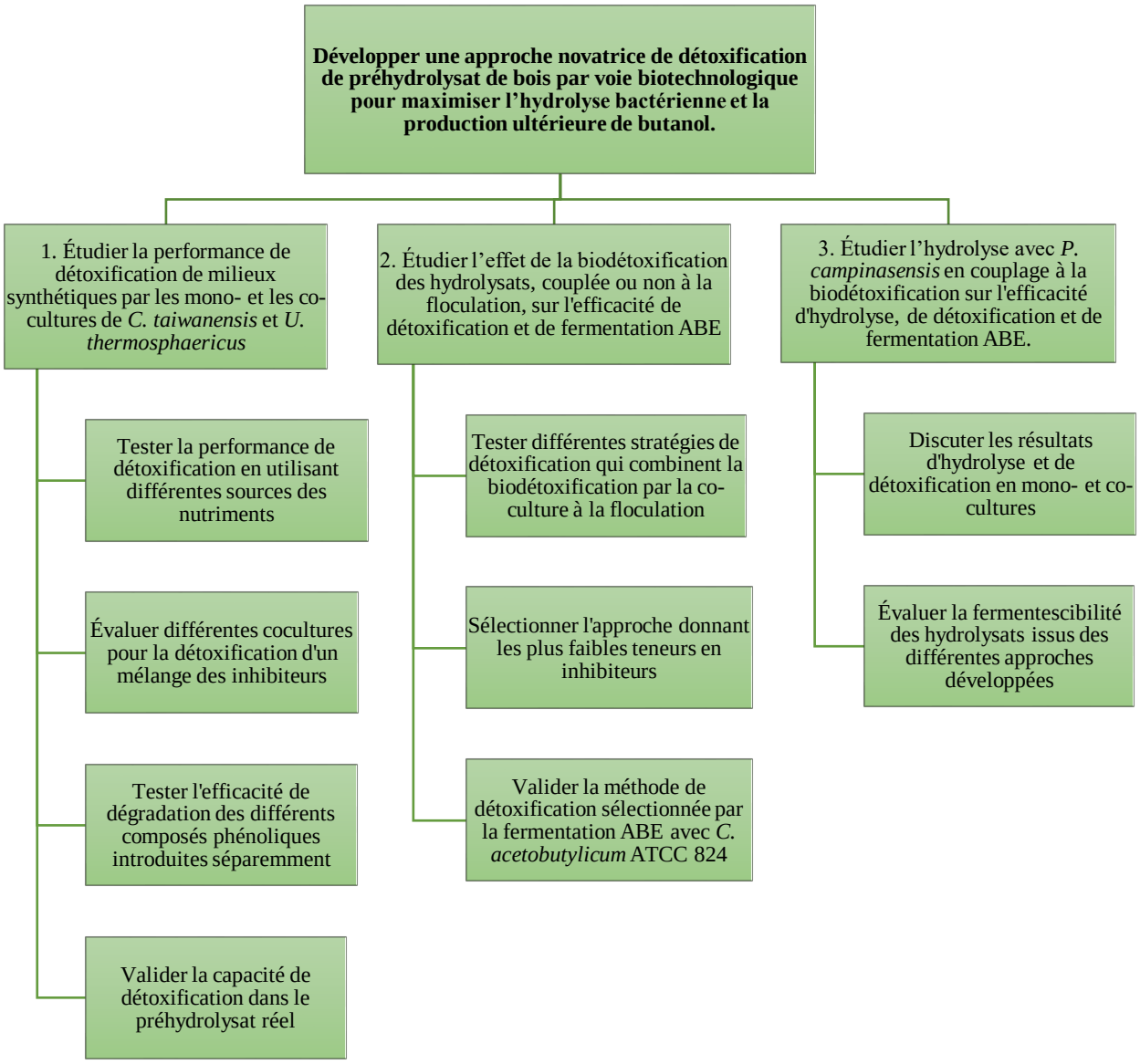



Figure 3-1 : Méthodologie générale du projet.

La méthodologie développée dans ces travaux de thèse contient trois phases majeures qui répondent aux trois objectifs spécifiques définis à la section 3.1.2. Les travaux développés dans les trois phases ont résulté en un ensemble de trois manuscrits scientifiques soumis à des journaux importants dans les domaines traités, et les résultats de chacun des sous-objectifs sont présentés dans des chapitres séparés. Les souches *U. thermosphaericus* et *C. taiwanensis* ont été sélectionnées dans ce projet pour la détoxification de la biomasse grâce aux rendements élevés de dégradation des différents inhibiteurs (composés phénoliques, furfural, 5-HMF, etc.), à la courte durée de traitement, à l'incapacité d'assimilation des sucres et à leur potentiel de production des catalases, oxydases et laccases principalement qui participent dans la dégradation des inhibiteurs.
Le premier manuscrit, présenté au Chapitre 4 s'intitule: "Detoxification of a hardwood hemicelluloses prehydrolysate by a co-culture of *Ureibacillus thermosphaericus* and *Cupriavidus taiwanensis*" et décrit la première phase du projet : « Étudier la performance de détoxification de milieux synthétiques par les mono- et les co-cultures de *C. taiwanensis* et *U. thermosphaericus* » et discute les principaux résultats. Dans ce travail, les milieux synthétiques ont été formulés de manière à simuler la composition des préhydrolysats de bois couramment utilisés. Les paramètres d'étude utilisés dans cette étude sont la source des nutriments, le type et la concentration des composés phénoliques (i.e. acide gallique, vanilline, catéchol et syringaldéhyde) et le type de la culture bactérienne utilisée (mono-cultures et différentes associations des bactéries en co-culture). À la fin de ce chapitre, on a testé la performance de détoxification de préhydrolysat de bois non dilué avec les mono- et les co-cultures d'étude. L'article 1 a été soumis au journal: "Fermentation".
Le deuxième manuscrit, présenté au Chapitre 5 s'intitule: "Combining chemical flocculation and bacterial co-culture of *Cupriavidus taiwanensis and Ureibacillus thermosphaericus* to detoxify a hardwood hemicelluloses hydrolysate and enable ABE fermentation leading to butanol" décrit les analyses et les résultats correspondants à la deuxième phase de la méthodologie : «Étudier l'effet de la biodétoxification des hydrolysats de bois, couplée ou non à la floculation et/ou à la nanofiltration, sur la performance de la fermentation ABE » . Le préhydrolysat utilisé dans ces études a été obtenu par explosion couplée à l'eau chaude. En comparaison avec d'autres méthodes de prétraitement comme l'utilisation des champignons qui permet également la délignification, l'explosion à la vapeur est caractérisée par sa courte durée et par des rendements élevés de solubilisation des hémicelluloses avec des faibles teneurs en lignine. Dans cette étude, la floculation était utilisée comme une étape supplémentaire de détoxification et la nanofiltration

comme une étape de concentration des sucres avant l'étape d'hydrolyse. La meilleure stratégie qui a fourni l'hydrolysat de plus faibles teneurs en inhibiteurs, et qui consiste en la floculation de l'hydrolysat suivie de la co-culture, était validée par la fermentation ABE avec *C. acetobutylicum* ATCC 824 pour la production de butanol. L'article 2 a été soumis au journal: "Biotechnology Progress".

Le troisième et dernier manuscrit, présenté au Chapitre 6 s'intitule: "Hydrolysis and detoxification of hemicellulosic hardwood prehydrolysate by sequential and simultaneous bacterial cultures for enhancement of butanol production", décrit la troisième phase du projet et vise à étudier l'efficacité d'hydrolyse de préhydrolysat de bois avec *P. campinasensis* et à évaluer le couplage de la biohydrolyse à la biodétoxification sur l'efficacité d'hydrolyse, de détoxification et de fermentation ABE. Cette étude était réalisée pour la première fois en utilisant le préhydrolysat de bois généré par explosion à la vapeur couplée à l'eau chaude. Dans l'article 3, sont présentés et comparés les résultats d'hydrolyse et de détoxification bactériennes, appliquées simultanément ou séquentiellement. Le procédé de détoxification employé correspond au procédé optimal déterminé dans l'article 2 (Chapitre 5) et qui consiste en la floculation de l'hydrolysat acide suivie de son biodétoxification avec la co-culture de deux bactéries. Finalement, les résultats de fermentation ABE des différents hydrolysats générées par les approches développées sont discutés. L'article 3 a été soumis au journal "Biochemical Engineering Journal".

# CHAPITRE 4 : ARTICLE 1: DETOXIFICATION OF A HARDWOOD HEMICELLULOSES PREHYDROLYSATE BY A CO-CULTURE OF UREIBACILLUS THERMOSPHAERICUS AND *CUPRIAVIDUS TAIWANENSIS*

## Fermentation

**Authors: Mariem Theiri [1,2], Mariya Marinova [3], Hassan Chadjaa [2] and Mario Jolicoeur [1,*]**

[1] Research Laboratory in Applied Metabolic Engineering, Department of Chemical Engineering, École Polytechnique de Montréal, J.-A. -Bombardier Pavilion, 2900 Édouard-Montpetit Blvd., Montréal, QC, H3T 1J4 Canada.

[2] Centre National en Électrochimie et en Technologies Environnementales, 5230, Boulevard Royal, Shawinigan, QC G9N 4R6, Canada.

[3] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, 13 General Crerar Crescent, Kingston, ON K7K 7B4.

***** Corresponding author: mario.jolicoeur@polymtl.ca; Tel.: 514-340-4711 ext. 4525.

## 4.1 Abstract

Hemicelluloses pre-hydrolysate, an attractive feedstock for biofuels production, contains inhibitors, along with fermentable sugars, which can hamper the efficient conversion of the lignocellulosic biomass. The use of a co-culture composed of *Ureibacillus thermosphaericus* and *Cupriavidus taiwanensis* to detoxify the pre-hydrolysate was investigated for the first time in this work. A preliminary study was performed in tryptic soy broth. First, an optimal temperature of 41°C was determined, favoring similar kinetics of mono- and co-cultures. Then a starting volumetric ratio of 1-unit *Cupriavidus taiwanensis* on 1.5-units *Ureibacillus thermosphaericus* was determined by Q-PCR for an equal number of the strains in the detoxification media. Afterwards, the optimal nutrients source able to detoxify phenolic compounds in synthetic mediums inoculated

with mono-cultures and a medium of metallic ions was selected. Then, the performance of phenolics degradation in a single-compounds solution and in a mixture solution was compared and the higher degradation (90% at 2.8 g/L of phenolics) was obtained using simultaneous co-culture. Finally, the detoxification performance of the tested cultures was confirmed on a real pre-hydrolysate, and a maximum degradation of 14% of the phenolics was observed using sequential addition of first *Ureibacillus thermosphaericus* followed by *Cupriavidus taiwanensis*.

**Keywords**

pre-hydrolysate; phenolic compounds; co-culture; detoxification; synthetic mediums

## 4.2 Introduction

Lignocellulosic biomass is considered as an attractive feedstock for producing biofuels and reducing green house gas emissions thanks to its abundance, sustainability, renewability, low cost and non-food criterion [1, 2]. However, the complex structure of lignocelluloses contributes to the biomass recalcitrance. This recalcitrant nature hampers the access of enzymes and chemicals to the carbohydrates they need to liberate fermentable reducing sugars. Sugars serve as precursors for producing value-added products. To alleviate the biomass recalcitrance and enhance the yield of fermentable sugars and thus the production of bioenergy and bio-based products, pre-treatment and hydrolysis steps are required. Many biomass pretreatment processes have been developed including steam explosion, acid hydrolysis, hot water extraction, etc. [3]. Depending on the operating conditions related to these processes, a variety of inhibitors are liberated together with fermentable sugars over a broad concentration range as a result of the lignocellulose components break-down. These inhibitors include phenolic compounds, furan aldehydes, organic acids and inorganic salts [4].

The presence of phenolic compounds among others syringaldehyde, vanillin, catechol, gallic acid, ferulic acid and p-coumaric acid, in lignocellulosic biomass, decreases the performance of the enzymatic hydrolysis [5-7]. Kim et al. [5] reported that the activity of ß-glucosidase from *Trichoderma reesi* was reduced by half in the presence of phenolic acids. In addition to inhibition and deactivation of cellulolytic enzymes [7, 8] phenolic compounds affect both cell growth and the yields of solvents production [9, 10]. Yao et al. [11] claimed that 1 g/L of syringaldehyde, ferulic acid and p-coumaric acid completely blocked cell metabolism and that no Acetone-Butanol-

Ethanol (ABE) were produced under these conditions. The presence of 0.5 g/L of ferulic acid prolonged the lag phase and decreased cell growth by 80% and butanol production by 90% with *Clostridium beijerinckii* NCIMB 8052, as observed by Ezeji et al. [12] In fact, phenolic compounds acted on solventogenic cells by interfering with their cell membrane and raising its fluidity, causing the leak of cellular contents [9, 10, 12]. Furfural and 5-(hydroxymethyl) furfural (5-HMF) resulting from the degradation of pentose and hexose sugars respectively during pretreatment and hydrolysis (i.e. acid hydrolysis) caused inhibition of cell growth (totally in presence of 4 g/L of furfural) [11]. At concentrations higher than 3 g/L, furan aldehydes decreased by 10% the ABE produced by *Clostridium acetobutylicum* ATCC 824 [9]. Acetic acid is an organic acid inhibitor generated from the break-down of acetyl groups of hemicelluloses [6, 13]. When used in ABE fermentation using *Clostridium beijerinckii* B592, this inhibitor induced acid crash at 60 mM of the undissociated form, thus contributing to cell death [6, 9]. Inorganic salts, for example sodium sulfate and sodium chloride, can be obtained from lignocellulosic material or alkali and acid solutions supplemented during pretreatment, hydrolysis or neutralization steps [6, 14]. They are potential inhibitors of ABE fermentation. Indeed, concentrations of sodium chloride higher than 10 g/L inhibited *Clostridium beijerinckii* BA101 [15].

The development of a detoxification procedure is crucial to counteract these inhibitory impacts and maximize the conversion of hemicellulosic biomass into value added products. Chemical (e.g. flocculation, alkali treatment, activated charcoal and anion exchange) and physical (e.g. nanofiltration and evaporation) methods were developed to eliminate inhibitors. However, these methods employ harsh operating conditions (dose of chemicals, temperature, etc.) in addition to fermentable sugars losses exceeding 25% with anion-exchange resin at pH 10 [16] and 30% with activated charcoal [17, 18]. These side effects, affecting negatively the economic viability of the process, may be alleviated thanks to a biological treatment using ligninolytic enzymes extracted from microorganisms or applying directly these strains. Although enzymatic detoxification is very effective leading to the degradation of 75% of the phenolic compounds in steam-exploded wheat straw, this treatment, besides its high cost, targets only phenolic compounds without affecting the concentration of other inhibitors. An alternative to chemical, physical and enzymatic methods of detoxification is the microbial treatment employing bacteria or fungi. This technique targets various inhibitors (phenolic compounds, furfural, 5-HMF, acetic acid, etc.) and has a low cost and a positive environmental impact. However, most of these microorganisms are characterized by

their low detoxification yields, long residence time and consumption of fermentable sugars. For instance, *Trichoderma reesei* consumed 35% of fermentable sugars in dilute acid hydrolysate of spruce to detoxify a maximum of 6% of phenolic compounds during 6 days [16]. Lignin loss in corn stover has reached 51.4% when pretreated with *Phanerochaete chrysosporium* NRRL-6370 for one month accompanied by hemicelluloses loss of 57% [19]. In contrast, *Cupriavidus taiwanensis* (*C. taiwanensis*) and *Ureibacillus thermosphaericus* (*U. thermosphaericus*) in detoxification were proven to be among the most effective strains due to their high performance in short duration (24 h), without consumption of fermentable sugars [20]. However, researchers demonstrated the low tolerance of *C. taiwanensis* to high concentrations of inhibitors. Many attempts, such as the use of fed-batch cultures or supplementation of inducers of phenols degradation, to enhance the performance of the bacteria did not achieve significant improvement of the detoxification of highly inhibitory biomass [21-24]. To enhance the tolerance of the strains, increase the yields of detoxification of a wider range of inhibitors and shorten the duration of treatment, the development of a co-culture composed of both strains (*C. taiwanensis* DSM 17343 and *U. thermosphaericus* NCIMB 13819) was investigated in this work. Previous studies demonstrated that a co-culture may affect the long duration of the biodetoxification process [25, 26]. A synthetic medium containing the inhibitors present in conventional pre-hydrolysates (steam explosion, acid, alkaline and hydrothermal methods, etc.) and a hemicelluloses pre-hydrolysate generated from a Kraft dissolving pulping process were detoxified in this work using an original co-culture strategy. Indeed, the consortium we studied proved its efficiency in the removal of a broader range of inhibitors than mono-cultures. To the best of our knowledge it is the first time that mixed cultures of *U. thermosphaericus* and *C. taiwanensis* were investigated for the detoxification of synthetic media composed of different concentrations of inhibitors and of non-diluted Kraft pre-hydrolysate.

The specific objectives of this work were to conduct:

- preliminary studies on Tryptic Soy Broth (TSB) for determination of the optimal growth temperature of mon- and co-cultures;
- an investigation of the effects of synthetic medium composition on the efficiency of phenolic compounds removal with pure and mixed cultures;

- a comparison of the performance of hardwood pre-hydrolysate detoxification by mono- and co-cultures.

## 4.3 Materials and Methods

### 4.3.1 Bacterial strains and culture conditions

*U. thermosphaericus* NCIMB 13819 was obtained from NCIMB Ltd. (YA, Scotland) and was activated in 3% *m/v* sterilized Tryptic Soy Broth (TSB) (Quelab Laboratories inc.; QB-39-5626) at 55 °C with agitation at 180 rpm until two deceleration points of Optical Density ($OD_{600}$) were observed. Glycerol 30% *v/v* was then added to prepare a stock culture frozen at -80 °C. For inoculum preparation, TSB (g/L: casein peptone 17, soy peptone 3, dipotassium phosphate 2.5, dextrose 2.5 and sodium chloride 5) was autoclaved at 121 °C for 15 min then inoculated with 0.1% *v/v* of the stock culture. The culture was let to grow for 10 h at 50 °C with agitation at 180 rpm in an orbital shaker (New Brunswick Scientific Innova 44, USA). For the determination of the incubation duration for the inoculum, growth curve was followed for 48 h and on that basis, a duration of 10 h was selected, corresponding to the mid to late exponential phase, for subsequent inoculation to the test media. 2 mL of the inoculum were inoculated to the media where detoxification experiments were performed. The dose of inoculum corresponded to an initial concentration of $0.2x10^8$ CFU (Colony Forming Unit)/mL.

*C. taiwanensis* DSM 17343 was purchased from DSMZ (Braunschweig, Germany). This strain was revived in R2A medium, consisting of (in g/L): yeast extract 0.5 (BD, catalog number: 212750), proteose peptone 0.5 (Nutri-Bact; QB-39-1910), casamino acids 0.5 (organotechnie), glucose 0.5 (Thermo Fisher Scientific D1610), soluble starch 0.5 (Fisher Chemical; S516-500), Na-pyruvate 0.3 (Fisher Bioreagents; 253 S-15629U), $K_2HPO_4$ 0.3 (Fisher Bioreagents; catalog number 19 L-15781U) and $MgSO_4 \cdot 7H_2O$ 0.05 (Acro Organics; catalog number 423905000). It was then incubated at 28 °C and 180 rpm in an orbital shaker (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA) until two deceleration points of Optical Density ($OD_{600}$) were observed. The stock culture was prepared from the R2A culture similarly as for *U. thermosphaericus*. For inoculum preparation, TSB was used, and the inoculum was allowed to grow for 10 h at 37 °C with agitation at 180 rpm. 1.33 mL of the inoculum were used to inoculate the detoxification media aiming at an initial concentration of $0.2x10^8$ CFU/mL.

In the case of the co-culture, the inocula were prepared separately. Then, 1.2 mL of *U. thermosphaericu*s inoculum were mixed simultaneously or sequentially (interval time 12 h) with 0.8 mL of *C. taiwanensis* inoculum.

### 4.3.2 Biomass preparation

#### 4.3.2.1 Synthetic media preparation

Synthetic media were composed of phenolic compounds (gallic acid, catechol, vanillin and syringaldehyde), furfural, acetic acid, salts ($Na_2SO_4$, KCl, NaCl, $CaCl_2$ and $H_3PO_4$), selectively used in this work, and a source of nutrients. The pH of media was adjusted to 7 with NaOH 4 M.

In the experiments using a mixture of inhibitors as a carbon source, phenolic compounds were introduced in variable amounts in the culture media (0.3 g/L; 2.8 g/L and 8 g/L). All other toxic compounds were supplemented with fixed concentrations: furfural 2 g/L, acetic acid 10 g/L and salts 0.25 M (total molarity of salts). These media were formulated to simulate the compositions of pre-hydrolysates frequently used in industrial processes [20, 21, 27-30]. The different types of phenolic compounds as well as the salts were introduced with the same concentration. In the case of single-compound solutions containing phenolic compounds as a sole carbon source in absence of salts, 2 g/L, 4 g/L and 8 g/L of phenolic compound were evaluated. These values were fixed based on the results reported from the detoxification of a mixture of inhibitors in this study.

Two sources of nutrients were first tested in mono-cultures: The first medium (Medium 1) contained a basal salt mixture (in g/L: $Na_2HPO_4$ 7, $KH_2PO_4$ 3, NaCl 0.5 and $NH_4Cl$ 1) and trace metal ions (in mg/L: $MgSO_4.7H_2O$ 580, $FeSO_4.7H_2O$ 7, $CaCl_2$.2H2O 53.6, $MnSO_4.H_2O$ 0.38, $CoCl_2.5H_2O$ 0.2 and $CuSO_4.5H_2O$ 0.043), while the second medium (Medium 2) contained (in g/L): yeast extract 5, bactopeptone 5, $K_2HPO_4$ 1 and $MgSO_4.7H_2O$ 0.2. A medium exhibiting higher performance of phenolic compounds degradation by different cultures was selected for the subsequent experiments.

#### 4.3.2.2 Hemicelluloses pre-hydrolysate preparation

The pre-hydrolysate was obtained by steam explosion coupled to hot water pretreatment of a mixture of 60% aspen and 40% maple. The generation of the liquid pre-hydrolysate was carried out in a pilot digestor at the FPInnovations facilities (Pointe-Claire, Québec, Canada). The details

of the pre-hydrolysis step can be found in the work of Ajao *et al.* [31]. The pre-hydrolysate was then delivered to the Centre National en Électrochimie et en Technologies Environnementales (CNETE). Its composition is shown in Table 4-1. The pH was adjusted from 3.5 to 7 with $Ca(OH)_2$ and the resultant medium was passed through a 1.5 µm filter to be used for the detoxification experiments.

Table 4-1 : Characterization of hemicellulosic pre-hydrolysate used in this study

| Components | Values |
|---|---|
| Total sugars (g/L) | 33 |
| Phenolic compounds (HPLC) (mg/L) | 155 |
| Total Organic Carbon (g/L) | 24.5 |
| Total Nitrogen (g/L) | 0.035 |
| Acetic acid (g/L) | 3.63 |
| Furfural (mg/L) | 518.3 |
| 5-HMF (mg/L) | 82.4 |
| Vanillin (mg/L) | 48.1 |
| Syringaldehyde (mg/L) | 109.5 |

### 4.3.3 Biodetoxification procedure

Biodetoxification experiments were performed in 500 mL baffled Erlenmeyer flasks with a 100 mL reaction volume. The medium was composed of a carbon source (synthetic inhibitors or hemicelluloses pre-hydrolysate) and a nutrients source, and cells were cultured at previously optimal temperature under agitation of 200 rpm in an orbital shaker (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA). The duration of the experiments was 24 h. All detoxification experiments were performed in duplicate and the results show the mean of the two values.

### 4.3.4 Analytical methods

To monitor cell growth, a UV-visible spectrophotometer (Eppendorf BioPhotometer plus) was used and the optical density was measured at 600 nm. A spectrophotometer (Pharmacia Biotech Novaspec®II, Piscatoway, NJ, USA) was used for the determination of reducing sugars' concentration at 570 nm using dinitrosalycilic acid (DNS) colorimetric method.

For bacterial enumeration, a "Petroff-hausser hemacytometer" (Fischer Scientific) with the recommended coverslip was used. A suspension culture was prepared using peptonized water

composed of 0.1% *m/v* peptone and 0.85% *m/v* NaCl. The suspension was adjusted for 20-100 cells/1 mm-square. Trypan Blue Solution (0.4%) is a dye obtained from Fisher Scientific which was used for the distinction between live and dead cells thanks to its absorption by dead cells and its exclusion by live cells. Cells were counted with bright light microscopy using the 40x objective. The four-corner group of 16 small squares of the hemacytometer were counted. Only cells located within squares considered or touching the left and lower edges of squares were enumerated. The number of cells per milliliter was calculated using the following Equation (1):

$$\text{Average count per square} \times \text{dilution factor} \times 8 \times 10^5. \quad (1)$$

And the mean deviation of three counts was determined.

The hemacytometer count method was compared to plate count method and results with both techniques are presented in 4.6.1 (Appendix A).

To investigate the variation of the relative number of bacteria in a co-culture with the initial ratio of the strains, defined volumes for each inoculum were mixed to a final volume of 1 mL. Thereafter, genomic DNA was extracted using Wizard Genomic DNA Purification Kit (PROMEGA (# item A1120). Country of origin: USA). NanoDrop™ 3300 Fluorospectrometer - Thermo Fisher Scientific (#ND-3300) was employed for DNA dosage before its amplification with the Quantitative Polymerase Chain Reaction (Q-PCR) approach using Fastmix reagent (PerfeCTa® SYBR® Green FastMix® Reaction Mixes, Quanta Biosciences). DNA amplification was conducted in MasterCycle Realplex de Eppendorf equipment. The forward and reverse primers used for Q-PCR are shown in Table 4-2.

Table 4-2 : Primers for DNA amplification of *C. taiwanensis* and *U. thermosphaericus* with Q-PCR

| **Primers** | ***C. taiwanensis*** | ***U. thermosphaericus*** |
|---|---|---|
| **Forward (sequence 5'-3')** | CGATACTGTCACCATCACCATTC (22 bp) | CACACACGCACGTATTCCTG (23 bp) |
| **Reverse (sequence 5'-3')** | GGTTTCGTTCGGATCGACATAG (21 bp) | CTTCACCGAGAAGGCGATTTA (22 bp) |

Prior to DNA amplification of the inoculum with Q-PCR, specificity tests of the primers were performed. The details of the optimisation tests are given in the section 4.6.2: Appendix B.

Phenolic compounds, furan aldehydes and acetic acid concentrations were determined with High Performance Liquid Chromatography (HPLC) (Agilent Technologies, Germany). In the case of phenolic compounds (gallic acid, catechol, vanillin and syringaldehyde), a diode array detector (DAD) at 313 nm and 280 nm and a Nucleosil C18 (150 X 4.6 mm) column were used. The solvent of separation 15% acetonitrile and 85% phosphoric acid 10 mM was fed into the system at 0.8 mL/min. 20 µL of the sample containing phenolic compounds were injected and mixed with the solvents then passed through C18 column at 35 °C. The retention times of gallic acid, catechol, vanillin and syringaldehyde were respectively 2.9 min, 5.5 min, 10.3 min and 12 min. Prior to the quantification of phenolic compounds by HPLC, standard solutions of gallic acid, catechol, vanillin and syringaldehyde were injected into the HPLC at known concentrations to establish a linear calibration curve with 0-10-50-150-200 mg/L for each phenolic compound. The chromatograms of the standards and phenolic compounds at an initial concentration of 8 g/L in synthetic medium before and after biodetoxification are shown in Section 4.6.24.6.3 (Appendix C). For furan aldehydes analysis by the same HPLC, a Nucleosil C18 (150 X 4.6 mm) column and a DAD at 280 nm were used. The elution solvent was a mixture of 84% of acetonitrile, 15% of deionized water and 1% of acetic acid. The acetic acid concentration was determined using Interstil ODS-3 column (150 X 4.6 mm). The flow rate of the solvent solution composed of 99% $KH_2PO_4$ and 1% acetonitrile was 1.25 mL/min and the column temperature was 40 °C.

The total nitrogen was determined by applying the Kjeldahl procedure with a Foss TecatorTM digester system and a KjettecTM 8200 auto distillation unit [32].

For measurement of the total organic carbon (TOC), an ASI-L autosampler and a TOC-L series analyzer from Shimadzu were used [14].

### 4.3.5 Statistical analyses

Statistical analyses were performed using Excel 365 software. Comparisons were done using a one-way analysis of variance (ANOVA) allowing the calculation of p-values to determine if results are significantly different.

## 4.4 Results and discussion

### 4.4.1 Optimisation of growth conditions on TSB medium for the detoxifying bacteria in pure and mixed cultures

#### 4.4.1.1 Effect of incubation temperature on cultures growth parameters

*C. taiwanensis* is able to grow at 28 °C, 30 °C and 37 °C [33], while the optimal temperature for *U. thermosphaericus* growth is 50 °C [21]. To develop an efficient co-culture, temperatures allowing approached growth and metabolism of different cultures should be determined.

Figure 4-1 shows growth curves and

Figure 4-2 illustrates pH curves of mono- and co-cultures at different reaction temperatures (37 °C, 41 °C and 45 °C). The variation of different parameters (kinetic parameters, C/N ratio, reducing sugars and ammoniacal nitrogen concentrations) after 24 h of incubation in TSB at different temperatures is shown in Table 4-3. The experiments described in this section were performed in duplicate and results are mean of two values.

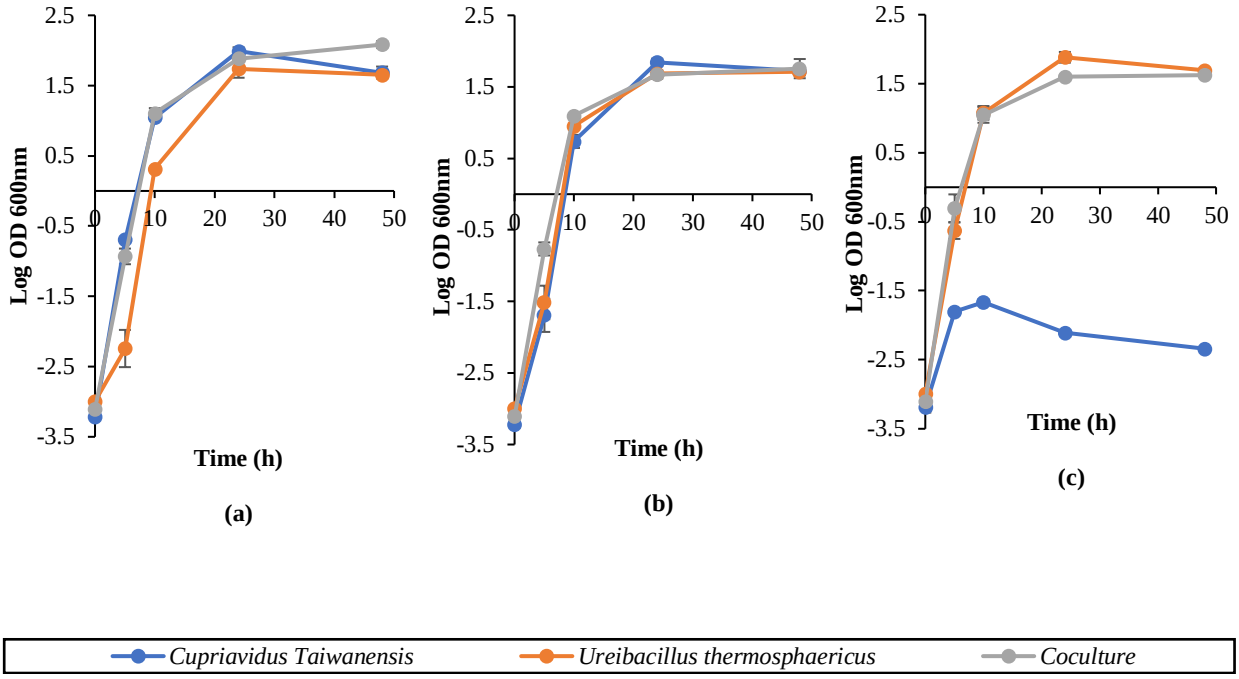



Figure 4-1 : Growth curves of mono- and co-cultures of *U. thermosphaericus* and *C. taiwanensis* represented at logarithmic scale at different incubation temperatures: (a) 37 °C; (b) 41 °C; (c) 45 °C. The growth medium was TSB (100 mL) with agitation at 180 rpm. The initial inoculum dose was 0.1% *v/v*.

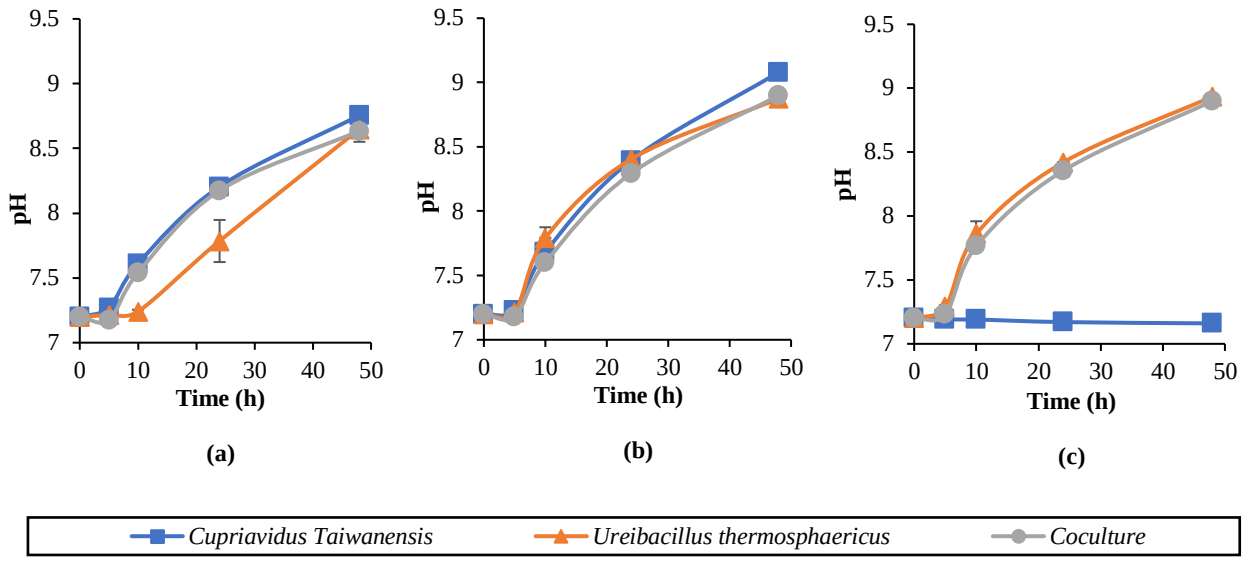



Figure 4-2 : Variation of pH of mono- and co-cultures composed of *U. thermosphaericus* and *C. taiwanensis* at different incubation temperatures (a) 37 °C; (b) 41 °C; (c) 45 °C. The growth medium was TSB (100 mL) with agitation at 180 rpm. The initial inoculum dose was 0.1% *v/v*.

Table 4-3 : Variation of different parameters after 24 h cultivation of mono- and co-cultures composed of *U. thermosphaericus* and *C. taiwanensis* at different incubation temperatures (37 °C, 41 °C and 45 °C). The growth medium was TSB (100 mL) with agitation at 180 rpm. The initial inoculum dose was 0.1% *v/v*.

| **Reaction temperature** | **Culture** | **Kinetic parameters** | | **C/N**[3] | **Glucose**[4] **(g/L)** | **Ammoniacal nitrogen production**[5] **(g/L)** |
|---|---|---|---|---|---|---|
| | | **$\mu_{max}$**[1] **($h^{-1}$)** | **$t_g$**[2] **(h)** | | | |
| 37 °C | *C. taiwanensis* | 0.43±0.01 | 1.63±0.02 | 3.15±0.05 | 2.32±0.07 | 0.26±0.00 |
| | *U. thermosphaericus* | 0.33±0.00 | 2.09±0.00 | 3.23±0.05 | 2.35±0.32 | 0.27±0.02 |
| | *Co-culture* | 0.42±0.02 | 1.65±0.09 | 3.85±0.00 | 2.27±0.14 | 0.30±0.05 |
| 41 °C | *C. taiwanensis* | 0.40±0.01 | 1.75±0.04 | 3.30±0.17 | 2.32±0.14 | 0.30±0.13 |
| | *U. thermosphaericus* | 0.39±0.00 | 1.76±0.01 | 3.31±0.01 | 2.27±0.07 | 0.36±0.02 |
| | *Co-culture* | 0.42±0.01 | 1.65±0.05 | 3.94±0.00 | 2.25±0.18 | 0.33±0.01 |
| 45 °C | *C. taiwanensis* | 0.28±0.01 | 2.50±0.12 | 4.04±0.12 | 3.00±0.00 | 0.17±0.03 |
| | *U. thermosphaericus* | 0.41±0.01 | 1.70±0.04 | 3.30±0.01 | 2.53±0.36 | 0.37±0.01 |
| | *Co-culture* | 0.42±0.00 | 1.67±0.02 | 3.73±0.23 | 2.42±0.72 | 0.38±0.00 |

[1] $\mu_{max}$ is the maximum specific growth rate of the strain and it corresponds to the slope of the linear part in the Ln (OD 600)=f(time) curve.

[2] $t_g$ is the generation time and was determined as $Ln2/\mu_{max}$.

[3] The initial C/N value was 3.89±0.06 prior to incubation.

[4] Residual glucose concentrations after 24 h. The initial concentration was 2.72 g/L prior to incubation.

[5] The production of ammoniacal nitrogen was calculated by the subtraction of its initial concentration from its final one.

At 37 °C, similar sigmoid curves of mono- and co-cultures were obtained. Although lag phases in

Figure 4-1 are not clearly distinguishable due to large sampling interval, a delay in growth for the mono-culture *U. thermosphaericus* in comparison with the other cultures can be noticed. *U. thermosphaericus* culture attained a maximum growth (OD $600_{nm}$: 5.7) after 24 h of incubation while maxima OD $600_{nm}$ of 7.33 and 8.04 were obtained respectively with *C. taiwanensis* and the co-culture after 24 h and 48 h respectively. As shown in Table 4-3, the growth rate of *U. thermosphaericus* at 37 °C was 0.33 $h^{-1}$ which was lower than those obtained with *C. taiwanensis* (0.43 $h^{-1}$) and the co-culture (0.42 $h^{-1}$). The generation time of *U. thermosphaericus* was 2.09 h compared to 1.63 h and 1.65 h with *C. taiwanensis* and the co-culture respectively. To better understand the metabolism of different cultures at these conditions, the variation of pH with the time was evaluated, as shown in

Figure 4-2. At the exponential phase, pH values increased significantly and at a higher extent in the mono-culture *C. taiwanensis* and the co-culture (

Figure 4-2). The pH increase might be attributed to the release of ammoniacal nitrogen, as observed in Table 4-3. Indeed, ammoniacal nitrogen can be produced through the degradation of peptone [34] contained in TSB. Adour *et al.* [35] demonstrated that peptone can be assimilated not only as a nitrogen source but also as a carbon source by *Geotrichum candidum* Geo17 and a similar metabolism can be hypothesized for our cultures. It has to be highlighted that ammoniacal nitrogen production was more pronounced with the co-culture compared to mono-cultures. These findings might be associated with the creation of a competitive environment in the presence of both strains, which could enhance the rate of carbon and nitrogen sources assimilation required for bacterial growth and maintenance. In terms of C/N values, the pure culture of *C. taiwanensis* exhibited lower C/N ratio than *U. thermosphaericus*. These findings could be explained by the higher carbon source (glucose and/or peptone) consumption with *C. taiwanensis*. With the co-culture, this ratio (C/N) remained constant after 24 h of incubation due to a higher residual TOC in the medium (8.82 g/L). This observation could be related to the high production of biomass with the co-culture. Sugars consumption (16.54%) with mixed cultures was higher in comparison with the results with pure cultures at 37 °C (Table 4-3). In summary, the reaction temperature (37 °C) was not favorable for *U. thermosphaercius* growth as deduced from the lower growth rate and the longer generation time.

Raising the incubation temperature to 41 °C was favorable for pure and mixed cultures to follow similar growth behaviors throughout the incubation time. In fact, from the growth curves at 41 °C represented in Figure 4-1, the maximum values of OD $600_{nm}$ obtained with mono- and co-cultures were close (averaged 5.65±0.14). Close growth rates around 0.4 $h^{-1}$ and generation times of about 1.75 h characterized pure cultures while the shortest generation time (1.65 h) was observed with the co-culture which may reveal the synergetic growth in mixed cultures. A similitude in pH curves of the different cultures was observed at 41 °C (Figure 4-2). Identically to 37 °C, pH increased significantly during the exponential phase due to the release of ammoniacal nitrogen. The ammoniacal nitrogen release was more pronounced at 41 °C than at 37 °C mostly with the pure culture *U. thermosphaericus* (Section 4.6.4: Appendix D. Table 4-7). With the co-culture, the highest C/N ratio (3.94) was obtained while the ratio was 3.3 in the case of pure cultures. This observation was due to lower final nitrogen in the co-culture than in pure cultures (2.23 g/L, 2.44 g/L and 2.32 g/L respectively in the co-culture, *C. taiwanensis* and *U. thermosphaericus* mono-cultures) which reveals the higher consumption of nitrogen in the co-culture.

The temperature increase to 45 °C led to the inhibition of *C. taiwanensis* growth. In fact, the growth rate decreased from 0.4 $h^{-1}$ at 37 °C and 41 °C to 0.28 $h^{-1}$ at 45 °C. The generation time, in its turn, increased from 1.63 h at 37 °C and 1.75 h at 41 °C to 2.5 h at 45 °C. Moreover, glucose was not consumed by *C. taiwanensis* at 45 °C as its concentration remained almost constant (Table 4-3). This inhibition may be attributed to unfavorable operating conditions for *C. taiwanensis* growth, which is a mesophilic bacterium growing optimally at moderate temperatures [24, 32, 35]. In the case of *U. thermosphaericus* culture, the parameters were still identical to those reported at 41 °C except for glucose consumption, which was reduced from 16.5% at 41 °C to approximately 7% at 45 °C. The maximum OD $600_{nm}$ of the mono-culture *U. thermosphaericus* at 45 °C was 6.58 obtained after 24 h. This value was higher than those obtained at 37 °C (5.71 after 24 h) and at 41 °C (5.54 after 48 h), albeit glucose consumption was reduced. This observation could be related to lower final TOC in the culture at 45 °C (7.61 g/L) which might be explained by higher consumption of peptone as carbon source for energy production in favorable operating conditions (45 °C). Regarding the co-culture metabolism at 45 °C, C/N ratio was reduced comparing to the results at 37 °C and 41 °C, albeit the final sugars concentration was higher (Table 4-3). In fact, at 45 °C, the co-culture exhibited similar metabolism as the mono-culture *U. thermosphaericus* regarding the growth and pH evolution, sugars consumption and ammoniacal nitrogen production. The co-culture

was thus dominated by *U. thermosphaericus* which could explain the decline in C/N ratio although the decrease in sugars consumption (due to peptone consumption as carbon source).

Based on these results, the mono- and co-cultures exhibited a high similitude for most kinetic properties at 41 °C. This reaction temperature was thus considered favorable for the co-cultivation of the two strains.

#### 4.4.1.2 Effect of initial bacteria ratio on the relative number in co-culture

In order to fix an equal initial number of strains in the co-culture, the starting ratio of bacteria was varied (Figure 4-3). The relative proportion of each strain in the co-culture was determined by the Q-PCR approach. The experiments were performed in duplicate and results are mean of two values.

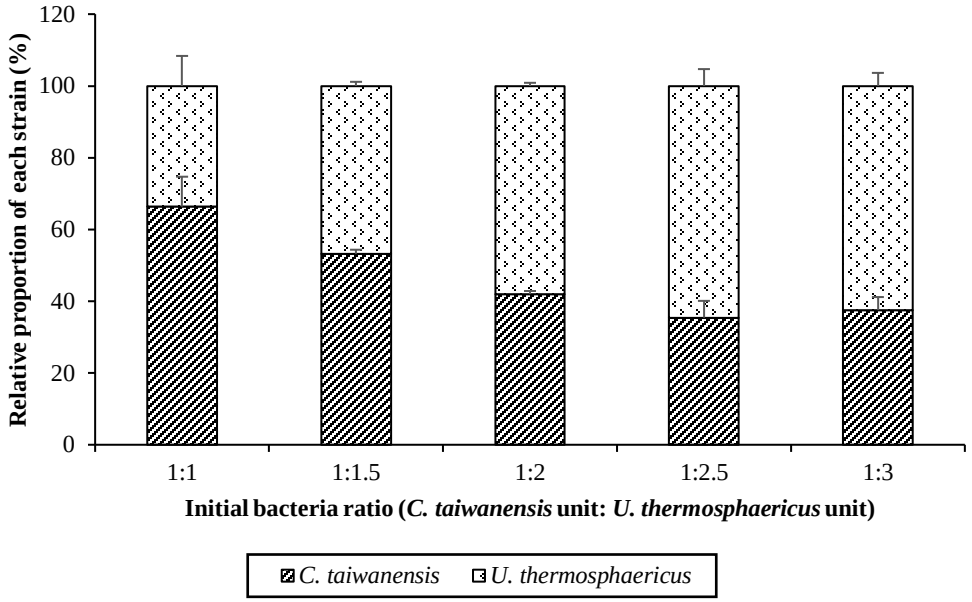



Figure 4-3 : Variation of the relative proportion of each strain with the starting bacteria ratio. The co-culture was composed of inoculum obtained from 10 h cultures (leading to equal cells number determined by hemacytometer method) on TSB at 37 °C for *C. taiwanensis* and 50 °C for *U. thermosphaericus* with agitation at 180 rpm. One unit was defined as a volumetric unit.

A "Petroff-hausser hemacytometer" was used in this study for bacteria enumeration. From the results presented in Figure 4-3, we can conclude that equal volumes of inocula (1-unit *C. taiwanensis* :1-unit *U. thermosphaericus*) did not lead to equal relative proportions in the co-culture. Although hemacytometer counting was more precise and rapid than plate count procedure, some imperfections characterize this method. In fact, au underestimation of the bacteria number

may be due to cells aggregation and inaccuracies of dilution and pipetting [37, 38]. Besides, the bacteria studied remained mobile after heating the suspension at 95 °C for 15 min. Errors in counting were thus likely to occur by visual observations [39, 40]. Figure 4-3 shows that a starting ratio of 1-unit *C. taiwanensis* :1.5-unit *U. thermosphaericus* gave an initial relative number close to 50%/50% in the co-culture. This ratio was thus selected for detoxification procedure with mixed cultures.

### 4.4.2 Detoxification procedure with mono- and co-culture

#### 4.4.2.1 Effect of nutrients source on the detoxification of a mixture of inhibitors

In this part of the study, two sources of nutrients, the composition of which were detailed in the "Material and methods" section, were tested for their impact on the performance of synthetic media detoxification with pure cultures *U. thermosphaericus* and *C. taiwanensis*. Synthetic media were composed of phenolic compounds introduced at different concentrations (0.3 g/L, 2.8 g/L and 8 g/L), furfural 2 g/L, acetic acid 10 g/L and salts 0.25 M.

Figure 4-4 shows the results for the degradation of phenolic compounds with pure cultures using different nutrients sources. At low concentration of phenolics (0.3 g/L) and in presence of basal salt medium and trace metal ions (medium 1) as nutrient source, both cultures (*C. taiwanensis* and *U. thermosphaericus*) exhibited weak performance of detoxification. In fact, the degradation of phenolic compounds did not exceed 13% with *U. thermosphaericus*. A plausible explanation of this observation was that the inhibitors were at a concentration that was below the threshold for expression of the detoxification genes. In presence of the nutrient source containing yeast extract, bactopeptone, $K_2HPO_4$ and $MgSO_4 \cdot 7H_2O$ (medium 2), negative values of removal were obtained, as illustrated in Figure 4-4. These negative values may be attributed to intermediates generated which have the same retention time as phenolics. Incomplete mineralisation could lead to the accumulation of intermediates in the medium. To confirm these assumptions, detoxification at higher concentrations of phenolic compounds were tested with both mediums.

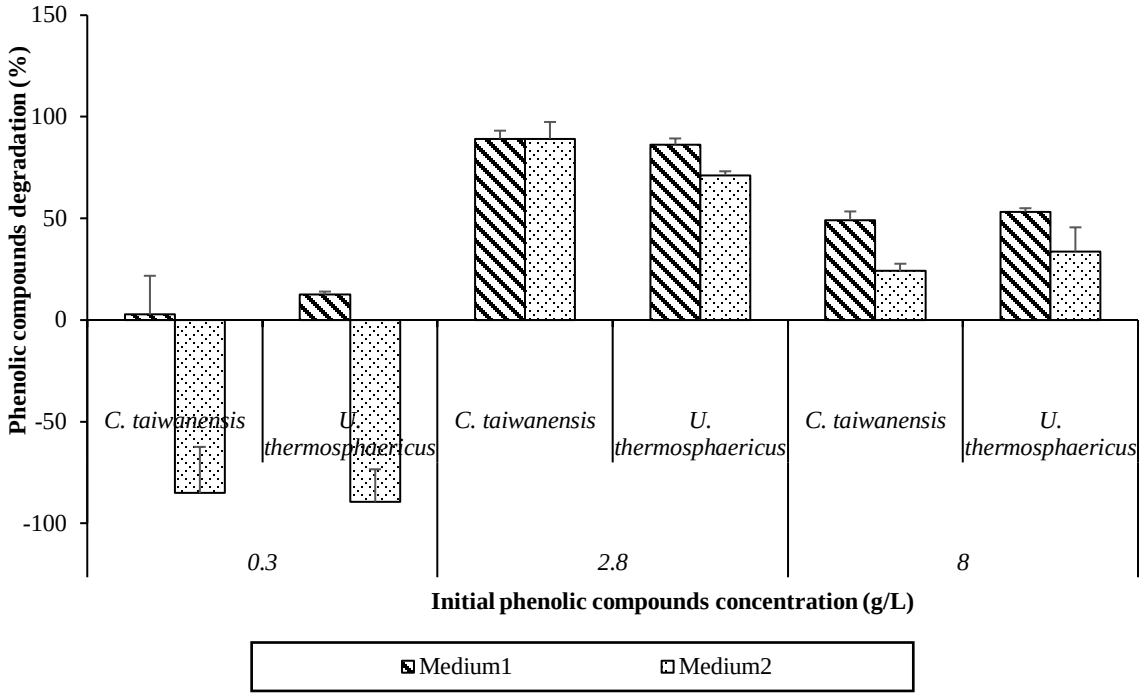



Figure 4-4 : Effect of nutrient sources (Medium 1 (basal salt medium and trace metal ions) and Medium 2 (yeast extract, bactopeptone, $K_2HPO_4$ and $MgSO_4 \cdot 7H_2O$)) on the performance of phenolic compounds degradation with *U. thermosphaericus* and *C. taiwanensis* at 41 °C and 200 rpm. The detoxification media were composed of: phenolic compounds introduced at different concentrations (0.3 g/L, 2.8 g/L and 8 g/L), furfural 2 g/L, acetic acid 10 g/L, salts 0.25 M and the nutrient source.

Raising the concentration of phenolic compounds to 2.8 g/L led to a considerable increase of the cultures' performances in terms of phenolics' degradation with both media. In fact, removal efficiency was close to 90% with *C. taiwanensis* in both mediums (Figure 4-4). With the *U. thermosphaericus* culture, the removal percentages were 86% and 71% in presence of medium 1 and medium 2 respectively. In conclusion, a significant decrease of phenolics was noticed at higher concentration of the inhibitor (2.8 g/L) compared to the results from cultures containing lower concentration (0.3 g/L). It proves that phenolics can act as a substrate for bacteria to stimulate the degradation of inhibitors and strengthens the previous assumption that lower concentrations of phenolics in the medium were insufficient to trigger biodetoxification.

By raising further the concentration of phenolic compounds to 8 g/L, high percentages of removal were also achieved. Indeed, 52% of phenolics were removed with *C. taiwanensis* using basal salt medium and trace metal ions as nutrients source (medium 1). Close removal values were obtained with *U. thermosphaericus* (55%) under the same operating conditions (8 g/L of phenolic compounds and medium 1 as nutrient source). In medium 2 (containing yeast extract, bactopeptone, $K_2HPO_4$ and $MgSO_4 \cdot 7H_2O$) the bacteria degraded phenolic compounds to a lesser extent: 24% and 34% for *C. taiwanensis* and *U. thermosphaericus* respectively (Figure 4-4). The better performance of degradation in medium 1 could be attributed to the presence of heavy metals for the detoxification procedure. Indeed, metallic ions can control osmotic pressure and stimulate the production of degrading enzymes. Furthermore, they participate in the preservation of the cell structure [41]. Farnet *et al.* [42] claim that sulfate salts as $CuSO_4$ and $MnSO_4$ lead to an increase in laccase activity as they can be cofactors for the enzymes. The positive ions can act as mediators required for the degradation of recalcitrant compounds. The $Cu^{2+}$ ions are known to act as potent catalysts in oxidation reactions [43]. They participate in the formation of phenoxy radicals, thus triggering degradation reactions [44]. Loow et al. [45] stated that metal ions may contribute to bond rupture thanks to the abundance of positive charges to pair with electrons. Medium 1 was thus selected for further experiments. It is important to notice that results of phenolic compound removal reported at 2.8 g/L (nearly 88% degraded in medium 1) were better than those obtained at 8 g/L (around 52% removal in medium 1). These results might be due to inhibitory concentrations of phenolic compounds at 8 g/L.

Once the optimal nutrients source was determined with mono-cultures, simultaneous and sequential (inoculation interval of 12 h) cultures of *C. taiwanensis* and *U. thermosphaericus* were tested at the optimal conditions (at 41 °C and in presence of medium 1) to detoxify mixed and single-compounds solutions of phenolic compounds.

#### 4.4.2.2 Effect of co-culture type on the detoxification of a mixture of inhibitors

In this part of study, we tested the simultaneous and sequential inoculations of bacteria in the co-culture for the detoxification of synthetic media composed of different concentrations of phenolic compounds (0.3 g/L, 2.8 g/L and 8 g/L), furfural (2 g/L), acetic acid (10 g/L) and salts (0.25 M).

Figure 4-5 shows the results for the degradation of phenolic compounds with different co-cultures at different initial concentrations. At 0.3 g/L, the concentration increased to 0.7 g/L and 0.8 g/L

respectively with *C. taiwanensis* followed by *U. thermosphaericus* and *U. thermosphaericus* followed by *C. taiwanensis* (Figure 4-5). The concentration of phenolic compounds determined by HPLC prior to inoculation was around 0.5 g/L. Simultaneous cultures, contrarily to sequential cultures, exhibited a decrease in phenolic compounds concentration to 0.4 g/L corresponding to a decline by 16.42%.

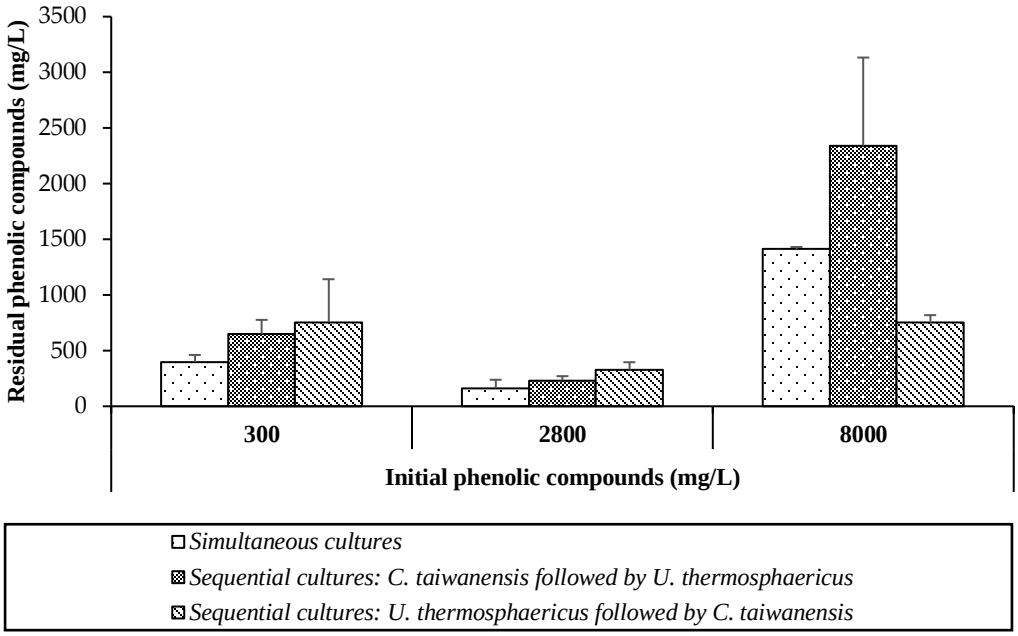



Figure 4-5 : Degradation of phenolic compounds (mg/L) introduced at different concentrations (0.3 g/L, 2.8 g/L and 8 g/L) in a mixture of inhibitors (furfural 2 g/L, acetic acid 10 g/L and salts 0.25 M) by simultaneous and sequential (inoculation interval: 12 h) cultures of *U. thermosphaericus* and *C. taiwanensis*. The cultures were incubated at 41 °C with agitation at 200 rpm.

To better understand the metabolism of the cells explaining these observations, the degradation of each phenolic compound in the mixture was evaluated. At 0.3 g/L of total phenolic compounds, gallic acid was almost totally removed after 24 h of treatment with different bacteria associations (Figure 4-6a). Syringaldehyde was degraded to a lower extent (46%, 49% and 7% respectively with simultaneous cultures, *C. taiwanensis* followed by *U. thermosphaericus* and *U. thermosphaericus* followed by *C. taiwanensis*). In terms of vanillin degradation, its concentration decreased by 3% and 32% respectively with simultaneous cultures and *C. taiwanensis* followed by *U. thermosphaericus*. While, it rose to 62 mg/L with *U. thermosphaericus* followed by *C. taiwanensis*

which corresponded to an increase by 17% from the initial concentration of vanillin. Catechol concentration was slightly reduced with simultaneous cultures (5%) even increased to 0.6 g/L and 0.7 g/L respectively with *C. taiwanensis* followed by *U. thermosphaericus* and the inverse sequence (Figure 4-6a). Overall, at 0.3 g/L of phenolic compounds, simultaneous cultures showed the increased affinity to: vanillin<catechol<syringaldehyde<gallic acid, while sequential cultures showed a higher preference to gallic acid followed by syringaldehyde, then vanillin and at lesser extent catechol. The gallic acid was almost totally removed with the different co-cultures while catechol, vanillin and syringaldehyde were better removed respectively with simultaneous cultures (5%), *C. taiwanensis* followed by *U. thermosphaericus* (32%) and *C. taiwanensis* followed by *U. thermosphaericus* (49%). The increase of phenolic compound concentration in this study, mostly of catechol, is similar to the results obtained in the study of Ajao *et al.* [46], where the efficiency of detoxification of phenolic compounds in single-compounds solutions or in a mixture was tested using laccase enzymes. The authors stated complete removal of gallic acid followed by syringaldehyde. However, vanillin and catechol were accumulated and negative values were reported [46]. A possible explanation of these findings is that gallic acid contains more hydroxyl group in its structure than other phenolics (syringaldehyde, vanillin and catechol) which makes it more accessible to enzymes produced by bacteria. Hence, it was observed that gallic acid was preferentially degraded by the different mixed cultures followed by syringaldehyde containing two methoxy groups, one hydroxyl group and one aldehyde group. An explanation for the apparent increase in vanillin concentration and catechol by the different consortia could be related to the formation of intermediates having the same retention times as the initial compounds.

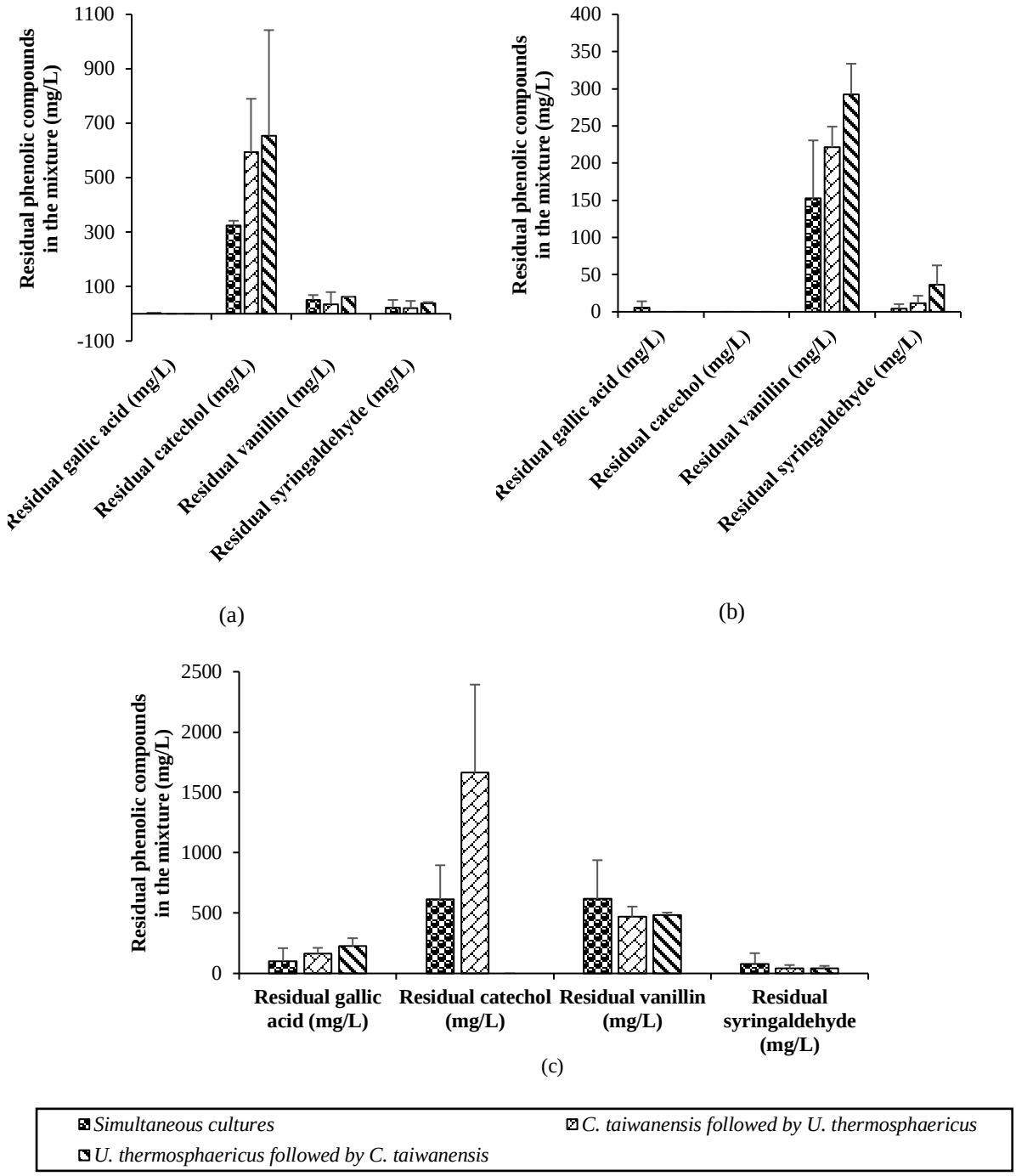



Figure 4-6 : Biodegradation of the different types of phenolic compounds (gallic acid, catechol, vanillin and syringaldehyde) in a mixture of inhibitors (furfural 2 g/L, acetic acid 10 g/L and salts 0.25 M) by simultaneous and sequential cultures of *U. thermosphaericus* and *C. taiwanensis* (inoculation interval: 12 h). The cultures were incubated at 41 °C with agitation at 200 rpm: (a) [Total phenolic compounds] =0.3 g/L; (b) [Total phenolic compounds] =2.8 g/L; (c) [Total phenolic compounds] =8 g/L.

By increasing the initial concentration of phenolic compounds to 2.8 g/L, higher degradation values up to 90%, 86% and 81% with simultaneous cultures, *C. taiwanensis* followed by *U. thermosphaericus* and *U. thermosphaericus* followed by *C. taiwanensis* were respectively obtained (Figure 4-5). The change of each phenolic compound concentration in the mixture is represented in Figure 4-6b. In fact, catechol and gallic acid were almost completely removed with the different co-cultures, followed by syringaldehyde and finally by vanillin. Syringaldehyde was best removed with simultaneous cultures and the sequence *C. taiwanensis* followed by *U. thermosphaericus* (around 97%). In the case of vanillin, higher degradation was obtained by simultaneous cultures (58%) then by sequential cultures *C. taiwanensis* followed by *U. thermosphaericus* (39%) and sequential cultures *U. thermosphaericus* and *C. taiwanensis* (20%) (Figure 4-6b). The lower percentages of vanillin degradation compared to the other phenolics reported in this study could be related to the structure of vanillin. Indeed, it contains one hydroxyl group and one methoxy group, thus a small number of oxidable groups. The detoxification performance of the co-cultures (in terms of phenolic compounds degradation) was close to the pure culture (Figure 4-4).

Further increase of the phenolic compounds concentration to 8 g/L led to 76%, 60% and 87% degradation with respectively simultaneous cultures, sequential cultures *C. taiwanensis* followed by *U. thermosphaericus* and sequential cultures *U. thermosphaericus* followed by *C. taiwanensis* (Figure 4-5). The compositional analysis of the mixture is provided in Figure 4-6c. Higher performances of gallic acid removal were obtained with simultaneous cultures and *C. taiwanensis* followed by *U. thermosphaericus* with an average of 92%±2.6. With the sequence of *U. thermosphaericus* followed by *C. taiwanensis*, gallic acid concentration decreased to around 226 mg/L, which corresponded to 87% decline from its initial values. Regarding the degradation of catechol, total removal was obtained with the sequence *U. thermosphaericus* followed by *C. taiwanensis* after 24 h, while 63% were degraded with simultaneous cultures. With the sequence *C. taiwanensis* followed by *U. thermosphaericus*, a change in catechol concentration was not observed (Figure 4-6c) with respect to the initial concentration (1.6±0.8 g/L). Syringaldehyde was degraded to the same extent with the different types of consortia (94%±2.3). Thus, at 8 g/L of phenolic compounds, the simultaneous cultures had a high affinity to syringaldehyde and gallic acid followed by catechol then vanillin. Under the same conditions (8 g/L as initial concentration of phenolic compounds), *C. taiwanensis* followed by *U. thermosphaericus* had a preference to syringaldehyde and gallic acid followed by vanillin and finally catechol. Sequential cultures of *U.*

*thermosphaericus* followed by *C. taiwanensis* showed higher preference for catechol, followed by syringaldehyde, then gallic acid and finally vanillin. It is important to note the increase in the degradation of phenolic compounds with the different types of co-cultures in comparison with the pure cultures (49% with *C. taiwanensis* and 53% with *U. thermosphaericus*) at 8 g/L of initial phenolic compounds. The compositional analysis of the different cultures showed similar performances of gallic acid, syringaldehyde and vanillin degradation with mono- and co-cultures (Section 4.6.4: Appendix D. Figure 4-28). Catechol removal was more pronounced with mixed cultures (i.e. Simultaneous cultures (63%) and *U. thermosphaericus* followed by *C. taiwanensis* (100%)) than pure cultures, which exhibited negative percentages (Section 4.6.4: Appendix D. Figure 4-28). These findings are important and highlight the interactions between the strains in the co-culture to degrade a broader range of inhibitors. It is worthy to note that from chromatograms of phenolic compounds in the culture media (see Section 4.6.3: Appendix C), new pics appeared revealing the generation of new products after biodegradation. However, the formed products were not identified due to the lack of certain standards in the laboratory research.

In summary, similarly to the results of degradation of phenolic compounds with mono-cultures, the co-cultures showed higher performance of detoxification at 2.8 g/L as initial concentration of phenolics, followed by 8 g/L and finally by 0.3 g/L where there is an apparent accumulation of percentages of removal of phenolics observed. In fact, as explained above, high concentration of phenolic compounds (8 g/L) could inhibit bacteria. At low concentration (0.3 g/L), bacterial competition could occur in the co-cultures due to substrates starvation (i.e. phenolic compounds).

In the first sets of detoxification experiments of synthetic media, the removal of phenolic compounds when mixed with other inhibitors (furfural, acetic acid and salts) was studied. However, it is important to determine the performance of mono- and co-cultures to detoxify individual phenolic compounds. Hence, the effects of the other inhibitors on the performance of phenolic compounds biodegradation could be determined. Detoxification experiments of single-component solutions of phenolic compounds with pure and mixed cultures were carried out.

#### 4.4.2.3 Detoxification of individual phenolic compounds

To investigate the performance of pure and mixed cultures for individual phenolic compound degradation, single-component solutions composed of different concentrations of phenolics (2 g/L, 4 g/L and 8 g/L) and nutrients source (Medium 1) were prepared. It should be noted that the co-culture type used for the detoxification of each single-component solution was selected based on best results reported in the mixture. Indeed, sequential inoculation of *U. thermosphaericus* followed by *C. taiwanensis* was performed for catechol solutions detoxification, while simultaneous cultures were applied for gallic acid, vanillin and syringaldehyde degradation.

Figure 4-7a gives a comparison of residual gallic acid in different bacterial cultures (mono- and co-cultures (i.e. simultaneous cultures)). At 2 g/L, gallic acid was completely removed with *U. thermosphaericus* and the co-culture. *C. taiwanensis* eliminated gallic acid to a final concentration of 206 mg/L. At 4 g/L, around 107 mg/L, 46 mg/L and 37 mg/L remained in *C. taiwanensis* and *U. thermosphaericus* pure cultures and in the co-culture respectively. At 8 g/L, we observed higher gallic acid concentration remained in pure cultures after 24 h of treatment: 821 mg/L and 259 mg/L with *C. taiwanensis* and *U. thermosphaericus* respectively. However, the co-culture exhibited lower final concentration of gallic acid than pure cultures (9 mg/L). In conclusion, simultaneous cultures showed higher performance of gallic acid removal at different initial concentrations of the inhibitor. Percentages removal with simultaneous cultures of individual gallic acid (around 100%) were higher than those obtained in a mixture of inhibitors containing a total concentration of phenolics of 8 g/L (90%) (Figure 4-6c). This result demonstrates high preference of the co-culture to gallic acid.

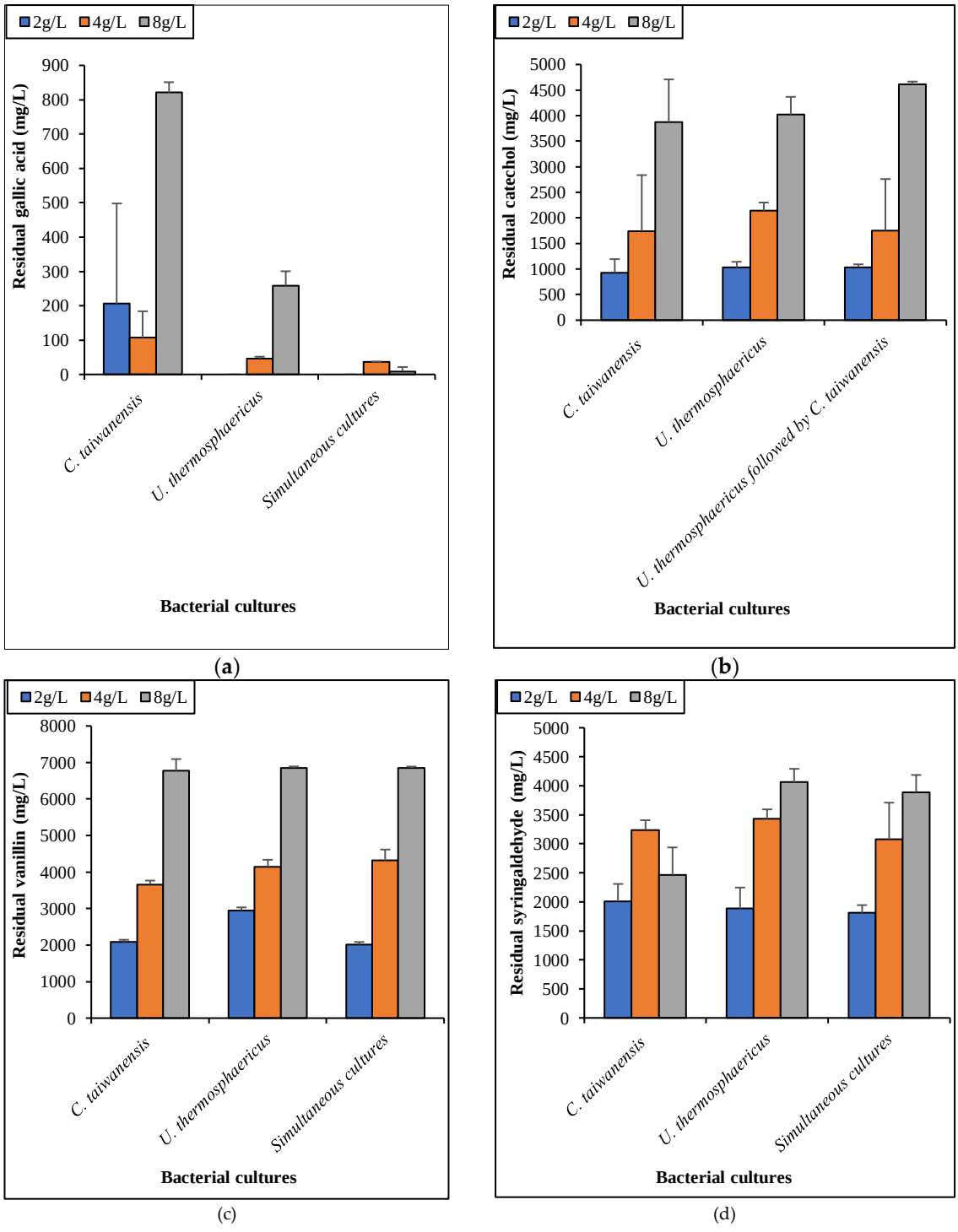



Figure 4-7 : Biodegradation of individual phenolic compounds by pure cultures (*U. thermosphaericus* and *C. taiwanensis*) and by their optimized co-culture. The cultures were incubated at 41 °C with agitation at 200 rpm: (a) residual gallic acid (mg/L); (b) residual catechol (mg/L); (c) residual vanillin (mg/L); (d) residual syringaldehyde (mg/L).

Figure 4-7b shows remaining catechol after 24 h of treatment with mono- and co-cultures (i.e. sequential cultures *U. thermosphaericus* followed by *C. taiwanensis* with an interval inoculation of 12 h) at different initial concentrations of the phenolic compound. At 2 g/L, pure and mixed cultures degraded catechol to a similar extent to an average of 997 mg/L. Nevertheless, catechol was totally eliminated with this sequence of cultures in a mixture of phenolic compounds where the total concentration was 2.8 g/L (Figure 4-6b). Increasing the initial concentration of catechol to 4 g/L gave similar performance of degradation with the mono-culture *C. taiwanensis* and sequential cultures *U. thermosphaericus* followed by *C. taiwanensis* with an average value of residual catechol of 1747 mg/L. At 8 g/L as initial concentration of catechol, pure cultures showed higher performance of degradation than the co-culture where residual catechol after 24 h of treatment was around 4609 mg/L; notwithstanding, catechol was completely removed from a mixture of total phenolic compounds with an initial concentration of 8 g/L (Figure 4-6c). It is worthy to note that new products could be generated (see Section 4.6.3: Appendix C). However, the formed products were not identified and their toxicity was not verified in this study. A possible explanation of these results is the requirement of bacteria for salts and to other carbon sources in the medium (vanillin, syringaldehyde, gallic acid, furfural, etc.) to stimulate higher production of enzymes degrading inhibitors.

Figure 4-7c shows residual vanillin after 24 h of treatment with mono- and co-cultures (i.e. simultaneous cultures) at different initial concentrations. At 2 g/L, the remaining concentration of vanillin after treatment with *C. taiwanensis* and the co-culture was 2047 mg/L. It is noteworthy that the final concentration of vanillin obtained with the co-culture in single-component solutions was 13-fold higher than that reported in a mixture of inhibitors (Figure 4-6a). A concentration of 2949 mg/L of vanillin were left in the mono-culture *U. thermosphaericus* at the same initial concentration of 2g/L. These values were close to the initial concentration of vanillin introduced, revealing the inability of these strains to counteract the inhibitory effects of the individual phenolic compound. At 4 g/L of vanillin, lower amounts remained in the mono-culture *C. taiwanensis* (3656 mg/L) in comparison with the culture of *U. thermosphaericus* (4144 mg/L) and the co-culture (4320 mg/L). At 8 g/L, the different cultures removed vanillin almost at the same extent until a final concentration of around 6824 mg/L after 24 h. It is important to highlight that the final concentration of vanillin contained in a mixture of inhibitors with an initial concentration of phenolic compounds of 8 g/L was approximately 617 mg/L (Figure 4-6c) after 24 h of treatment

with the co-culture. The obtained value was 11-fold lower than the value reported in single-components solution at the same operating conditions (at 41 °C and in presence of 8 g/L of phenolic compounds). These results confirm the need of bacteria from other carbon sources (furfural, other phenolic compounds, etc.) in the medium to stimulate the production of enzymes degrading inhibitors.

Figure 4-7d shows the residual syringaldehyde, following the treatment with different bacterial cultures (mono- and co-cultures (i.e. simultaneous cultures)) at different initial concentrations (2 g/L; 4 g/L and 8 g/L). At 2 g/L, the final concentrations of syringaldehyde after bacterial treatments were 2009 mg/L, 1886 mg/L and 1810 mg/L respectively with *C. taiwanensis*, *U. thermosphaericus* and the co-culture. These values were close to the initial concentration of syringaldehyde in the medium (2 g/L), demonstrating a low performance of degradation at this inhibitory level. Only 4.27 mg/L of syringaldehyde remained from 2.8 g/L of phenolics in a mixture of inhibitors after 24 h of treatment with simultaneous cultures. This result confirms the requirement of other types of inhibitors as carbon sources. In fact, genome sequencing of *C. taiwanensis* demonstrated the ability of the strain to produce catalase and oxidase enzymes [33]. For the strain of *U. thermosphaericus*, laccase, peroxidase and catalase enzymes can be produced [47]. These enzymes participate in the oxidation of inhibitors. At 4 g/L, 3239 mg/L of syringaldehyde remained after treatment with *C. taiwanensis*, 3431 mg/L following biodetoxification with *U. thermosphaericus* and 3079 mg/L with simultaneous cultures. These residual concentrations are close to the initial values, indicating a low performance of detoxification with the different cultures in the presence of 4 g/L of syringaldehyde in the medium. At 8 g/L, a reduction of syringaldehyde concentration to 2466 mg/L with *C. taiwanensis*, 4062 mg/L with *U. thermosphaericus* and 3892 mg/L with simultaneous cultures was observed. Compared to the results from lower initial concentrations of syringaldehyde (2 g/L and 4 g/L), degradation performances with the different cultures at 8 g/L were higher (Figure 4-7d). This observation was in agreement with the results obtained in a mixture of inhibitors. In fact, phenolic compounds were potential substrates for the studied bacteria requiring higher concentrations for their metabolism. Furthermore, it was observed that, at 8 g/L of syringaldehyde, *C. taiwanensis* exhibited higher performance of degradation than *U. thermosphaericus* and the co-culture. This could be explained by the higher affinity of the strain and its enzymes to syringaldehyde at high concentration (i.e. 8 g/L). Yet, the performance of biodegradation in single-component solutions is

still lower than that observed in a mixture containing 8 g/L of total phenolic compounds where an average of 94% of syringaldehyde were eliminated.

To summarize, in single-component solutions, bacterial cultures degraded preferentially gallic acid, in particular at initial concentrations of 2 g/L and 4 g/L. The second most readily degraded substrate was catechol. Weak performances of the different cultures in terms of degradation of vanillin at different concentrations and syringaldehyde at 2 g/L and 4 g/L were observed. These concentrations were inhibitory to bacteria in absence of inducers of degrading enzymes production. In fact, the presence of phenolics, furfural, salts, etc. creates a synergy effect, stimulating the production of enzymes [46] or reacting directly (as in the case of salts) with phenolics to generate radicals and then initiate oxidation reactions [48]. The higher affinity to gallic acid, followed by catechol, then syringaldehyde and vanillin could be related to the number of alcohol groups targeted by laccase enzymes. Reduced molecules are oxidized to free radicals, contributing to further polymerisation or degradation [49]. These observations led the study of the performance of mono- and co-cultures to detoxify the hemicelluloses pre-hydrolysate containing a mixture of inhibitors.

### 4.4.3 Optimization of hemicelluloses pre-hydrolysate detoxification

In this part of the study, the performance of the different cultures, tested for the detoxification of synthetic media was evaluated in the case of non-diluted hemicelluloses pre-hydrolysate detoxification. The pre-hydrolysate was obtained from steam explosion coupled with hot water pretreatment. Pure cultures (*C. taiwanensis* and *U. thermosphaericus*) and their co-cultures (simultaneous cultures and sequential cultures with an interval inoculation of 12 h) were used for biodetoxification. The concentration of the different types of phenolic compounds (gallic acid, catechol, vanillin and syringaldehyde) was determined by HPLC before and after treatment of 24 h and the percentages of removal at 41 °C and 200 rpm were calculated. The results were reported in Figure 4-8.

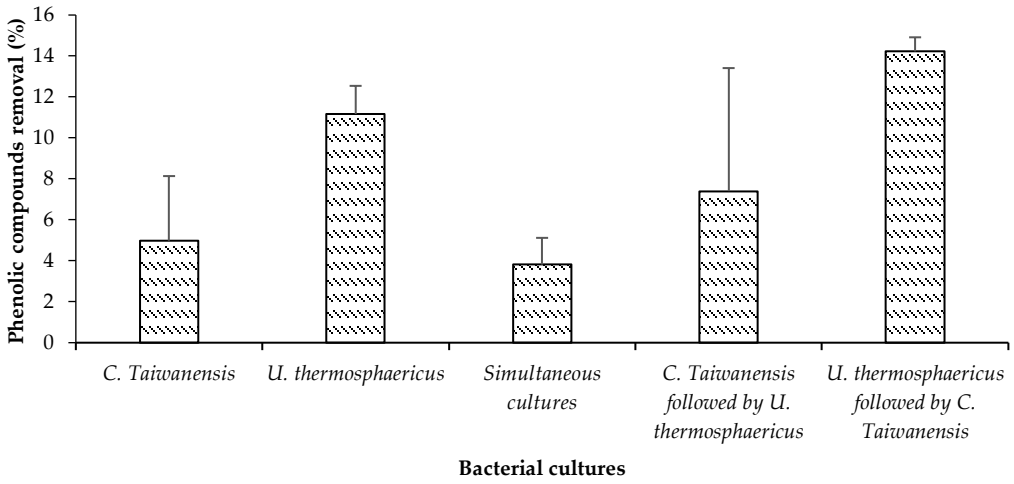



Figure 4-8 : Degradation of phenolic compounds in non-diluted hemicelluloses pre-hydrolysate by pure and mixed cultures. The cultures were incubated at 41 °C with agitation at 200 rpm for 24 h. Experiments were performed in duplicates ($p \leq 0.05$).

With the mono-culture *C. taiwanensis*, a 5% reduction of the phenolic compounds was obtained after 24 h. This percentage was doubled by applying the pure culture *U. thermosphaericus*. Inoculating both strains simultaneously contributed to 3.81% of degradation. Whereas, by inoculating bacteria sequentially, degradation of phenolics increased to 7.4% and 14 % respectively with the sequence *C. taiwanensis* followed by *U. thermosphaericus* and the sequence *U. thermosphaericus* followed by *C. taiwanensis*. *U. thermosphaericus* proved its higher performance of degrading phenolic compounds in the pre-hydrolysate than *C. taiwanensis*. To the best of our knowledge, we investigated for the first time in this study the ability of degrading monomeric phenolic compounds with the strain *U. thermosphaericus*. Okuda *et al.* [20] used this bacterium for the detoxification of wood hydrolysate and reported only furfural and HMF degradation results. The poor degradation performance of *C. taiwanensis* may be related to an intolerance of this strain to the concentration of phenolic compounds in the pre-hydrolysate. In fact, *C. taiwanensis* was investigated for the phenol degradation and a maximum of 900 mg/L [22], 2200 mg/L [24] and 3200 mg/L [23] were degraded with batch culture supplemented with glycerol, fed-batch and continuous culture respectively. The initial concentration of phenolic compounds in the pre-hydrolysate used in our study was 155 mg/L as determined by HPLC. A possible explanation of this result was the co-presence of other inhibitors in the pre-hydrolysate at high concentration among others furfural, acetic acid, salts, 5-HMF, etc. as detailed in Table 4-1 and synergetic

inhibition could exist. The lowest results of degradation were obtained when inoculating simultaneously both strains despite the better removal with *U. thermosphaericus* inoculated individually. This finding might be due to the dominance of *C. taiwanensis* characterized by its low detoxification ability of the pre-hydrolysate preventing thus the contributions of *U. thermosphaericus*. Furthermore, in such mixed cultures, a bacterial death of *C. taiwanensis* was expected due to extreme inhibitory environment. Dead cells limited the ability of *U. thermosphaericus* to access to its substrates. The best results obtained with the sequence *U. thermosphaericus* followed by *C. taiwanensis* might be attributed first to the access of *U. thermosphaericus* and its enzymatic system to nutriments and phenolic compounds and secondly to the degradation of toxic compounds to less toxic products or to non-inhibitory concentration for *C. taiwanensis*. To better understand the metabolism of phenolic compounds, the phenolics in the treated pre-hydrolysate were characterized and the results were illustrated in

Figure 4-9. 4.65% and 5.19% of vanillin and syringaldehyde were respectively removed using the mono-culture of *C. taiwanensis*. *U. thermosphaericus* removed 16.2% of vanillin and 9% of syringaldehyde, whereas the simultaneous cultures eliminated 1.85% of vanillin and 4.67% of syringaldehyde. Sequential cultures *C. taiwanensis* followed by *U. thermosphaericus* led to 12.3% and 5.22% of vanillin and syringaldehyde elimination. Better results were obtained with the sequential cultures *U. thermosphaericus* followed by *C. taiwanensis* where vanillin and syringaldehyde removal were respectively 27 % and 11%. From these findings, *U. thermosphaericus* mono-culture and sequential cultures *U. thermosphaericus* followed by *C. taiwanensis* exhibited higher removal of phenolic compounds.

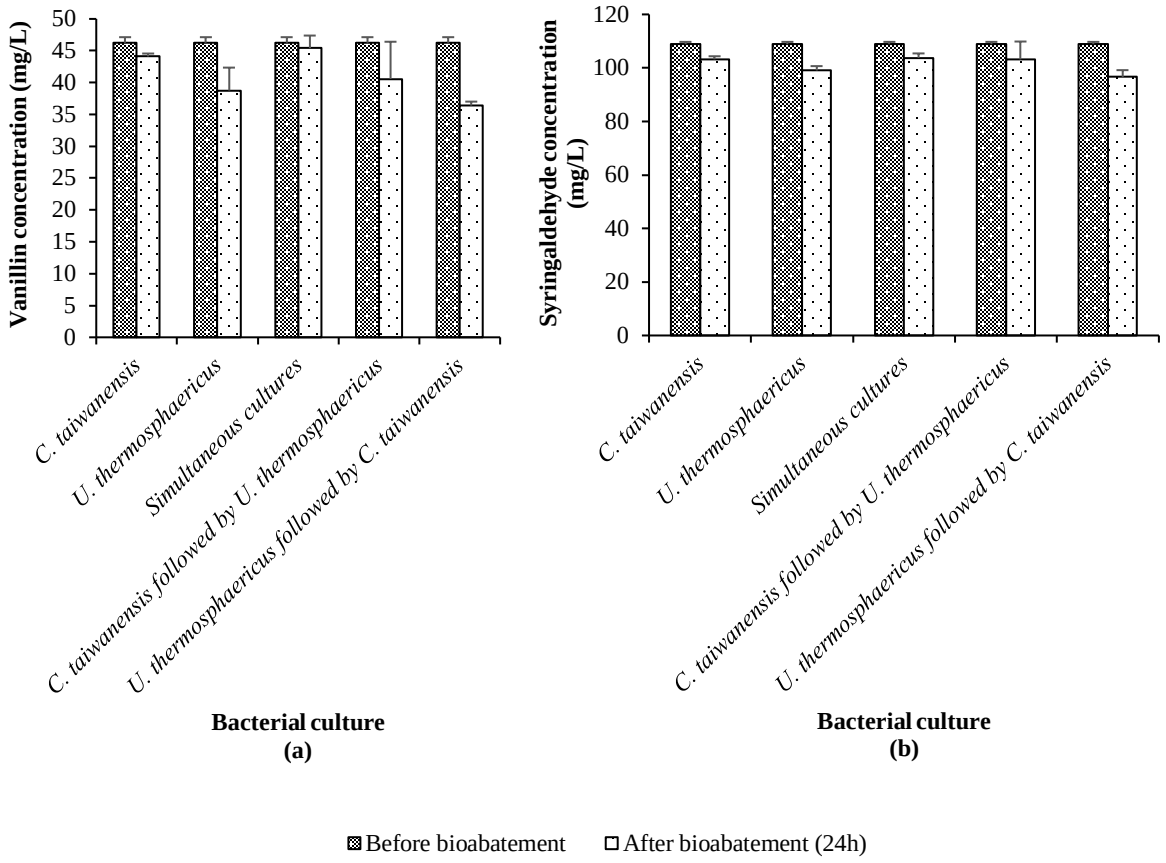



Figure 4-9 : Biodegradation of vanillin and syringaldehyde present in the hemicelluloses pre-hydrolysate by pure and mixed cultures. The cultures were incubated at 41 °C with agitation at 200 rpm for 24 h: (a) vanillin degradation; (b) syringaldehyde degradation.

## 4.5 Conclusions

In this work, an optimized co-culture composed of *C. taiwanensis* and *U. thermosphaericus* was developed and tested for its efficiency in key phenolic compounds degradation (gallic acid, catechol, vanillin and syringaldehyde). At an initial concentration of phenolics of 2.8 g/L, the new co-culture exhibited an excellent removal ability of phenolic compounds in synthetic mediums, up to 90% with simultaneous cultures. A higher affinity to gallic acid and catechol, then syringaldehyde and vanillin was observed with the different co-cultures at 2.8 g/L of phenolic compounds. Pure, simultaneous and sequential cultures were investigated in the case of the pre-hydrolysate detoxification and best results in terms of phenolic compounds degradation were obtained with the sequential cultures *U. thermosphaericus* followed by *C. taiwanensis*. Nevertheless, low percentages of reduction were reported (14%) for the pre-hydrolysate

detoxification. Thus, additional parameters should be evaluated in order to optimize the co-culture and enhance its performance in lignocelluloses detoxification.

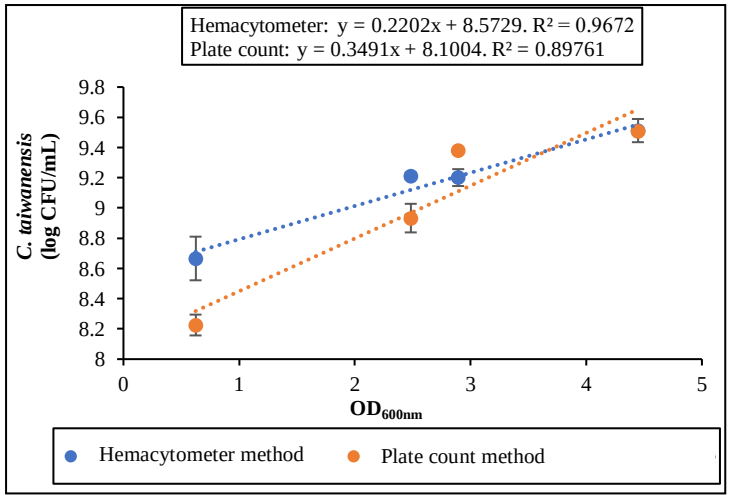
**Author Contributions:** Mariem Theiri, Hassan Chadjaa, Mariya Marinova and Mario Jolicoeur conceived and designed the experiments; Mariem Theiri performed the experiments; All the authors contributed to the analysis of the data and the discussion of the obtained results. Mariem Theiri and Mariya Marinova wrote the paper. All the authors have read and approved the final manuscript.

**Acknowledgments:** This work was supported by a grant from the College-University I2I Program of the Natural Sciences and Engineering Research Council of Canada (NSERC) (grant number 437803-12) of Dr. M. Marinova, the NSERC Industrial Research Chair for Colleges in Bioprocess and Fermentation Technology of Dr. H. Chadjaa, and the NSERC Discovery Grant (# 380070-08) of Prof. M. Jolicoeur. The authors wish to express their gratitude to FPInnovations for supplying the pre-hydrolysate samples and to the Centre National en Électrochimie et Technologies Environnementales (Shawinigan, QC) for hosting the experimental work and especially to Mme Nancy Déziel, Jean-François Lemay, Patrick Quessy and Christophe Damour for their help.

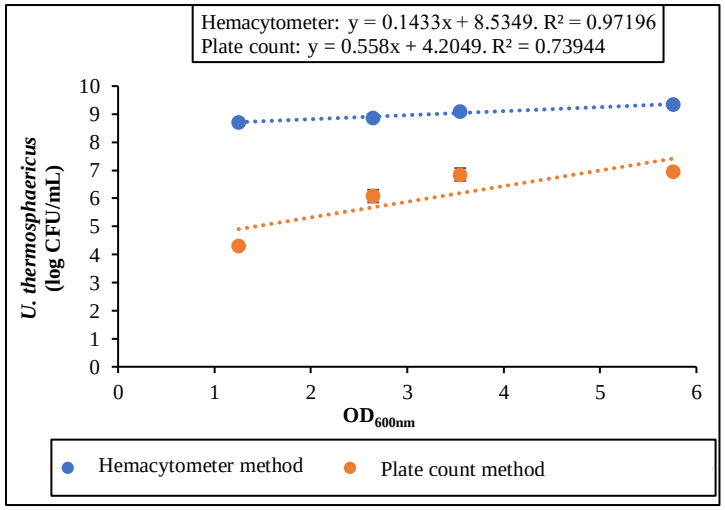
**Conflicts of Interest:** The authors declare no conflict of interest.

## 4.6 Appendices

### 4.6.1 Appendix A. Cells count of *U. thermosphaericus* and *C. taiwanensis* using hemacytometer and plate count methods




**(a)**




**(b)**

Figure 4-10 : Counting results of *C. taiwanensis* et *U. thermosphaericus* with hemacytometer and plate count[1] methods: (a) *C. taiwanensis* count; (b) *U. thermsophaericus* count.

[1] Plate count method consists firstly on serial dilution of samples up to 20 à 300 cells. Then, 100 µL aliquots from dilutions were pipetted on the surface of each three agar plates. Sterile beads were used to spread the sample droplet over the surface. Finally, the plates were incubated at 37 °C for *C. taiwanensis* and 50 °C for *U. thermosphaericus*. Colonies were counted after 24 h incubation.

## 4.6.2 Appendix B. Summary steps for the monitoring of *U. thermosphaericus* and *C. taiwanensis* inocula by Q-PCR

### 4.6.2.1 Selection and design of target genes in *C. taiwanensis* and *U. thermosphaericus* genome

Table 4-4 : Primers of 16S rRNA genes for *U. thermosphaericus* and *C. taiwanensis*

| Primer designation | (F/R)[i] | Strain | Protein | Sequence 5'-3' | $T_m$ (°C) | % G:C | Primer size (bp) | PCR product (bp) |
|---|---|---|---|---|---|---|---|---|
| Myr001 | F | *U. thermosphaericus* | meso-diaminopimelate dehydrogenase | GCAGAATC ATAGCGGA ATGCCTC | 58,2 | 52,2 | 23 | 141 |
| Myr002 | R | *U. thermosphaericus* | meso-diaminopimelate dehydrogenase | GCATAAGC TACCAGAG CGCTAG | 57,4 | 54,5 | 22 | 141 |
| Myr003 | F | *U. thermosphaericus* | meso-diaminopimelate dehydrogenase | CACACACG CACGTATT CCTG | 62 | 40,9 | 22 | 128 |
| Myr004 | R | *U. thermosphaericus* | meso-diaminopimelate dehydrogenase | CTTCACCG AGAAGGCG ATTTA | 62 | 47,6 | 21 | 128 |

Table 4-4 : Primers of 16S rRNA genes for *U. thermosphaericus* and *C. taiwanensis* (continued)

| Primer designation | (F/R)[i] | Strain | Protein | Sequence 5'-3' | $T_m$ (°C) | % G:C | Primer size (bp) | PCR product (bp) |
|---|---|---|---|---|---|---|---|---|
| Myr005 | F | *U. thermosphaericus* | glycerol-3-phosphate dehydrogenase | GAGCGTTC ATTCGCGT AACT | 63 | 50 | 20 | 102 |
| Myr006 | R | *U. thermosphaericus* | glycerol-3-phosphate dehydrogenase | CGAACTCC TTCAACGA CCATAC | 63 | 50 | 22 | 102 |
| Myr007 | F | *C. taiwanensis* | Aminohydrolase | CGATACTG TCACCATC ACCATTC | 55.4 | 48 | 23 | 142 |
| Myr008 | R | *C. taiwanensis* | Aminohydrolase | GGTTTCGT TCGGATCG ACATAG | 55.5 | 50 | 22 | 142 |
| Myr009 | F | *C. taiwanensis* | dienelactone hydrolase | TGCGACAT CGAGACCA AC | 62 | 55.6 | 18 | 104 |
| Myr010 | R | *C. taiwanensis* | dienelactone hydrolase | ATCACGAC GATGCGAT CC | 62 | 55.6 | 18 | 104 |
| Myr011 | F | *C. taiwanensis* | Allophanate hydrolase | CAAGATCG GCGTGGTG AT | 62 | 55.6 | 18 | 127 |
| Myr012 | R | *C. taiwanensis* | Allophanate hydrolase | GTAGCGTG CCAGTTCC TC | 62 | 61.1 | 18 | 127 |

[i] F/R: Forward/Reverse

### 4.6.2.2 Optimisation assays of Q-PCR amplifications of the target genes

Prior to tackle with Q-PCR analysis, the specificity of primers and their optimal $T_m$ were determined. The genes were amplified by PCR then migrated on agarose gel to visualize the genes strips.

For Q-PCR amplification, the genomic DNA of each strain was amplified with different sets of primers as detailed in Table 4-5. The mix Q-PCR was as follows:

| | |
|---|---|
| - 0.25 µL primer Forward | 2.75 µL |
| - 0.25 µL primer Reverse | 2.75 µL |
| - 5.00 µL Fasta Mix (Quanta Bioscience) | 55.0 µL |
| - 3.50 µL DEPC water | 38.5 µL |
| 9.0 µL total mix in the wells of PCR plate | |

- 1.00 µL DNA in the corresponding wells.

The Q-PCR program used was:

- 4 min @ 95°C
- 10 " @ 95 °C
- 30 " @ 60 °C } 40 x
- 1 ' @ 95 °C
- Ramp 15 " @ 52 °C to 95°C, during 20 min
- End, hold to 10 °C

A Real-Time 96-Well PCR Plate (eppendorf™ 951022055) was used for Q-PCR assays and the wells were filled as detailed in Figure 4-11.

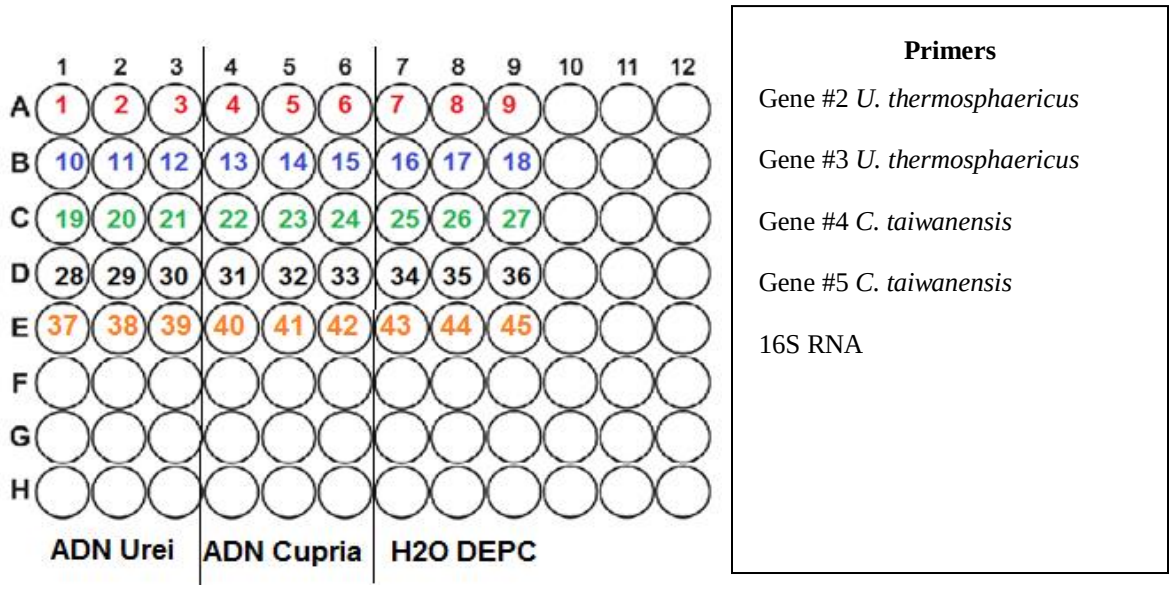



Figure 4-11 : Distribution of mix Q-PCR in plate wells.

After genes amplification, the specificity of primers was validated by migration on agarose gel and primers corresponding to intensified strips were selected.

Table 4-5 : Optimisation tests for the primers specificity of *U. thermosphaericus* and *C. taiwanensis*. The tests were performed in triplicates using Q-PCR equipment

| Q-PCR number | Target of the primers | Primers F/R | $T_m$ (°C) | Genomic DNA[i] | Theory |
|---|---|---|---|---|---|
| **1** | Gene #2 *U. thermosphaericus* | (Myr 003 + 004) | 60 | *U. thermosphaericus* | Positif |
| **2** | Gene #2 *U. thermospahericus* | (Myr 003 + 004) | 60 | *C. taiwanensis* | Negatif |
| **3** | Gene #2 *U. thermospahericus* | (Myr 003 + 004) | 60 | Negative control[ii] | Negatif |
| **4** | Gene #3 *U. thermospahericus* | (Myr 005 + 006) | 60 | *U. thermospahericus* | Positif |
| **5** | Gene #3 *U. thermospahericus* | (Myr 005 + 006) | 60 | *C. taiwanensis* | Negatif |
| **6** | Gene #3 *U. thermospahericus* | (Myr 005 + 006) | 60 | Negative control[ii] | Negatif |
| **7** | Gene #4 *C. taiwanensis* | (Myr 007 + 008) | 60 | *U. thermospahericus* | Negatif |
| **8** | Gene #4 *C. taiwanensis* | (Myr 007 + 008) | 60 | *C. taiwanensis* | Positif |
| **9** | Gene #4 *C. taiwanensis* | (Myr 007 + 008) | 60 | Negative control[ii] | Negatif |
| **10** | Gene #5 *C. taiwanensis* | (Myr 009 + 010) | 60 | *U. thermospahericus* | Negatif |
| **11** | Gene #5 *C. taiwanensis* | (Myr 009 + 010) | 60 | *C. taiwanensis* | Positif |
| **12** | Gene #5 *C. taiwanensis* | (Myr 009 + 010) | 60 | Negative control[ii] | Negatif |

[i] 10 ng of genomic DNA of the strains were used per well of Q-PCR plate.
[ii] The negative control was composed of UltraPure™ DEPC (diethylpyrocarbonate)-treated Water.

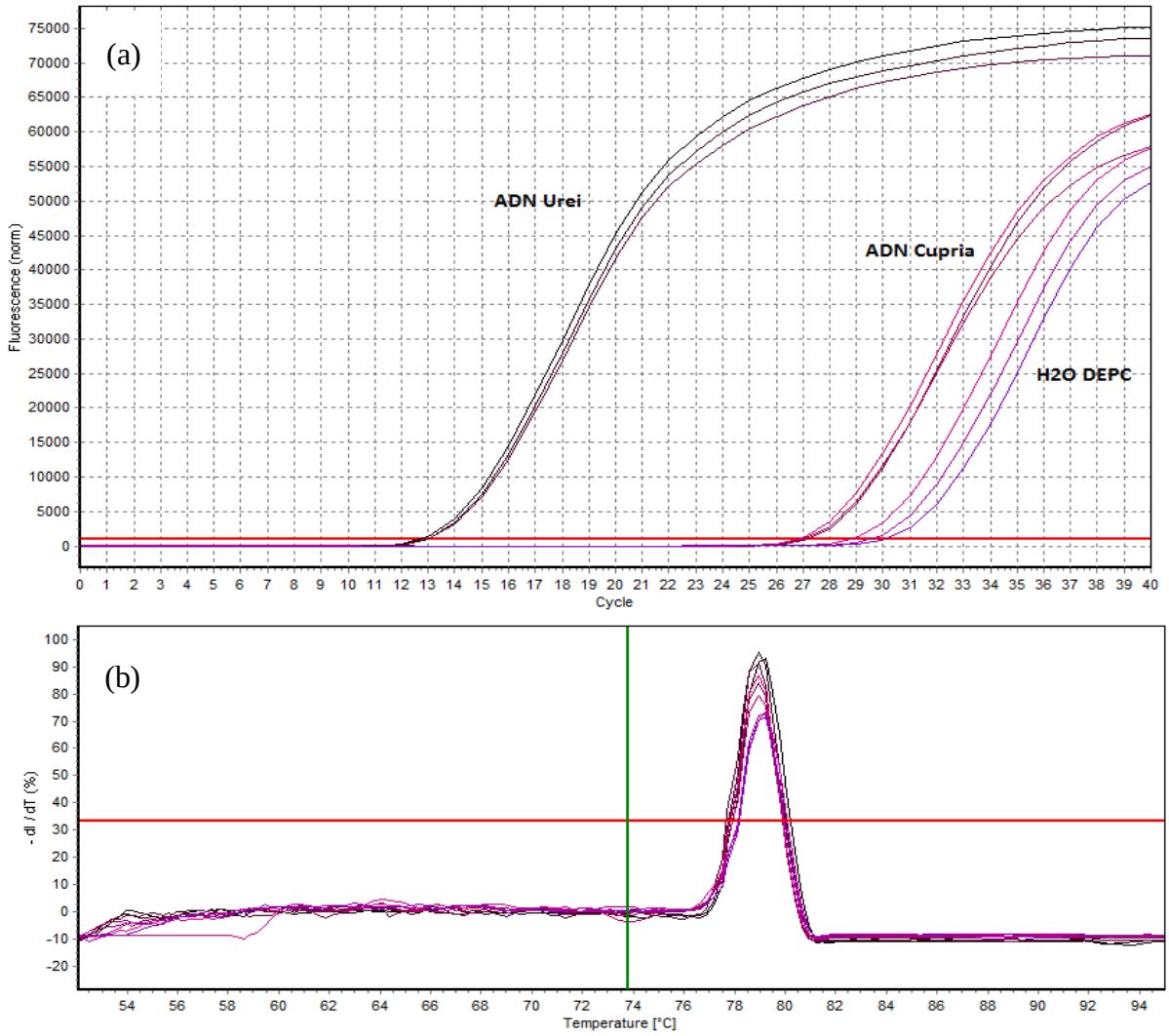



Figure 4-12 : Amplification and melting curves with SYBR Green I in a real time PCR of *U. thermosphaericus* and *C. taiwanensis* inocula and DEPC water using the primers specific for the gene of *U. thermosphaericus* expressing meso-diaminopimelate dehydrogenase: (a) Amplification curves; (b) Melting curves. The Q-PCR assays were conducted in triplicates for each sample.

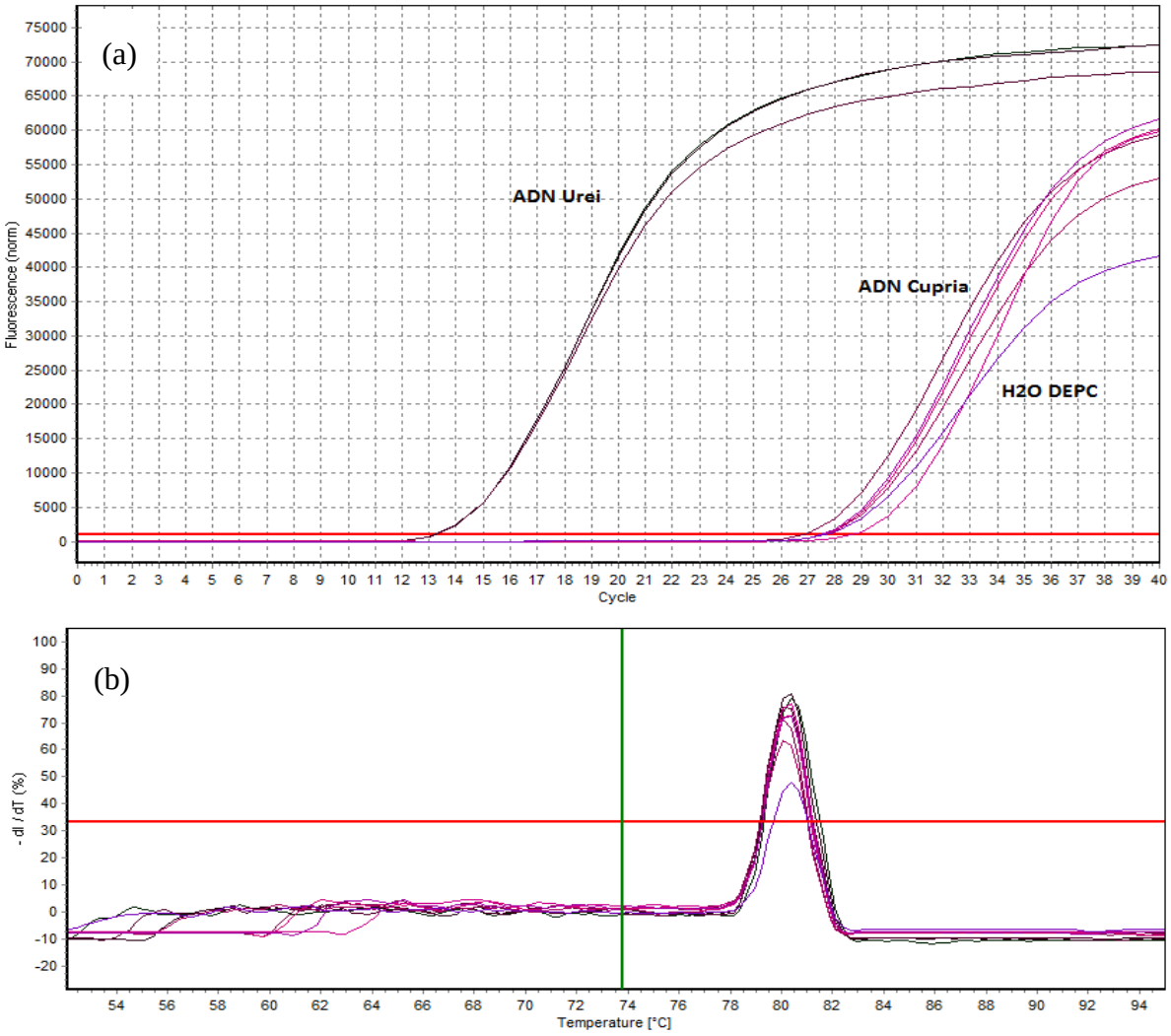



Figure 4-13 : Amplification and melting curves with SYBR Green I in a real time PCR of *U. thermosphaericus* and *C. taiwanensis* inocula and DEPC water using the primers specific for the gene of *U. thermosphaericus* expressing glycerol-3-phosphate dehydrogenase: (a) Amplification curves; (b) Melting curves. The Q-PCR assays were conducted in triplicates for each sample.

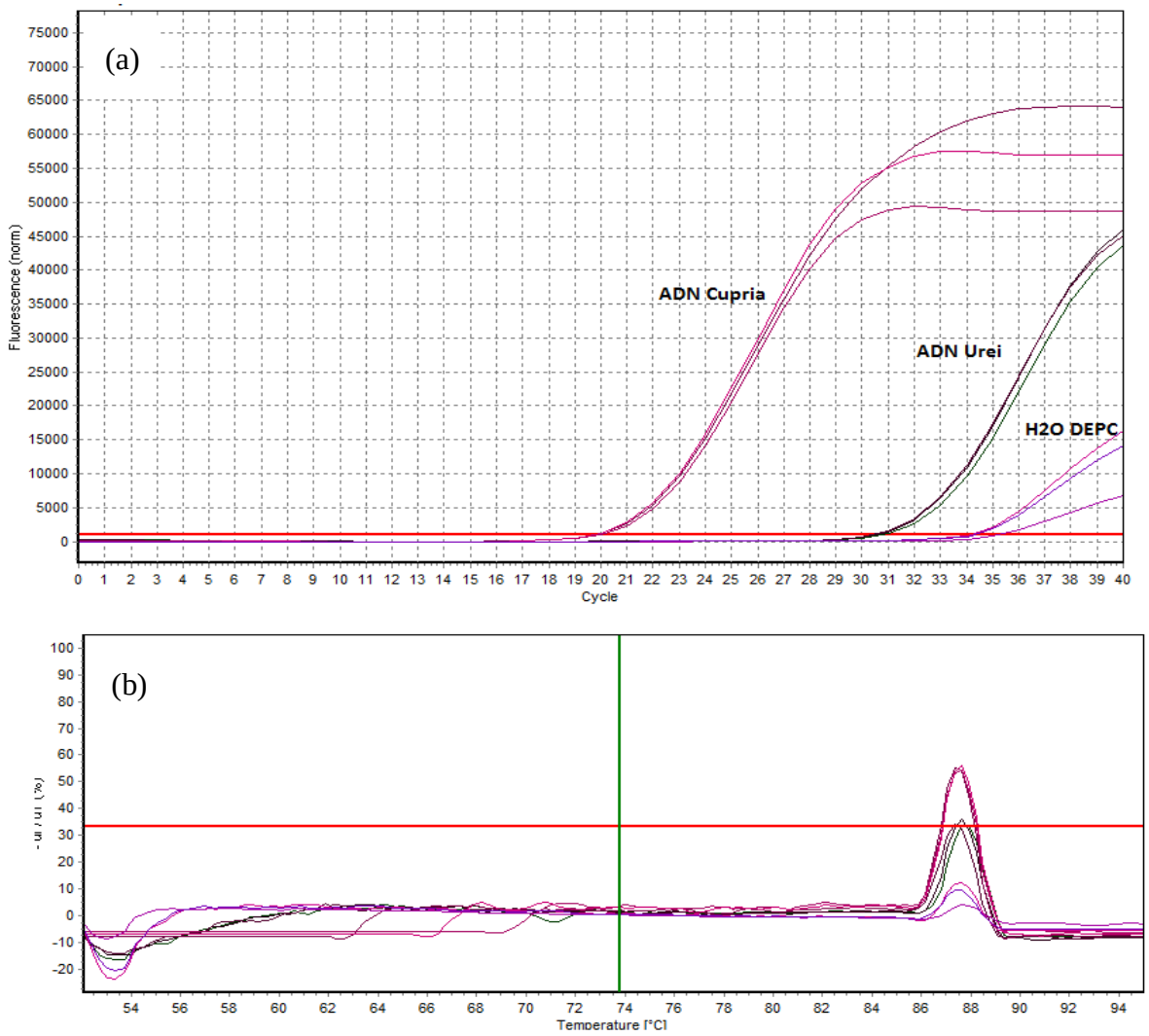



Figure 4-14 : Amplification and melting curves with SYBR Green I in a real time PCR of *U. thermosphaericus* and *C. taiwanensis* inocula and DEPC water using the primers specific for the gene of *U. thermosphaericus* expressing aminohydrolase: (a) Amplification curves; (b) Melting curves. The Q-PCR assays were conducted in triplicates for each sample.

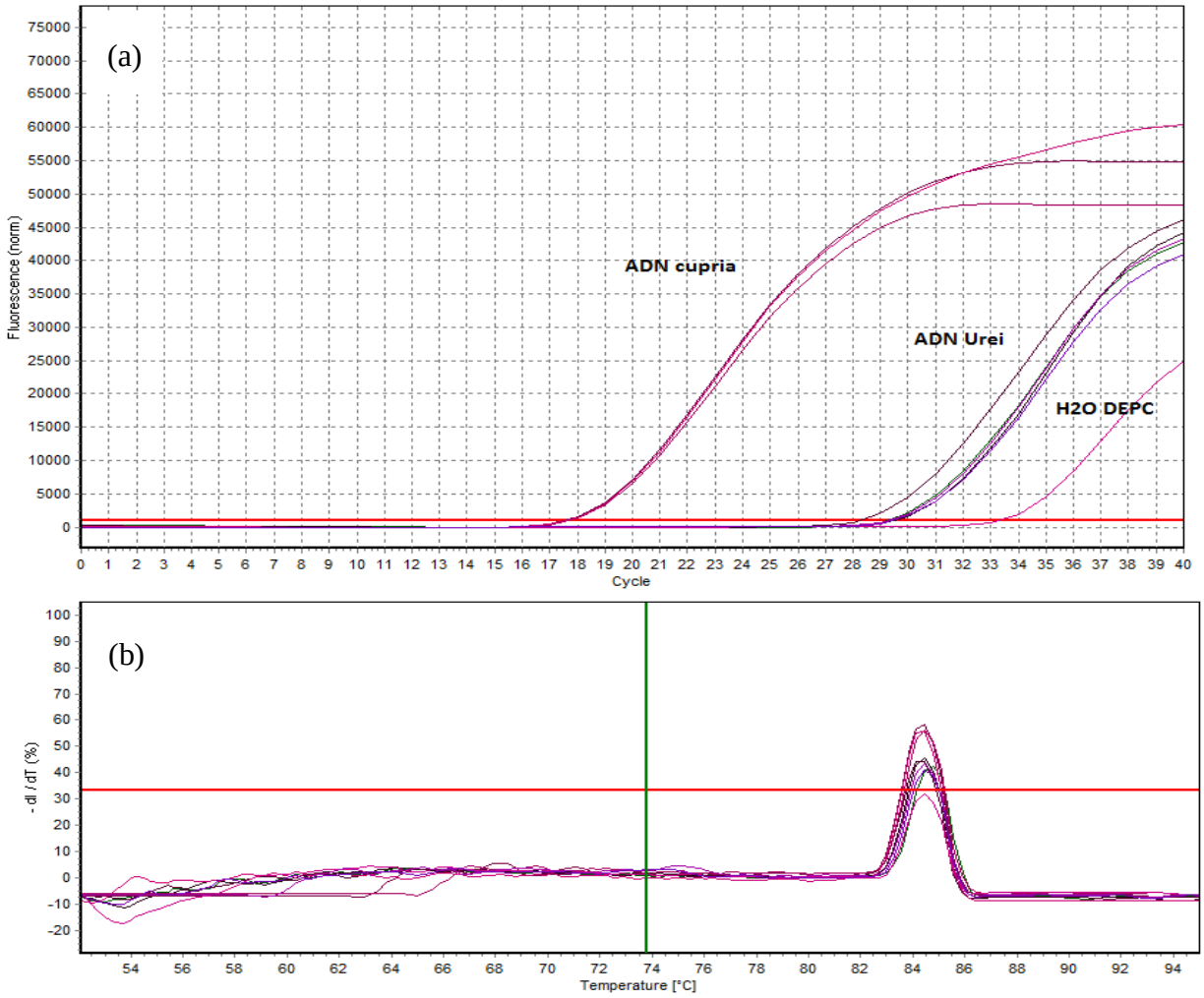



Figure 4-15 : Amplification and melting curves with SYBR Green I in a real time PCR of *U. thermosphaericus* and *C. taiwanensis* inocula and DEPC water using the primers specific for the gene of *U. thermosphaericus* expressing dienelactone hydrolase: (a) Amplification curves; (b) Melting curves. The Q-PCR assays were conducted in triplicates for each sample.

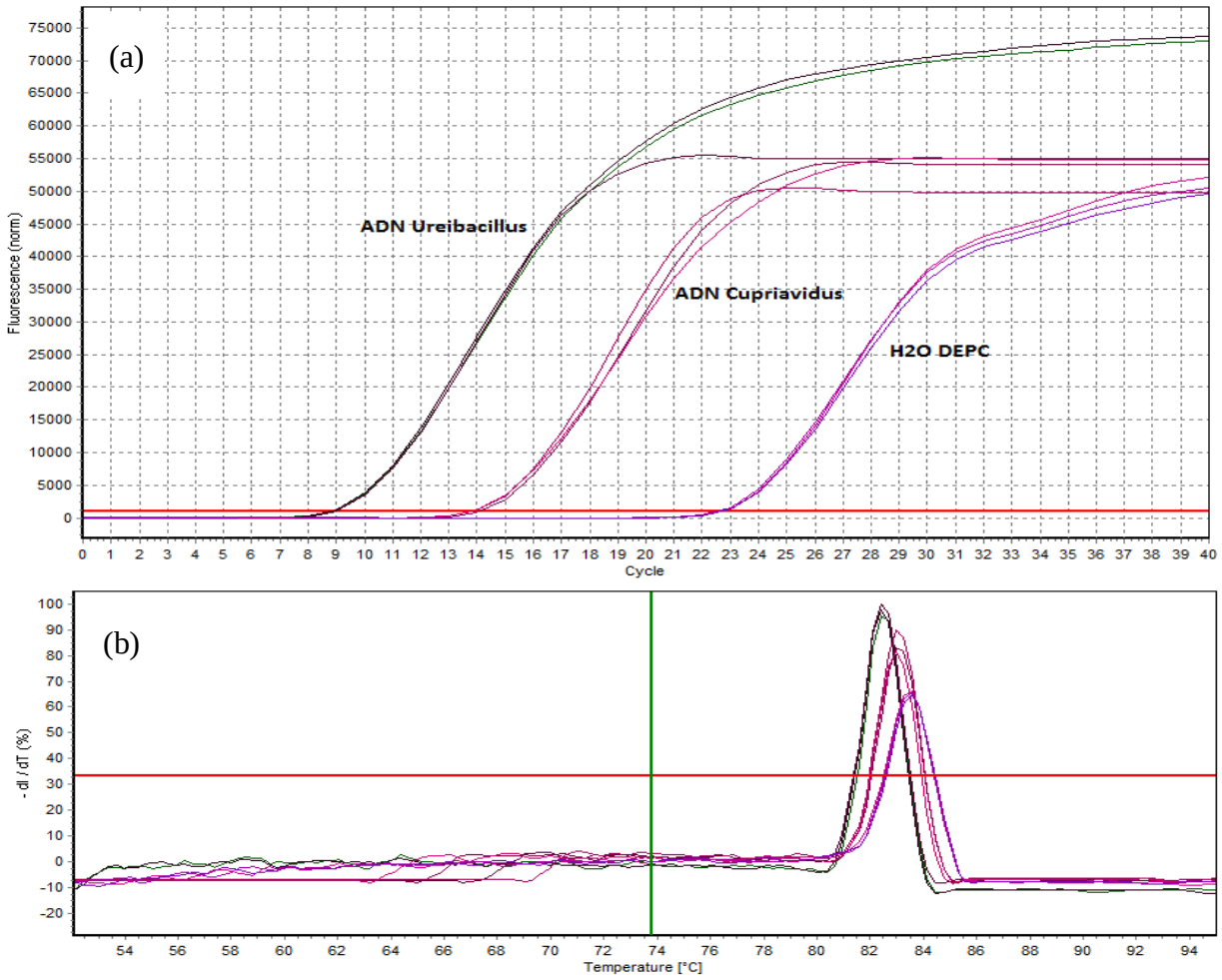



Figure 4-16 : Amplification and melting curves with SYBR Green I in a real time PCR of *U. thermosphaericus* and *C. taiwanensis* inocula and DEPC water using the primers specific for the gene of total RNA 16S: (a) Amplification curves; (b) Melting curves. The Q-PCR assays were conducted in triplicates for each sample.

After selectivity assays of primers were achieved, $T_m$ corresponding to the hybridation temperature of the tested primers was determined. For that, three $T_m$ values 58 °C, 60 °C and 62 °C were tested using Q-PCR then validated by migration on agarose gel. A $T_m$ value of 60 °C was further selected for more intensified strips. Finally, the primers for the target genes of meso-diaminopimelate dehydrogenase and aminohydrolase were respectively selected for the amplification of *U. thermosphaericus* and *C. taiwanensis* and an optimal $T_m$ value of 60 °C was determined.

### 4.6.2.3 Gel extraction, purification and quantification of pure DNA gene

*4.6.2.3.1 PCR amplification of the different genes*

Table 4-6 : Amplifications of the different genes of *U. thermosphaercius* and *C. taiwanensis* using PCR equipment

| PCR number | Target of the primers | Primers F/R | $T_m$ (°C) | Genomic DNA | Theory |
|---|---|---|---|---|---|
| **1** | Gene #2 *U. thermosphaericus* | (Myr 003 + 004) | 60 | *U. thermosphaericus* | Positif |
| **2** | Gene #3 *U. thermospahericus* | (Myr 005 + 006) | 60 | *U. thermospahericus* | Positif |
| **3** | Gene #4 *C. taiwanensis* | (Myr 007 + 008) | 60 | *C. taiwanensis* | Positif |
| **4** | Gene #5 *C. taiwanensis* | (Myr 009 + 010) | 60 | *C. taiwanensis* | Positif |

**Mix PCR:**

- 1.0 µL primer F
- 1.0 µL primer R
- 1.0 µL dNTP mix 10 mM
- 5.0 µL Stand buffer 10X
- 0.5 µL Taq DNA polymerase
- 39.5 µL sterile water DEPC
- 2.00 µL genomic DNA at 1 ng/µL

Total 50 µL

**PCR program:**

- 5 min @ 95°C
- 30 sec @ 95°C
- 30 sec @ **60°C**
- 30 sec @ 72°C

33 cycles

- 5 min @ 72°C

*4.6.2.3.2 Migration on agarose gel of the amplified genes*

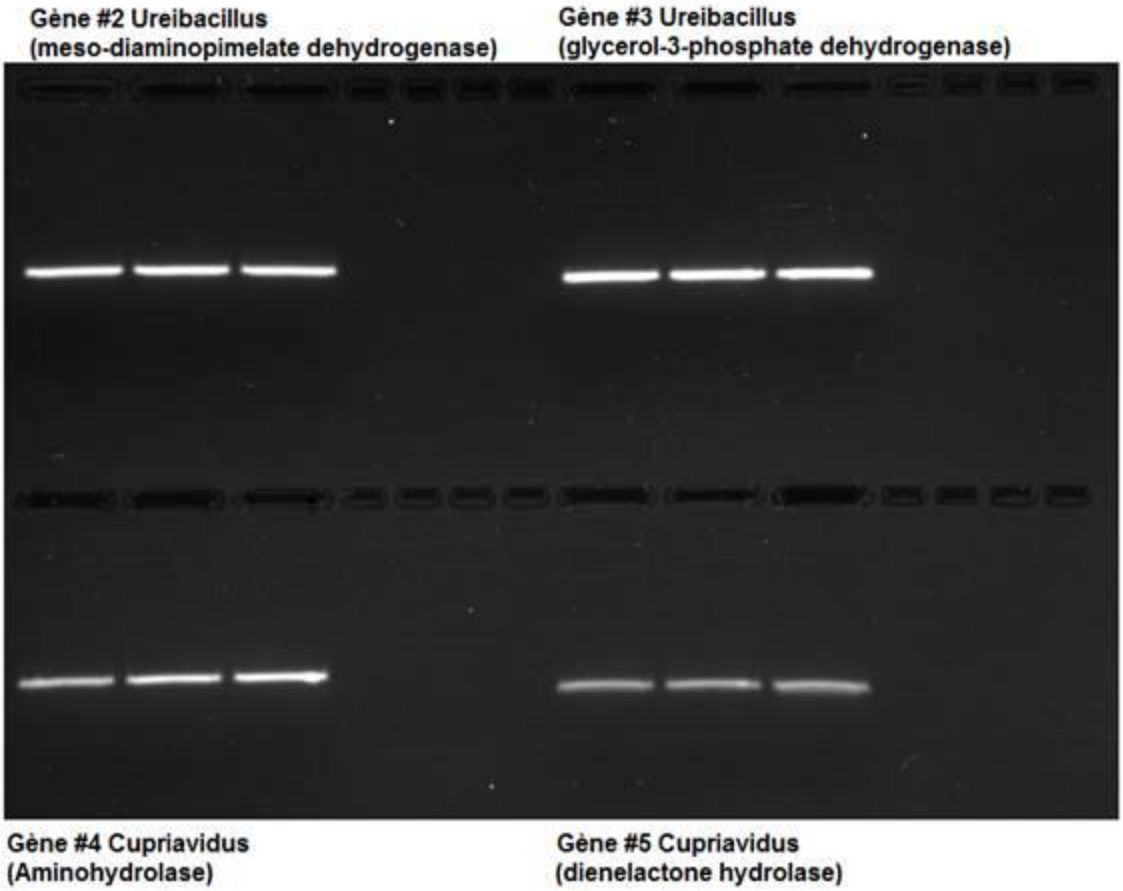



Figure 4-17 : Migration of the different genes of *U. thermosphaericus* and *C. taiwanensis* on agarose gel.

*4.6.2.3.3 Gel extraction*

The three strips of each gene were cut with a scalpel, and then purified using EZNA Gel extraction kit from OMEGA Biotek Company. The DNA aliquots were then quantified with spectrophotometer at 260 nm and the obtained values are:

**Gene #2 *U. thermosphaericus:*** 32.2 ng/µL

**Gene #3 *U. thermosphaericus:*** 29.9 ng/µL

**Gene #4 *C. taiwanensis:*** 30.8 ng/µL

**Gene #5 *C. taiwanensis:*** 20.1 ng/µL

The following step was to calculate the molecular weight of each gene.

#### 4.6.2.4 Calculations of molecular weights

The calculated molecular weights of *U. thermosphaericus* genes are as follow:

| | |
|---|---|
| **MW Sequence F (Single strand)** | 39 497.64 Da (g/mol) |
| **MW Sequence R (Single strand)** | 39 618.76 Da (g/mol) |
| **MW Total (Double strand)** | 79 116.40 Da (g/mol) |
| **Size of gene (pb)** | 128 pb |
| **Weight (g) of 1 mol of gene ($6.023x10^{23}$ genes)** | 79 116 g |
| **Weight of 1 gene (g)** | $1.31 \times 10^{-19}$ g |

The calculated molecular weights of *C. taiwanensis* genes are as follow:

| | |
|---|---|
| **MW Sequence F (Single strand)** | 43 803.12 Da (g/mol) |
| **MW Sequence R (Single strand)** | 43 994.26 Da (g/mol) |
| **MW Total (Double strand)** | 87 797.78 Da (g/mol) |
| **Size of gene (pb)** | 142 pb |
| **Weight (g) of 1 mol of gene ($6.023x10^{23}$ genes)** | 87 797 g |
| **Weight of 1 gene (g)** | $1.46 \times 10^{-19}$ g |

#### 4.6.2.5 Determination of standard curves and Q-PCR analysis

Once the molecular weights of genes were determined, serial dilutions were realized to establish standard curves (Figure 4-18 and Figure 4-19), then Q-PCR analysis were performed. The duration of the analysis was 1h30.

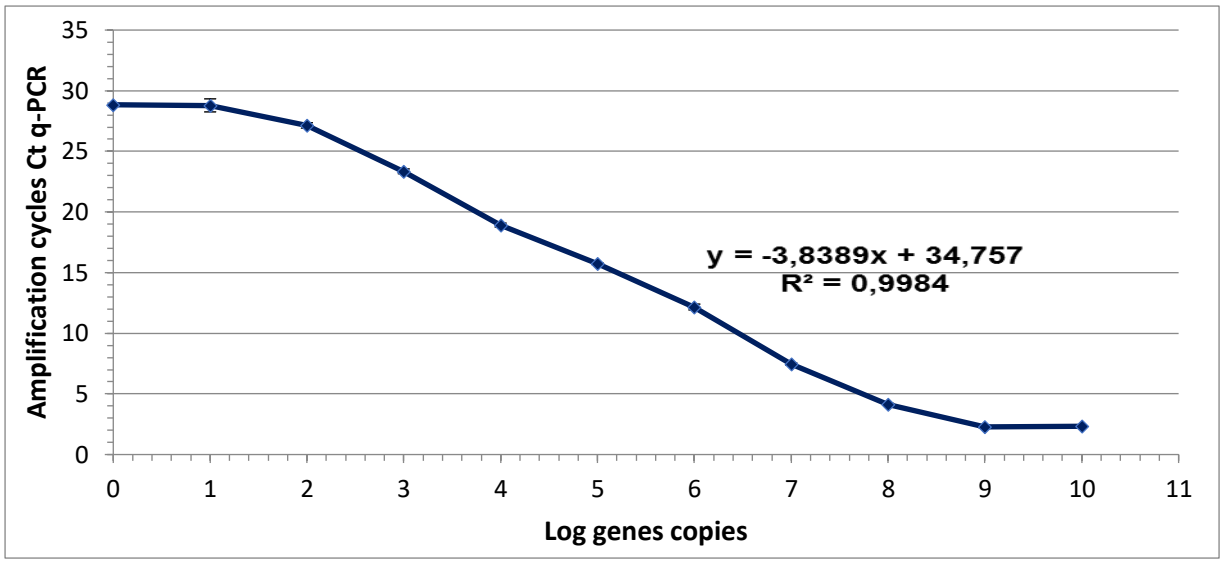



Figure 4-18 : Standard curve Q-PCR (SYBR GREEN) of the gene mdd of *U. thermosphaericus*.

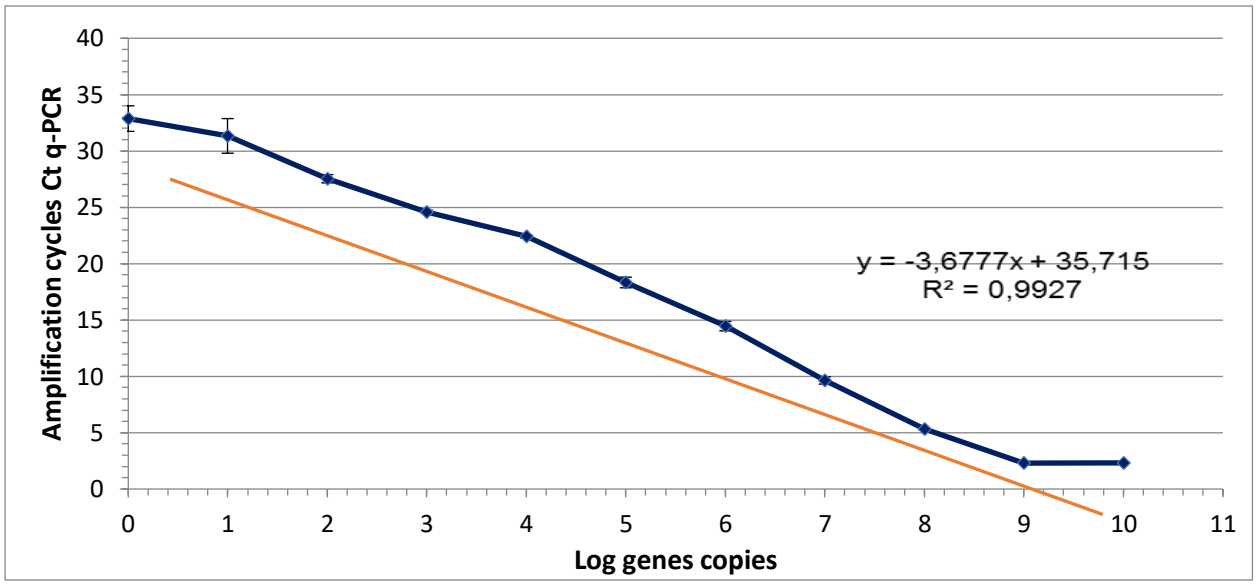



Figure 4-19 : Standard curve Q-PCR (SYBR GREEN) of the gene aminohydrolase of *C. taiwanensis*.

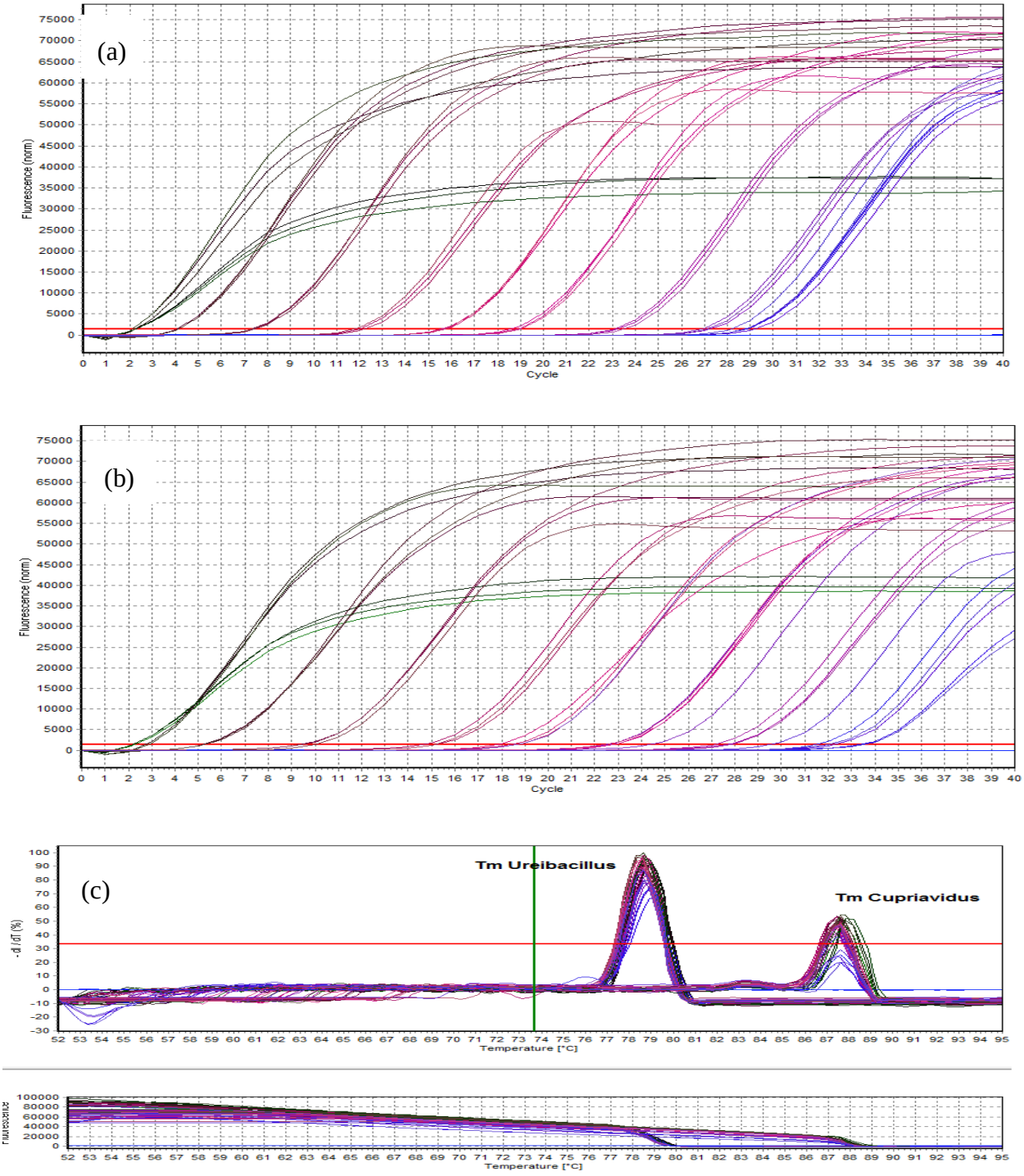



Figure 4-20 : Amplification and melting curves with SYBR Green I in a real time PCR of *U. thermosphaericus* and *C. taiwanensis* inocula and DEPC water using the primers specific for the gene of total RNA 16S: (a) Amplification curves of *U. thermosphaericus*; (b) Amplification curves of *C. taiwanensis*; (c) Melting curves. The Q-PCR assays were conducted in triplicates for each sample.

### 4.6.3 Appendix C. Chromatograms of phenolic compounds in detoxified synthetic media

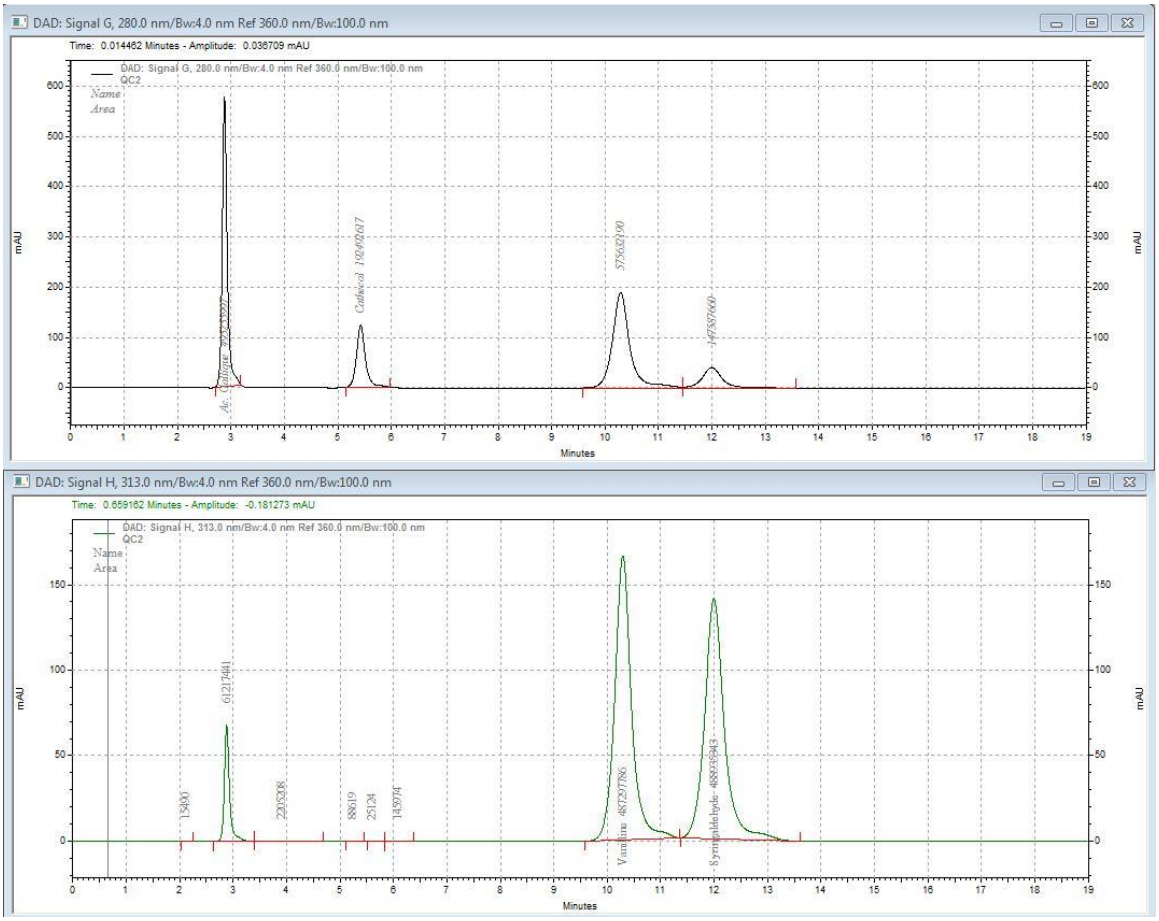



Figure 4-21 : Chromatogram of the standards of gallic acid, catechol, vanillin and syringaldehyde.

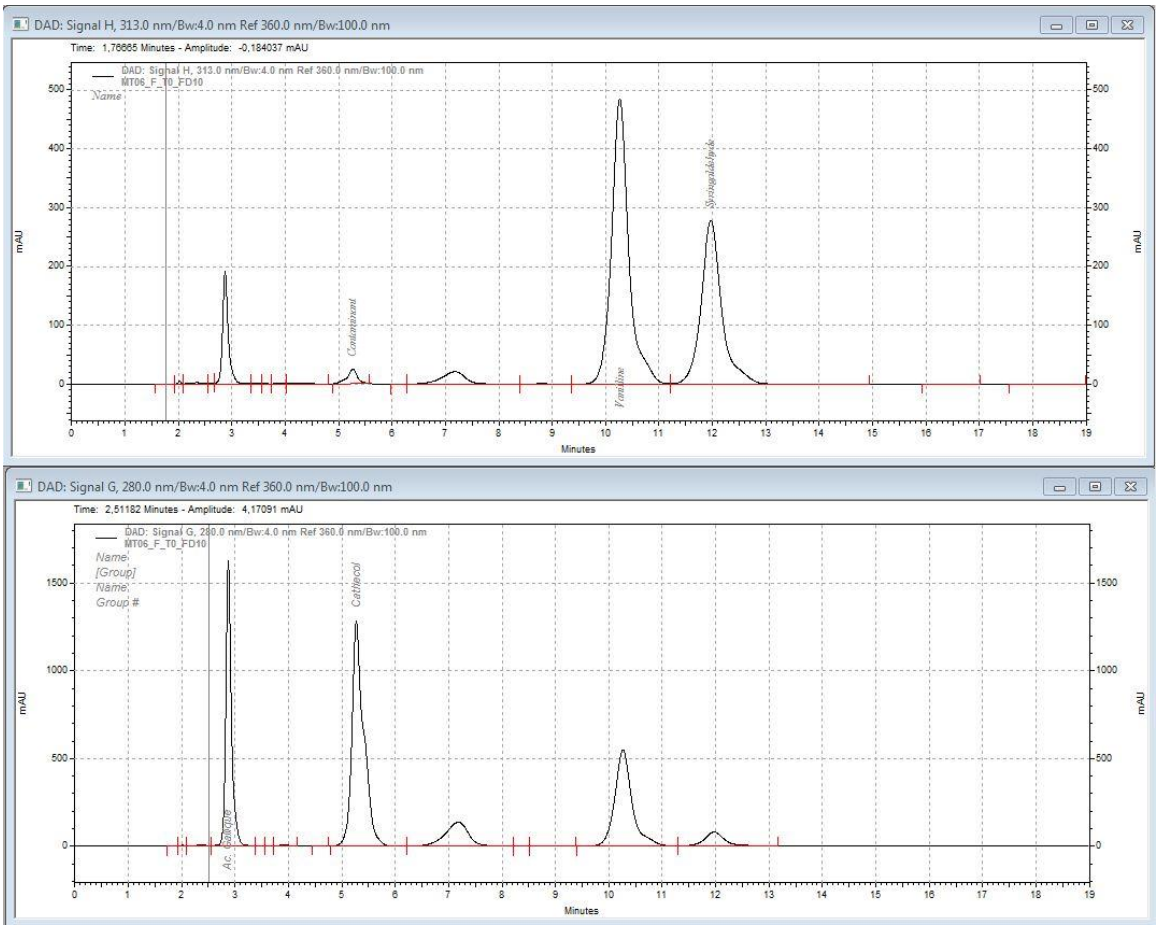



Figure 4-22 : Chromatogram of phenolic compounds in the initial composition of synthetic medium composed of: 2 g/L furfural, 10 g/L acetic acid, 0.25 M salts and 8 g/L phenolic compounds.

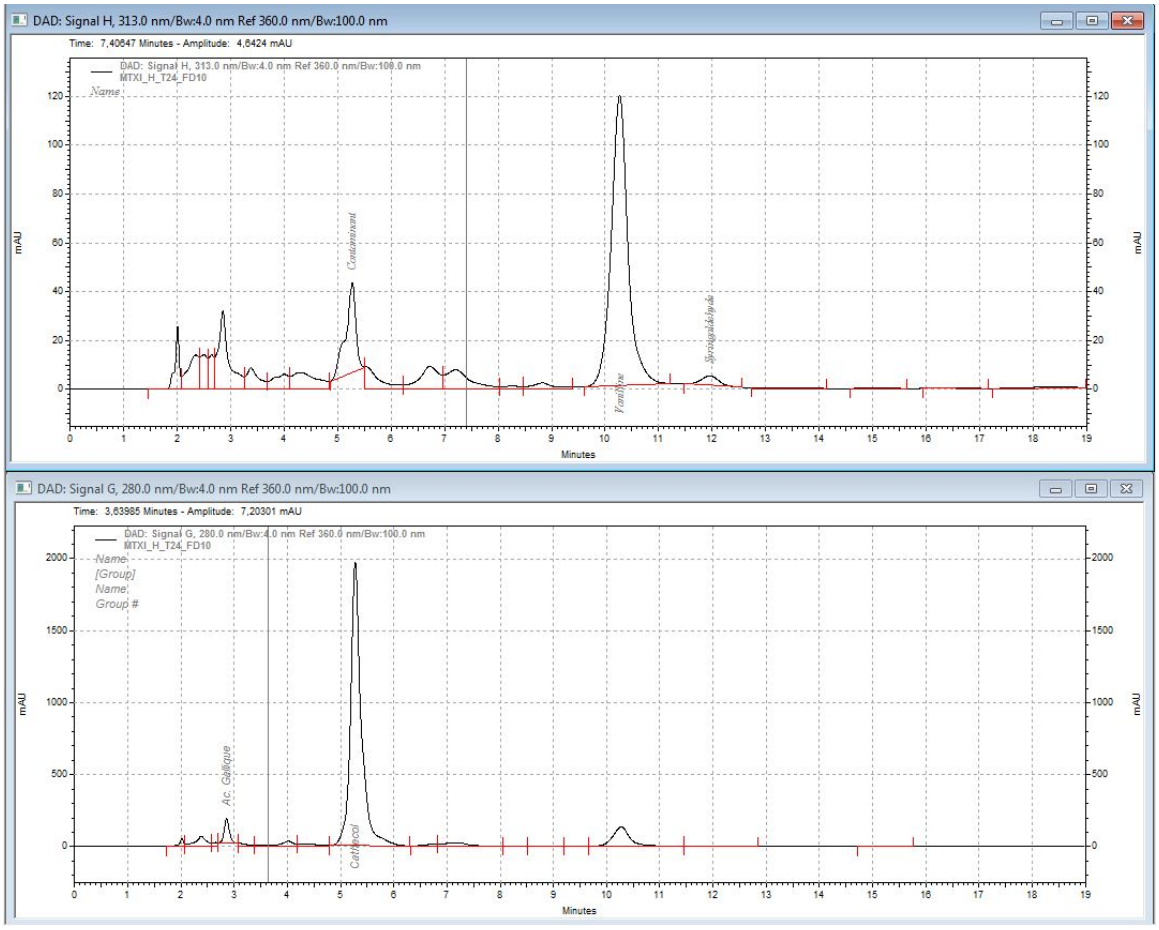



Figure 4-23 : Chromatogram of phenolic compounds in the detoxified synthetic medium (after 24 h) with simultaneous cultures. The initial composition of synthetic medium was: 2 g/L furfural, 10 g/L acetic acid, 0.25 M salts and 8 g/L phenolic compounds.

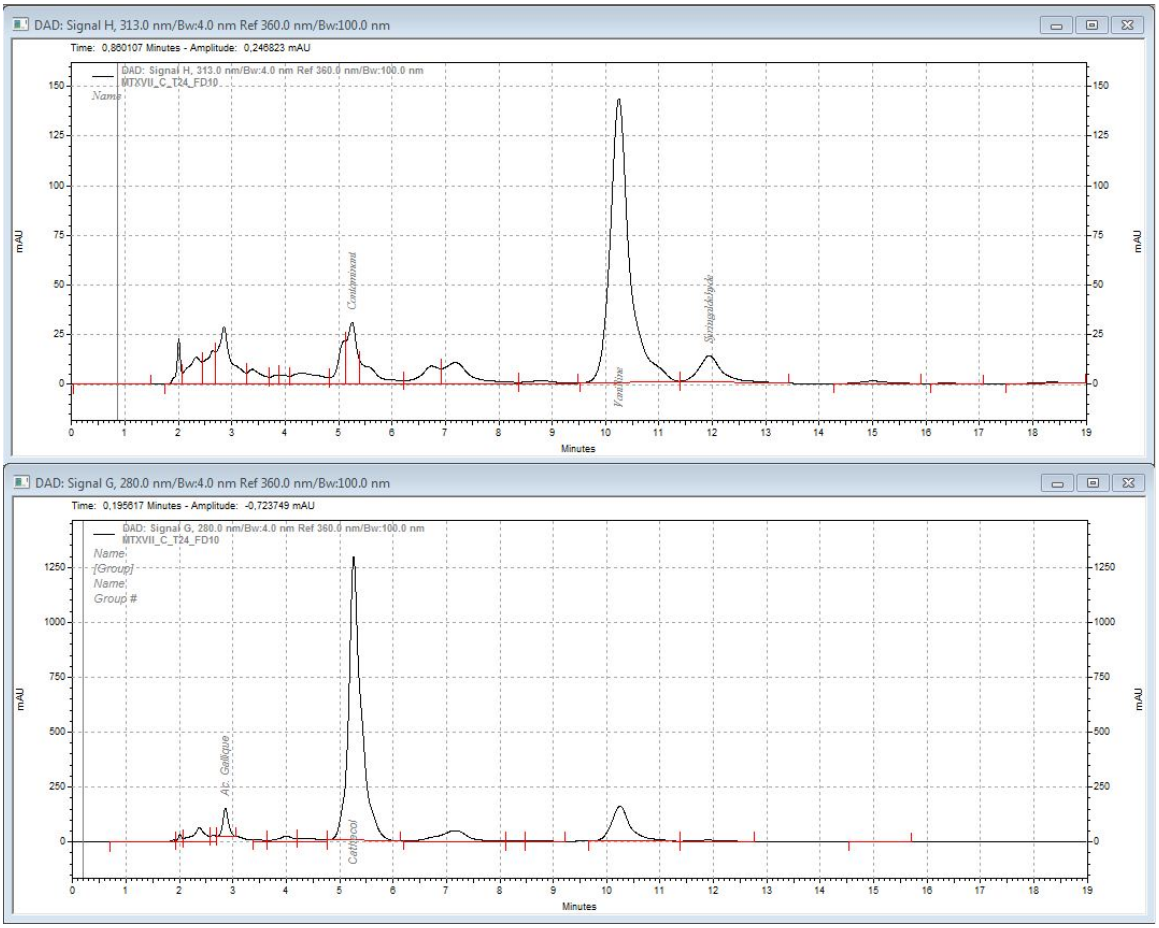



Figure 4-24 : Chromatogram of phenolic compounds in the detoxified synthetic medium (after 24 h) with sequential cultures *C. taiwanensis* followed by *U. thermosphaericus*. The initial composition of synthetic medium was: 2 g/L furfural, 10 g/L acetic acid, 0.25 M salts and 8 g/L phenolic compounds.

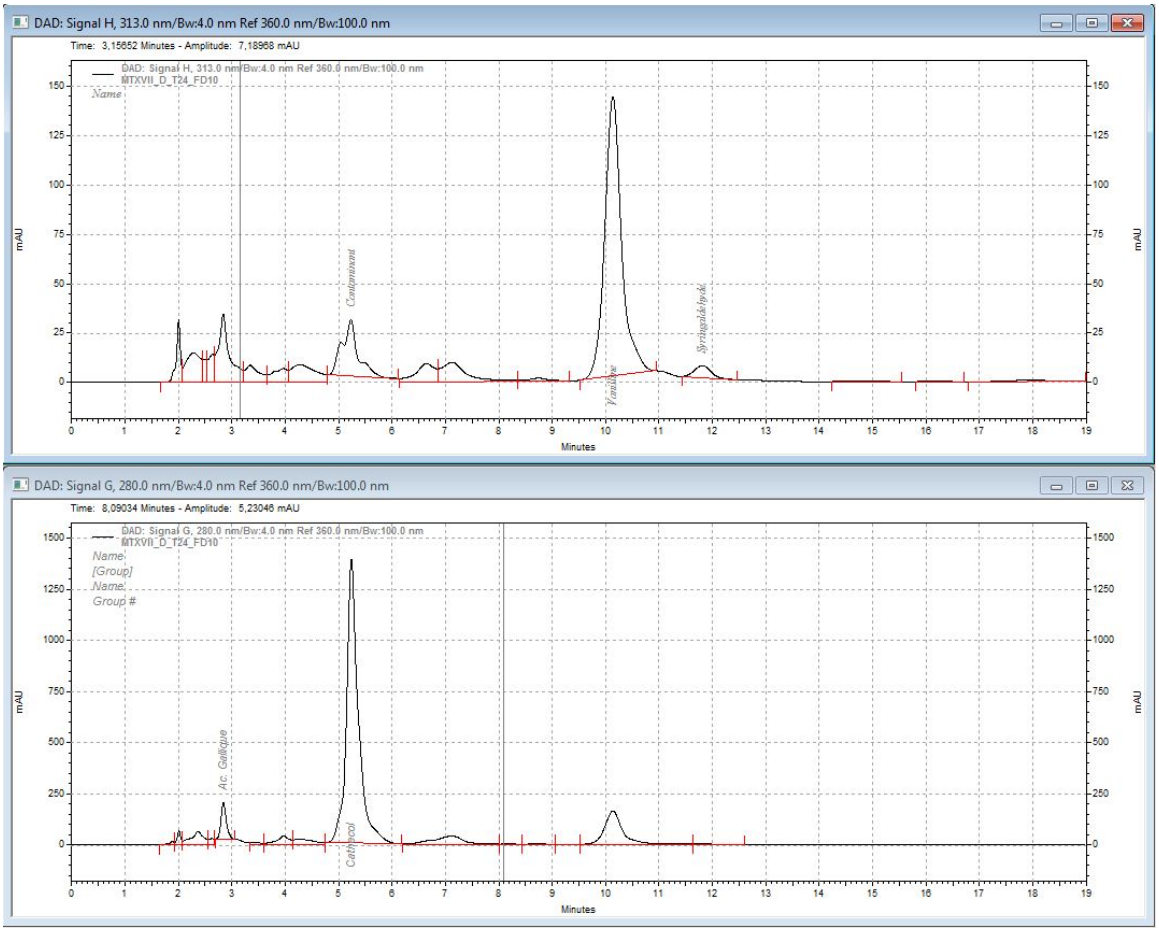



Figure 4-25 : Chromatogram of phenolic compounds in the detoxified synthetic medium (after 24 h) with sequential cultures *U. thermosphaericus* followed by *C. taiwanensis*. The initial composition of synthetic medium was: 2 g/L furfural, 10 g/L acetic acid, 0.25 M salts and 8 g/L phenolic compounds.

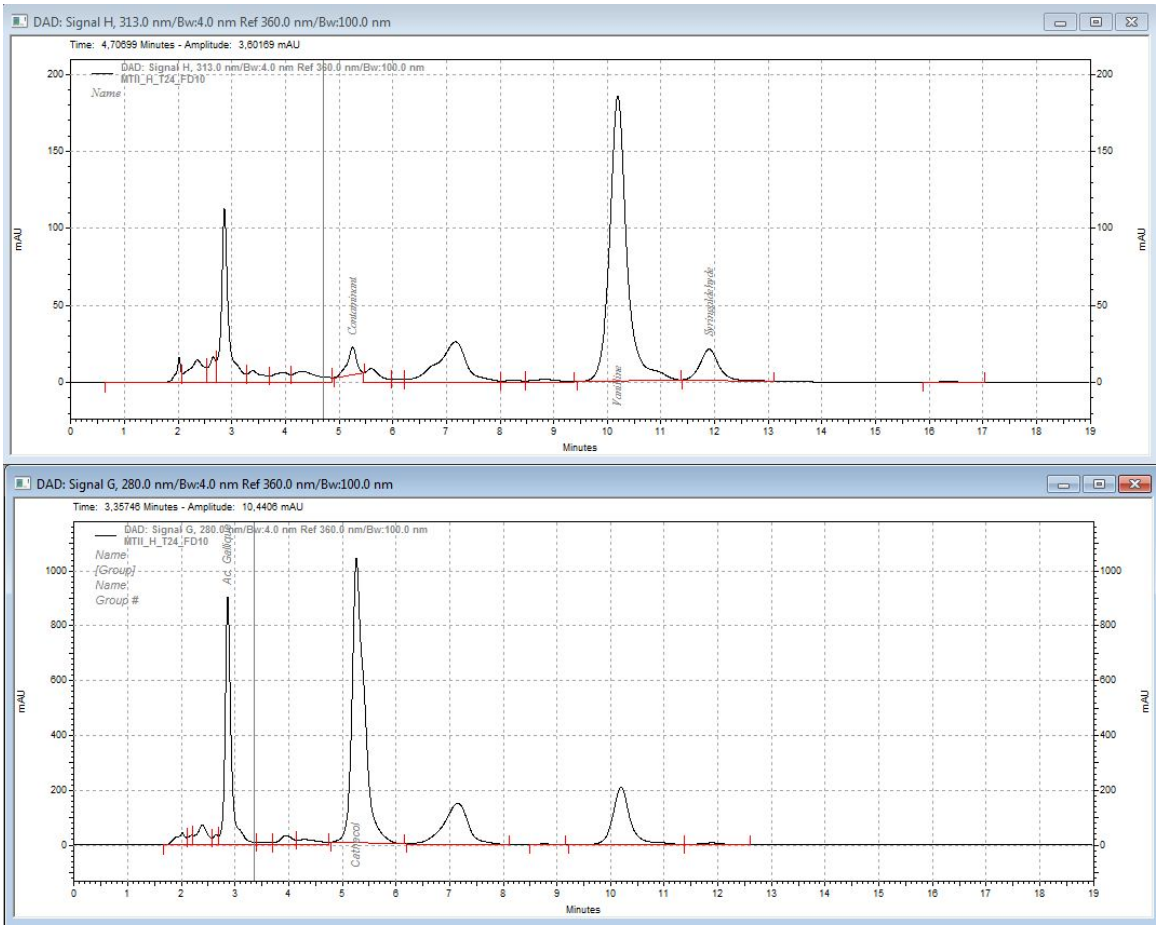



Figure 4-26 : Chromatogram of phenolic compounds in the detoxified synthetic medium (after 24 h) with the monoculture *C. taiwanensis*. The initial composition of synthetic medium was: 2 g/L furfural, 10 g/L acetic acid, 0.25 M salts and 8 g/L phenolic compounds.

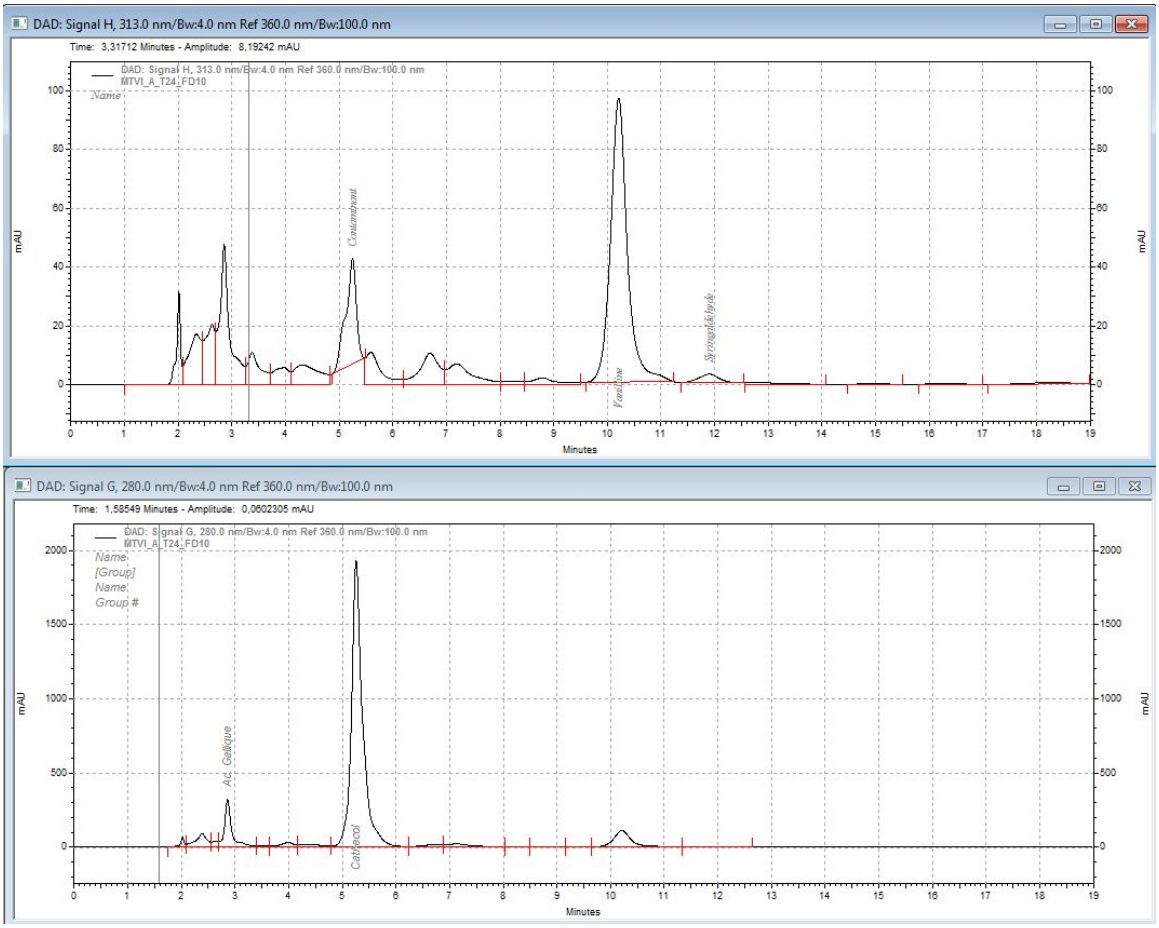



Figure 4-27 : Chromatogram of phenolic compounds in the detoxified synthetic medium (after 24 h) with the monoculture *U. thermosphaericus*. The initial composition of synthetic medium was: 2 g/L furfural, 10 g/L acetic acid, 0.25 M salts and 8 g/L phenolic compounds.

### 4.6.4 Appendix D. Complementary results

Table 4-7 : Variation of ammoniacal nitrogen in TSB cultures for 24 h at different incubation temperatures

| Reaction temperature | Culture | Initial concentration (mg/L) | Final concentration after 24 h (mg/L) |
|---|---|---|---|
| 37 °C | *C. taiwanensis* | 354.38±0.00 | 610.19±1.02 |
| | *U. thermosphaericus* | 354.38±0.00 | 624.88±15.83 |
| | Co-culture | 354.38±0.00 | 653.52±48.13 |
| 41°C | *C. taiwanensis* | 354.38±0.00 | 656.76±133.97 |
| | *U. thermosphaericus* | 354.38±0.00 | 711.86±18.53 |
| | Co-culture | 354.38±0.00 | 688.46±13.58 |
| 45°C | *C. taiwanensis* | 354.38±0.00 | 525.62±26.81 |
| | *U. thermosphaericus* | 354.38±0.00 | 726.39±13.22 |
| | Co-culture | 354.38±0.00 | 735.66±0.62 |

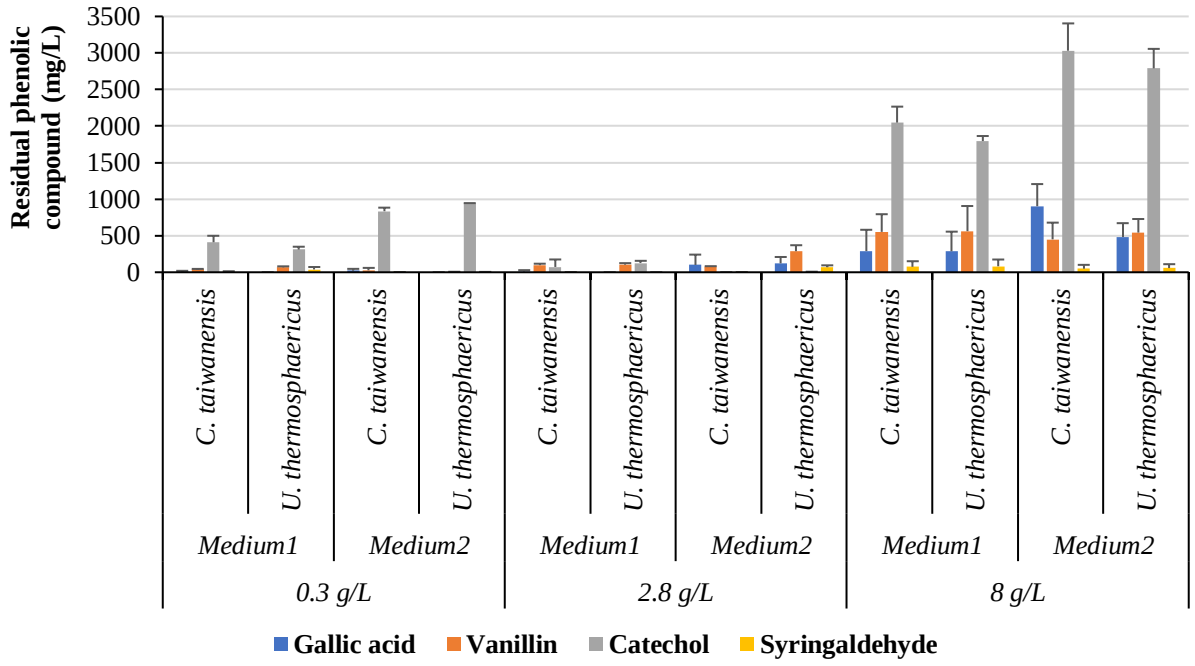



Figure 4-28 : Residual phenolic compound in monocultures at different initial concentration of total phenolics (0.3 g/L, 2.8 g/L and 8 g/L) and with different nutrient sources (Medium 1 (basal salt medium and trace metal ions) and Medium 2 (yeast extract, bactopeptone, $K_2HPO_4$ and $MgSO_4 \cdot 7H_2O$)). The detoxification experiments were performed in duplicates at 41 °C and 200 rpm for 24 h and results are mean of two values.

## 4.7 References

[1] J. A. Quintero, J. Moncada, and C. A. Cardona, "Techno-economic analysis of bioethanol production from lignocellulosic residues in Colombia: a process simulation approach," Bioresource technology, vol. 139, pp. 300-307, 2013.

[2] M. Vargas-Garcı, F. Suárez-Estrella, M. López, and J. Moreno, "Effect of inoculation in composting processes: modifications in lignocellulosic fraction," Waste Management, vol. 27, no. 9, pp. 1099-1107, 2007.

[3] H. K. Sharma, C. Xu, and W. Qin, "Biological Pretreatment of Lignocellulosic Biomass for Biofuels and Bioproducts: An Overview," Waste and Biomass Valorization, journal article August 28 2017.

[4] M. Schroyen, S. W. Van Hulle, S. Holemans, H. Vervaeren, and K. Raes, "Laccase enzyme detoxifies hydrolysates and improves biogas production from hemp straw and miscanthus," Bioresource technology, vol. 244, pp. 597-604, 2017.

[5] Y. Kim, E. Ximenes, N. S. Mosier, and M. R. Ladisch, "Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass," Enzyme and Microbial Technology, vol. 48, no. 4-5, pp. 408-415, 2011.

[6] R. Kumar, S. Singh, and O. V. Singh, "Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives," Journal of industrial microbiology & biotechnology, vol. 35, no. 5, pp. 377-391, 2008.

[7] E. Ximenes, Y. Kim, N. Mosier, B. Dien, and M. Ladisch, "Inhibition of cellulases by phenols," Enzyme and Microbial Technology, vol. 46, no. 3-4, pp. 170-176, 2010.

[8] E. Ximenes, Y. Kim, N. Mosier, B. Dien, and M. Ladisch, "Deactivation of cellulases by phenols," Enzyme and microbial technology, vol. 48, no. 1, pp. 54-60, 2011.

[9] K. Liu et al., "Butanol production from hydrothermolysis-pretreated switchgrass: Quantification of inhibitors and detoxification of hydrolyzate," Bioresource technology, vol. 189, pp. 292-301, 2015.

[10] V. Ujor, C. V. Agu, V. Gopalan, and T. C. Ezeji, "Glycerol supplementation of the growth medium enhances in situ detoxification of furfural by *Clostridium beijerinckii* during butanol fermentation," Applied microbiology and biotechnology, vol. 98, no. 14, pp. 6511-6521, 2014.

[11] D. Yao et al., "Robustness of Clostridium saccharoperbutylacetonicum for acetone-butanol-ethanol production: Effects of lignocellulosic sugars and inhibitors," Fuel, vol. 208, pp. 549-557, 2017.

[12] T. Ezeji, N. Qureshi, and H. P. Blaschek, "Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation," Biotechnology and bioengineering, vol. 97, no. 6, pp. 1460-1469, 2007.

[13] G. Yang, M. S. Jahan, L. Ahsan, L. Zheng, and Y. Ni, "Recovery of acetic acid from pre-hydrolysis liquor of hardwood kraft-based dissolving pulp production process by reactive extraction with triisooctylamine," Bioresource technology, vol. 138, pp. 253-258, 2013.

[14] F. Mechmech, H. Chadjaa, M. Rahni, M. Marinova, N. B. Akacha, and M. Gargouri, "Improvement of butanol production from a hardwood hemicelluloses hydrolysate by combined sugar concentration and phenols removal," Bioresource technology, vol. 192, pp. 287-295, 2015.

[15] N. Qureshi, A. Lolas, and H. Blaschek, "Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101," Journal of industrial Microbiology and Biotechnology, vol. 26, no. 5, pp. 290-295, 2001.

[16] S. Larsson, A. Reimann, N.-O. Nilvebrant, and L. J. Jönsson, "Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce," Applied biochemistry and biotechnology, vol. 77, no. 1-3, pp. 91-103, 1999.

[17] O. A. Ajao, "Development of Hemicelluloses Biorefineries for Integration Into Kraft Pulp Mills," École Polytechnique de Montréal, 2014.

[18] J. Shen, I. Kaur, M. M. Baktash, Z. He, and Y. Ni, "A combined process of activated carbon adsorption, ion exchange resin treatment and membrane concentration for recovery of dissolved organics in pre-hydrolysis liquor of the kraft-based dissolving pulp production process," Bioresource Technology, vol. 127, pp. 59-65, 2013.

[19] B. C. Saha, N. Qureshi, G. J. Kennedy, and M. A. Cotta, "Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis," International Biodeterioration & Biodegradation, vol. 109, pp. 29-35, 2016.

[20] N. Okuda, M. Soneura, K. Ninomiya, Y. Katakura, and S. Shioya, "Biological detoxification of waste house wood hydrolysate using *Ureibacillus thermosphaericus* for bioethanol production," Journal of bioscience and bioengineering, vol. 106, no. 2, pp. 128-133, 2008.

[21] C. Asada, C. Sasaki, T. Takamatsu, and Y. Nakamura, "Conversion of steam-exploded cedar into ethanol using simultaneous saccharification, fermentation and detoxification process," Bioresource technology, vol. 176, pp. 203-209, 2015.

[22] B.-Y. Chen, W.-M. Chen, and J.-S. Chang, "Optimal biostimulation strategy for phenol degradation with indigenous rhizobium *Ralstonia taiwanensis*," Journal of hazardous materials, vol. 139, no. 2, pp. 232-237, 2007.

[23] B.-Y. Chen, C.-Y. Lin, and S.-Y. Hsu, "Toxicity assessment upon augmented biostimulation source to indigenous rhizobium *Cupriavidus taiwanensis*," Journal of hazardous materials, vol. 163, no. 1, pp. 143-151, 2009.

[24] B.-Y. Chen, J.-W. You, Y.-T. Hsieh, and J.-S. Chang, "Feasibility study of exponential feeding strategy in fed-batch cultures for phenol degradation using *Cupriavidus taiwanensis*," Biochemical Engineering Journal, vol. 41, no. 2, pp. 175-180, 2008.

[25] N. Conde-Martínez, A. Acosta-González, L. E. Díaz, and E. Tello, "Use of a mixed culture strategy to isolate halophilic bacteria with antibacterial and cytotoxic activity from the Manaure solar saltern in Colombia," BMC microbiology, vol. 17, no. 1, p. 230, 2017.

[26] R. K. Pettit, "Mixed fermentation for natural product drug discovery," Applied microbiology and biotechnology, vol. 83, no. 1, pp. 19-25, 2009.

[27] N. Qureshi, B. C. Saha, R. E. Hector, and M. A. Cotta, "Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyzed wheat straw: production of butanol from hydrolysate using *Clostridium beijerinckii* in batch reactors," Biomass and bioenergy, vol. 32, no. 12, pp. 1353-1358, 2008.

[28] B. C. Saha, "Hemicellulose bioconversion," Journal of Industrial Microbiology and Biotechnology, vol. 30, no. 5, pp. 279-291, 2003.

[29] X. Z. Theodore Radiotis, Michael Paice,and Vince Byrne, "Optimizing Production of Xylose and Xylooligomers from Wood Chips," 570 Saint-Jean Blvd., Pointe-Claire, QC, H9R 3J9 CanadaPRR 1929, October 2010 2010.

[30] Y. B. Theodore Radiotis, Xiao Zahng, Michael Paice, Richard Berry, and Tom Browne, "Pre-Extraction of Hemicelluloses from Softwood Prior to Kraft Pulping," 570 Saint-Jean Blvd., Pointe-Claire, QC, Canada, H9R 3J9PSR 623, April 2009 2009.

[31] O. Ajao, M. Rahni, M. Marinova, H. Chadjaa, and O. Savadogo, "Retention and flux characteristics of nanofiltration membranes during hemicellulose prehydrolysate concentration," Chemical Engineering Journal, vol. 260, pp. 605-615, 2015.

[32] J. Bremner, "Total Nitrogen 1," Methods of soil analysis. Part 2. Chemical and microbiological properties, no. methodsofsoilanb, pp. 1149-1178, 1965.

[33] W.-M. Chen et al., "*Ralstonia taiwanensis* sp. nov., isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient," International Journal of Systematic and Evolutionary Microbiology, vol. 51, no. 5, pp. 1729-1735, 2001.

[34] S.-S. Cheng, S.-M. Chang, and S.-T. Chen, "Effects of volatile fatty acids on a thermophilic anaerobic hydrogen fermentation process degrading peptone," Water science and technology, vol. 46, no. 4-5, pp. 209-214, 2002.

[35] L. Adour, C. Couriol, and A. Amrane, "Differentiation Between Amino Acids Used as Carbon and Energy Sources During Growth of *Geotrichum candidum* Geo17," Food Technology and Biotechnology, vol. 43, no. 1, pp. 85-89, 2005.

[36] W.-M. Chen, C.-H. Wu, E. K. James, and J.-S. Chang, "Metal biosorption capability of *Cupriavidus taiwanensis* and its effects on heavy metal removal by nodulated *Mimosa pudica*," Journal of Hazardous Materials, vol. 151, no. 2-3, pp. 364-371, 2008.

[37] G. E. Imade, O. Towobola, A. S. Sagay, and J. Otubu, "Discrepancies in sperm count using improved Neubauer, Makler, and Horwells counting chambers," Archives of andrology, vol. 31, no. 1, pp. 17-22, 1993.

[38] M. J. ZINAMAN, M. L. UHLER, E. Vertuno, S. G. FISHER, and E. D. CLEGG, "Evaluation of computer-assisted semen analysis (CASA) with IDENT stain to determine sperm concentration," Journal of andrology, vol. 17, no. 3, pp. 288-292, 1996.

[39] L. M. Sandhaus, C. A. Dillman, W. P. Hinkle, J. M. MacKenzie, and G. Hong, "A New Automated Technology for Cerebrospinal Fluid Cell CountsComparison of Accuracy and Clinical Impact of GloCyte, Sysmex XN, and Manual Methods," American journal of clinical pathology, vol. 147, no. 5, pp. 507-514, 2017.

[40] Y. Wang, M. Castillo-Keller, E. Eustance, and M. Sommerfeld, "Early detection and quantification of zooplankton grazers in algal cultures by FlowCAM," Algal Research, vol. 21, pp. 98-102, 2017.

[41] K. Ma and Z. Ruan, "Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi," Bioresource technology, vol. 175, pp. 586-593, 2015.

[42] A. Farnet, G. Gil, and E. Ferre, "Effects of pollutants on laccase activities of *Marasmius quercophilus*, a white-rot fungus isolated from a Mediterranean schlerophyllous litter," Chemosphere, vol. 70, no. 5, pp. 895-900, 2008.

[43] S. Eghbaliferiz and M. Iranshahi, "Prooxidant activity of polyphenols, flavonoids, anthocyanins and carotenoids: updated review of mechanisms and catalyzing metals," Phytotherapy Research, vol. 30, no. 9, pp. 1379-1391, 2016.

[44] C. Zhou et al., "Effect of Common Metal Ions and Anions on Laccase Catalysis of Guaiacol and Lignocellulosic Fiber," BioResources, vol. 12, no. 3, pp. 5102-5117, 2017.

[45] Y.-L. Loow et al., "Recent advances in the application of inorganic salt pretreatment for transforming lignocellulosic biomass into reducing sugars," Journal of agricultural and food chemistry, vol. 63, no. 38, pp. 8349-8363, 2015.

[46] O. Ajao, M. Le Hir, M. Rahni, H. Chadjaa, and M. Marinova, "Comparative biocatalytic degradation of Kraft prehydrolysate phenolic fermentation inhibitors using bacteria-derived laccase," Wood science and technology, vol. 51, no. 3, pp. 585-599, 2017.

[47] K. Glendinning, L. Macaskie, and N. Brown, "Mercury tolerance of thermophilic *Bacillus* sp. and *Ureibacillus* sp," Biotechnology letters, vol. 27, no. 21, pp. 1657-1662, 2005.

[48] M. E. Brown and M. C. Chang, "Exploring bacterial lignin degradation," Current opinion in chemical biology, vol. 19, pp. 1-7, 2014.

[49] R. Allard-Massicotte, H. Chadjaa, and M. Marinova, "Phenols Removal from Hemicelluloses Pre-Hydrolysate by Laccase to Improve Butanol Production," Fermentation, vol. 3, no. 3, p. 31, 2017.

# CHAPITRE 5 : ARTICLE 2: COMBINING CHEMICAL FLOCCULATION AND BACTERIAL CO-CULTURE OF *CUPRIAVIDUS TAIWANENSIS* AND *UREIBACILLUS THERMOSPHAERICUS* TO DETOXIFY A HARDWOOD HEMICELLULOSES HYDROLYSATE AND ENABLE ABE FERMENTATION LEADING TO BUTANOL

**Biotechnology Progress**

**Authors: Mariem Theiri [1,2], Mariya Marinova [3], Hassan Chadjaa [2] and Mario Jolicoeur [1,*]**

[1] Research Laboratory in Applied Metabolic Engineering, Department of Chemical Engineering, École Polytechnique de Montréal, J.-A. -Bombardier Pavilion, 2900 Édouard-Montpetit Blvd., Montréal, QC, H3T 1J4 Canada.

[2] Centre National en Électrochimie et en Technologies Environnementales, 5230, Boulevard Royal, Shawinigan, QC G9N 4R6, Canada.

[3] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, 13 General Crerar Crescent, Kingston, ON K7K 7B4.

***** Corresponding author: mario.jolicoeur@polymtl.ca; Tel.: 514-340-4711 ext. 4525.

## 5.1 Abstract

Butanol, a fuel presenting superior characteristics to ethanol, can be produced via Acetone-Butanol-Ethanol (ABE) fermentation using lignocellulosic biomass as culture medium base. However, many inhibitors present in the hydrolysate limit the fermentation process. In this work, we present for the first time a detoxification technology combining flocculation and biodetoxification within a bacterial co-culture composed of *Ureibacillus thermosphaericus* and *Cupriavidus taiwanensis*. We first investigated co-culture based strategies to detoxify filtered and unfiltered hydrolysates. The best results of detoxification were obtained for a two-step approach combining flocculation to biodetoxification. This sequential process led to a final phenolic

compounds concentration of 1.4 g/L, a value close to the minimum inhibitory level observed for flocculated hydrolysate (1.1 g/L). The generated hydrolysate was then fermented with *Clostridium acetobutylicum* ATCC 824 for 120 h. A final butanol production of 8 g/L was obtained although the detoxified hydrolysate was diluted to reach 0.3 g/L of phenolics to ensure non-inhibitory conditions.

**Keywords:** pre-hydrolysate; inhibitors; flocculation; co-culture; butanol.

## 5.2 Introduction

Canada is the largest exporter of pulp and paper products and it is the second global producer after the United States [1, 2]. However, the global decrease in paper demand associated with the expansion of electronic media is strongly affecting this industrial sector [3]. To overcome the difficulties, the industry has mostly shifted from the conventional Kraft process to a Kraft dissolving pulp process to broaden its revenue sources and this strategy requires the integration of a pre-treatment step, upstream of pulp production, to facilitate hemicelluloses removal and the recovery of cellulose fibers [4]. The hemicellulose-enriched stream provides a true opportunity for integrating a biorefinery within pulp and paper production sites [5], thus enlarging the range of potential value-added products, including butanol [6-11]. Butanol, a four carbon alcohol, is considered a promising liquid fuel in comparison with ethanol [12] thanks to its 33% higher energy content [13], lower hygroscopy and vapor pressure, as well as its complete miscibility with gasoline [12, 14-17]. Although butanol can be chemically synthesised, its biological production via ABE fermentation using low cost sugars is an economically sound and environmentally friendly alternative [18]. Hemicelluloses generated from the pre-treatment step in the Kraft dissolving pulp process are composed of different polysaccharides [19], that can serve as carbon source in ABE fermentation. The hydrolysis step needs to be implemented downstream to the pre-treatment to convert the abundant hemicellulosic oligo-saccharides into fermentable monosaccharides [20]. The hydrolysis treatments are however often accompanied by the liberation of toxic compounds such as acetic acid, furfural, 5-hydroxymethylfurfural (5-HMF) and phenolic compounds, which can all have detrimental effects on the fermentation bioprocess and impede the conversion of carbon sources (e.g. xylose) to the desired products (e.g. butanol) [21]. It is worthy to note that furanic aldehydes can improve the ABE fermentation at low concentrations (i.e. 2-3 g/L of furfural and 5-HMF respectively can be reduced to their alcohols by *C. acetobutylicum* ATCC 824), while high

concentrations up to ~6 and 3.5 g/L of furfural and 5-HMF respectively are harmful to the solventogenic strain [22]. Furthermore, the accumulation of solvents including the product of interest, butanol, also presents a high level of toxicity for the fermentation process [23]. Novel strategies have been investigated to remove toxic compounds from the hemicelluloses hydrolysates and/or to limit their inhibitory effects. Several detoxification technologies were developed based on physico-chemistry, such as over-liming, activated carbon, ion exchange resins and evaporation, or on biotechnological processes [24]. The cost-effectiveness of the former is limited by low yields of detoxification, high energy demand, large volumes of effluents, sugar losses, inhibitory compounds generation, etc. [25]. The latter are based on enzymes (mainly laccases and peroxidases) that degrade phenolic compounds with high efficiency (up to 77%) [6]. Laccases are of particular interest because they do not require the addition of compounds such as hydrogen peroxide which are required when using lignin peroxidases [26]. However, these enzymatic treatments are limited to phenolics, and enzyme production, and purification and concentration can significantly increase the global process costs. Conversely, the use of specific microorganisms producing such detoxifying enzymes de novo offers a broader spectrum of action, including phenolic compounds, furfural, 5-HMF, and others [27].

Several factors must be considered when developing an efficient microbial detoxification process for lignocellulosic materials. Indeed, yields of inhibitors removal, type and extent of target toxic compounds, microbial tolerance, sugar consumption, treatment duration, nutritional requirements [26] and conditions under which the detoxification enzymes are expressed are of paramount importance for a cost-effective and efficient detoxification process. For instance, the *Amorphotheca resinae* ZN1 strain has been used for solid lignocellulosic pre-hydrolysate detoxification but suffers from a low rate of inhibitor removal (treatment up to 7 days can be required). He *et al.* [28] also reported high xylose loss of 34% during the process. Similarly, the *Penicillium* sp. strain *apw-tt2* was reported to degrade up to 28% of cellulose and 66% of lignin [29]. As for the *Coniochaeta ligniaria* NRRL30616 strain, despite its high performances for the degradation of furan aldehydes (furfural and 5-HMF) and phenolic compounds (100%, 69% and 46% in 25 h, respectively), the strain lacks capacity for laccases production and may cause a high loss of xylose (up to 74%) as reported by Kim *et al.* [30]. Among the numerous microorganisms that have been tested, *Ureibacillus thermosphaericus* (*U. thermosphaericus*) and *Cupriavidus taiwanensis* (*C. taiwanensis*) appear to be of high interest as potent bacteria for detoxification. In

fact, these two strains are known for their high efficiency in removing phenolic compounds in a timely manner (24 h) and for their production of a broad range of lignocellulolytic enzymes, including laccases, tyrosinase, peroxidases, catalases and oxidases. *U. thermosphaericus* also efficiently degrades furfural and 5-HMF with minor sugar consumption (<5%) [31, 32]. Unfortunately, the two bacteria strains exhibit a low tolerance to high concentrations of inhibitors, which has limited their commercial usage [31].

To overcome these limitations, the co-culture of *U. thermosphaericus* and *C. taiwanensis* was investigated for the first time (to the best of our knowledge) to detoxify both concentrated and non-concentrated hardwood hemicelluloses hydrolysates. The rationale was based on the observation that consortia of coexisting microorganisms are abundantly found in nature [33, 34] and that they can lead to the complete degradation of inhibitors in lignocellulosic biomass, faster than monocultures [34]. Moreover, the range of target inhibitors can be expanded through cooperative interactions between microorganisms [35]. The eco-efficiency of a co-culture-based process is emphasised using low-sugars consuming microorganisms. In fact, consumption of sugars by the co-culture is expected to not exceed 10% as *U. thermosphaericus* was not able to consume more than 5% of sugars in pure culture but no information is available in the literature about the values of sugar consumption by *C. taiwanensis*. A maximum of 5% of sugar consumption by *U. thermosphaericus* might be slightly increased in the co-culture. In order to enhance removal levels, a second detoxification method of flocculation was integrated within the co-culture process. In this project, physical (i.e. nanofiltration) and chemical (i.e. flocculation) treatments were tested in combination with biodetoxification by the selected co-culture of *U. thermosphaericus* and *C. taiwanensis*. The efficiency of the best detoxification method, yielding hydrolysate with lower concentration of inhibitors, was then validated by ABE fermentation with *Clostridium acetobutylicum* ATCC 824 (*C. acetobutylicum* ATCC 824) for butanol production. Overall, a novel efficient strategy for enhanced butanol production from hemicelluloses hydrolysate based on the combination of a flocculation step with biodetoxification by a co-culture of *U. thermosphaericus* and *C. taiwanensis* is reported in this article.

## 5.3 Materials and Methods

### 5.3.1 Bacterial strains and culture conditions

In this study, *U. thermosphaericus* NCIMB 13819 and *C. taiwanensis* DSM 17343 were used for the detoxification of filtered and non-filtered hydrolysates, and *C. acetobutylicum* ATCC 824 was used for ABE production.

*U. thermosphaericus* NCIMB 13819 was purchased from NCIMB Ltd. (YA, Scotland) and was activated in 3% (w/v) sterilized Tryptic Soy Broth (TSB) (Quelab Laboratories inc.; catalog number QB-39-5626) at 55 °C and 180 rpm. To maintain stock culture, cells were grown until two deceleration points were obtained and 50 mL aliquots were spun down in falcon tubes. The supernatant was removed and replaced with a glycerol 30% (v/v) solution (Fisher Bioreagents; catalog number 666 L-13751U). Aliquots (0.5 mL) were then dispensed into 1 mL cryogenic vials and placed at -20 °C before freezing at -80 °C. For detoxification's inoculum preparation, TSB (containing (in g/L): casein peptone 17, soy peptone 3, dipotassium phosphate 2.5, dextrose 2.5 and sodium chloride 5) [36-38] was sterilized by autoclave at 121 °C for 15 min. After cooling, the medium was inoculated with 0.1% (v/v) of stock culture. The inoculum was grown for 10 h at 50 °C and 180 rpm in an orbital shaker (New Brunswick Scientific Innova 44, USA) to a final OD between 3.8 and 4.1.

*C. taiwanensis* DSM 17343 was obtained from DSMZ (Braunschweig, Germany) and revived in a R2A medium (comprising (in g/L): yeast extract 0.5 (Becton, Dickinson and Company (BD), cat. # 212750), proteose peptone 0.5 (Nutri-Bact; catalog number QB-39-1910), casamino acids 0.5 (organotechnie), glucose 0.5, soluble starch 0.5 (Fisher Chemical; catalog number S516-500), Na-pyruvate 0.3 (Fisher Bioreagents; catalog number 253 S-15629U), $K_2HPO_4$ 0.3 (Fisher Bioreagents; catalog number 19 L-15781U), $MgSO_4{\cdot}7H_2O$ 0.05 (Acros Organics; catalog number 423905000)) at 28 °C with agitation at 180 rpm in an orbital shaker (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA) until an $OD_{600}$ between 1.6 and 1.9 was reached. The stock culture was treated by addition of glycerol 30% (v/v) to the pellets of R2A culture, followed by a storage at -80 °C. The inoculum was prepared in the TSB medium inoculated with 0.1 % (v/v) from the stock culture then grown at 37 °C and 180 rpm (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA) for 10 h to a final OD between 2.8 and 3.

*C. acetobutylicum* ATCC 824 was acquired from the American Type Culture Collection (ATCC) and was grown under anaerobic conditions in sterilized Reinforced Culture Medium (RCM) (Nutri-Bact; catalog number QB-39-3724) (containing (in g/L): tryptose 10, beef extract 10, glucose 5, yeast extract 3, soluble starch 0.5, sodium chloride 5 and L-cysteine-HCl 0.5) at 37 °C and 110 rpm until an $OD_{600}$ of 1.9-2. RCM was prepared by boiling the medium added with 0.1% (v/v) of resazurin (Sigma; catalog number 1001824725). In order to eliminate the oxygen in the medium, a purge with a gas mixture of 80% $N_2$ and 20% $CO_2$ was applied. 1% (v/v) of $Na_2S$ (5% (w/v)) was added to the medium if necessary. The RCM was further sterilized by autoclave at 121 °C for 15 min. For stock culture preparation, glycerol 30% (v/v) was used then the stock culture was stored at -80 °C. For preparation of the ABE fermentation's inoculum, sterilized RCM was inoculated with 0.2% (v/v) of stock culture. The inoculum was grown at 37 °C with agitation of 110 rpm in an orbital shaker (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA) until an $OD_{600}$ between 0.6 and 0.9. The inoculum cultures were performed in 100 mL screw capped Schott bottles (Retrace code: 10045637, Germany) filled with 50 mL of the culture medium. The bottles were sealed with a rubber septum (Butyl Septa 20 mm, USA) and an aluminium cap (Z114146-100EA, Wheaton closed-top seals, Tear-off seals only, diam. 20mm, PCode 1001743418, USA) and were used to ensure anaerobic conditions during growth.

### 5.3.2 Preparation of the hydrolysate

Prior to the ABE fermentation, six strategies were evaluated for the detoxification of hardwood hemicelluloses pre-hydrolysate (Figure 5-1). First, the co-culture performance for the detoxification of an acid hydrolysate (strategy A) was assessed. Second, a chemical treatment by flocculation of the hydrolysate was integrated prior to the co-culture treatment (strategy B). Third, the order of detoxification methods was switched, starting with the biodetoxification of the hydrolysate, followed by flocculation (strategy C). Due to the low concentration of sugars in the acid hydrolysate (24.45 ± 0.31 g/L), a concentration step with nanofiltration was added prior to the hydrolysis. For each of the three strategies (A-C), detailed above, the effect of a nanofiltration step on the removal performance for phenolic compounds and furans aldehydes was evaluated.

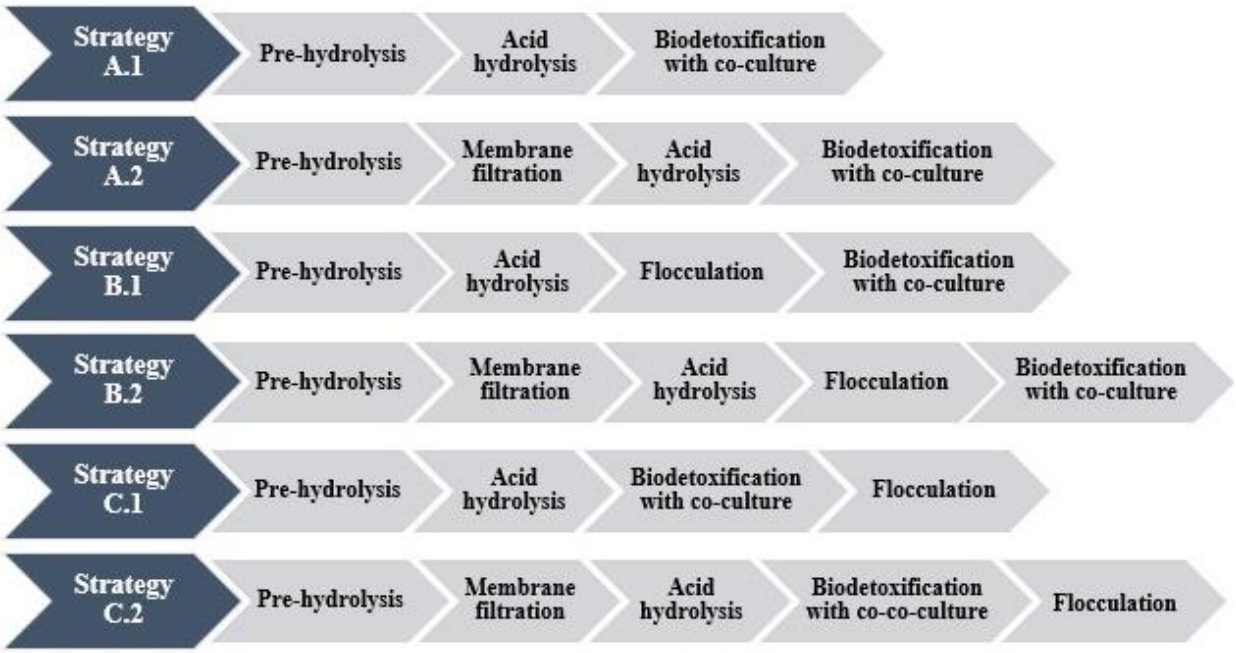



Figure 5-1 : Sequential process strategies (A-C) developed for the detoxification and concentration of wood pre-hydrolysate.

Prior to performing the detoxification experiments following the three strategies (Figure 5-1), the variation of the order and the interval of bacteria inoculation in the co-culture was investigated and is presented in the Section 5.7 (Appendix). The objective of these experiments was to define a system to establish the co-culture, which allows higher degradation of inhibitors. For that, three intervals of inoculation: 0 h, 12 h and 24 h were tested in the pre-hydrolysate and the order of *C. taiwanensis* and *U. thermosphaericus* inoculation was varied in each set of experiments (i.e. inoculation intervals of 12 h and 24 h). The different cultures were performed at 41 °C and 200 rpm during which the residual concentration of phenolic compounds was determined with the Folin Cioacalteu method. The variation of the phenolic compounds concentration was investigated, as they are considered strong inhibitors of the ABE fermentation with *C. acetobutylicum* ATCC 824 [6]. From the results described in the Section 5.7 (Appendix), the highest percentage removal of phenolic compounds was 25% and was obtained at 84 h of incubation using the sequential inoculation with a 24 h delay in the inoculation of *C. taiwanensis*.

#### 5.3.2.1 Pre-hydrolysis

The hemicelluloses pre-hydrolysate (composition provided in Table 5-1), was generated by steam explosion coupled with hot water. A mixture composed of 60% aspen and 40% maple wood chips was used as a raw material for pre-hydrolysis. The pre-hydrolysis step was carried out in a 56 L pilot digester at FPInnovations (Pointe-Claire, Québec, Canada). A bucket with lid was filled with 15 L of the generated pre-hydrolysate and then sent to the Centre National en Électrochimie et en Technologies Environnementales (CNETE) where it was stored at 4 °C. The details of the pre-treatment procedure were given in the paper of Ajao *et al.* [39].

Table 5-1 : Characterization of hemicelluloses pre-hydrolysate used in this study

| Component | Values |
|---|---|
| Monomeric xylose (g/L) | 3.2 |
| Phenolic compounds (UV/Vis) (g/L) | 4.5 |
| Total Nitrogen (g/L) | 0.035 |
| Acetic acid (g/L) | 3.63 |
| Furfural (g/L) | 0.518 |
| 5-HMF (g/L) | 0.082 |
| Vanillin (mg/L) | 48.1 |
| Syringaldehyde (mg/L) | 109.5 |

#### 5.3.2.2 Acid hydrolysis

A hydrolysis step was required in order to convert oligosaccharides into fermentable monosaccharides. Polysaccharides hydrolysis was achieved using sulfuric acid solution ($H_2SO_4$) which was proved to be efficient at industrial scale for its high yields of hydrolysis and low operational costs compared to enzymatic hydrolysis. Acid hydrolysis was permormed using a 1.5 wt% sulfuric acid solution (Caledon Laboratories Ltd., Ontario, Canada) at 121 °C for 60 min. The obtained hydrolysate was then filtered using a Whatman glass microfiber filter of 1.5 µm porosity (GE Healthcare Life Sciences; 934-AH$^{TM}$; diameter 70 mm, 100 circles; catalog number 1827-070) to eliminate the formed precipitate.

#### 5.3.2.3 Coagulation-flocculation using ferric sulfate

The flocculation experiments were performed in jars. First, a solution containing 200 g/L of the flocculating agent iron (III) sulfate (97%, Sigma-Aldrich, Co., St. Louis, MO, USA) was mixed with the hydrolysate until a ratio of 1 g iron/1 g phenolic compounds was reached under stirring at 150 rpm for 10 min. Subsequently, $Ca(OH)_2$ was added for pH adjustment to 7 (+/- 2%). The agitation speed was then reduced to 50 rpm for 30 min to stimulate the flocculation of destabilized colloids. Finally, a 2 h sedimentation step of the floc particles, followed by a filtration step through a 1.5 µm Whatman filter were performed to collect the treated hydrolysate.

#### 5.3.2.4 Nanofiltration

To concentrate the hemicelluloses pre-hydrolysate, nanofiltration was applied using a NF270 organic membrane (molecular weight cut-off 200-400 Da from Dow chemical, USA). In batch mode, the pre-hydrolysate was fed into a lab-scale SEFA CF II (GE Osmonics) cross-flow flat sheet membrane test unit. A volumetric concentration factor of 3.125 was obtained after recovering 0.8 L of concentrate from 2.5 L of biomass material.

#### 5.3.2.5 Biodetoxification procedure

For the detoxification step, 500 mL of baffled Erlenmeyer flasks were filled with 100 mL hydrolysate. The pH was adjusted to 7 with $Ca(OH)_2$ prior to autoclaving. The nutrients source, composed of basal salt medium (g/L: $Na_2HPO_4$ 7, $KH_2PO_4$ 3, NaCl 0.5 and $NH_4Cl$ 1) and trace metal ions (mg/L: $MgSO_4 \cdot 7H_2O$ 580, $FeSO_4 \cdot 7H_2O$ 7, $CaCl_2 \cdot 2H_2O$ 53.6, $MnSO_4 \cdot H_2O$ 0.38, $CoCl_2 \cdot 5H_2O$ 0.2 and $CuSO_4 \cdot 5H_2O$ 0.043) was added after sterilisation to the autoclaved hydrolysate. To generate the co-culture, 1.2 mL of *U. thermosphaericus* was inoculated into the flasks, followed by 0.8 mL of *C. taiwanensis* 24 h later. Bacterial cultures were incubated at 41 °C with a 200 rpm agitation in an orbital shaker (INFORS HT Multitron). The detoxification experiments were performed in duplicates and the resulting bio-detoxified hydrolysates were then autoclaved at 121 °C for 5 min. The negative control to confirm the effect of the detoxification procedure was the non-detoxified hydrolysate obtained after acid hydrolysis.

The percentage of the inhibitor compound degradation at a defined stage of detoxification was calculated following Equation (1):

$$\%\ \boldsymbol{Degradation} = \frac{\boldsymbol{Initial\ concentration - Final\ concentration}}{\boldsymbol{Initial\ concentration}} \times \mathbf{100} \quad (1)$$

The initial concentration represents the concentration of the inhibitor just before a defined stage of treatment, while the final concentration represents the residual concentration of the inhibitor after the defined step of detoxification.

### 5.3.3 ABE fermentation

The ABE fermentation experiments were performed in a 250-mL screw-capped Schott bottles using a 200 mL reaction volume. Xylose (60 g/L), was added to the diluted detoxified and non-detoxified hydrolysates, and used as a carbon source. Yeast extract (5 g/L) served as a nitrogen source for the ABE fermentation. For the hydrolysate fermentation, the xylose concentration was maintained at 60 g/L by supplementation with commercial xylose. As a consequence, the fermentation media were richer in xylose than glucose, as the initial pre-hydrolysate contains mainly xylose [40].

The different media were separately boiled in the presence of 0.1% (v/v) resazurin and purged with a gas mixture of 80% $N_2$ and 20% $CO_2$ for 5 min. Thereafter, they were sterilized in an autoclave at 121 °C for 15 min and then mixed in 250-mL screw-capped Schott bottles. The bottles, slightly open, were then placed in anaerobic jars containing Gas Pak envelopes (BD Gas Pak ™ EZ Anaerobe Container System, Franklin Lakes, NJ, USA) with indicators (BD BBL ™ Dry Anaerobic Indicator Strips, Franklin Lakes, NJ, USA) for 48 h to establish an anaerobic environment. Finally, solutions of vitamins (para-aminobenzoic acid 1 mg/L, thiamin 1 mg/L and biotin 0.01 mg/L), buffers ($KH_2PO_4$ 0.5 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.5 g/L and ammonium acetate 2.2 g/L) and minerals ($MgSO_4{\cdot}7H_2O$ 0.2 g/L, $MnSO_4{\cdot}H_2O$ 0.01 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L and NaCl 0.01 g/L) as set by Ezeji *et al.* [41], filtered and sterilized through a 0.22 µm filter, were added prior to inoculation with 5% (v/v) of C. acetobultylicum pre-culture and incubation at 37 °C and 110 rpm for 120 h in an orbital shaker (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA). The concentrations of vitamins, buffers and minerals detailed above correspond to the final concentrations in the fermentation medium. The fermentation procedure was adopted from the work of Mechmech *et al.* [9]. The fermentation experiments in the present study were performed in duplicates.

### 5.3.4 Analytical methods

#### 5.3.4.1 Spectrophotometric analysis

Cell growth was measured by absorbance at 600 nm with a UV-visible spectrophotometer (Eppendorf BioPhotometer plus). Another UV-visible spectrophotometer (Pharmacia Biotech Novaspec®II, Piscatoway, NJ, USA) was used for the determination of the total phenolic compounds concentration at 725 nm following a reaction with the Folin Cioacalteu reagent (Sigma-Aldrich, Canada Ltd.) The Folin Cioacalteu method was adapted from Singleton and Rossi [42]. The phenolic compounds analyses were performed in duplicates.

#### 5.3.4.2 HPLC analysis

A High performance liquid chromatography system (HPLC) (Agilent Technologies, Germany) with a diode array detector (DAD) at 313 nm and 280 nm and a Nucleosil C18 (150 X 4.6 mm) column was used to determine the concentration of the individual phenolic compounds (gallic acid, catechol, vanillin and syringaldehyde). A solution of 15% acetonitrile and 85% phosphoric acid 10 mM was used as a separation solvent. The same HPLC system equipped with a Nucleosil C18 (150 X 4,6 mm) column and a Diode Array Detector (DAD) at 280 nm was used for furans analysis. The elution solvent was composed of 84% acetonitrile, 15% deionized water and 1% acetic acid. An EC Nucleodur RP-$NH_2$ column (250 mm x 4.6 mm, 5 µm) and a refractive index detector were used for sugars analysis by the HPLC. In this case, a mixture of 75% acetonitrile and 25% deionized water was fed at 1.5 mL/min into the system for the separation of simple sugars at 40 °C. It is worthy to note that only xylose concentration was determined by HPLC, without considering the other sugars, usually available in the hemicelluloses hydrolysates (e.g. glucose, mannose, arabinose, galactose). In fact, Ajao *et al.* [ 40] used in their study a pre-hydrolysate provided by the same supplier (FPInnovations) and determined a ratio of pentose to hexose of 4:1 by using the same HPLC equipment. Based on the data from the pre-hydrolysate characterisation done by the authors [43], the pre-hydrolysate contained only 0.74 g/L of glucose, while xylose concentration was 2.55 g/L. Since xylose was proved to be the dominant monosaccharide released from hardwood hemicelluloses [40] only its content was quantified. Organic acids were analyzed using the same HPLC equipped with DAD (Diode Array Detector) and Interstil ODS-3 column (150 _ 4.6 mm) at

40 °C. The mobile phase, fed at 1.25 mL/min, was a mixture of 99% $KH_2PO_4$ at pH 2.8 and 1% acetonitrile [9].

#### 5.3.4.3 GC-MS analysis

To monitor the production of solvents (acetone, butanol and ethanol) during the ABE fermentation, a gas chromatography (GC 7890A, Agilent Technologies) analysis was performed with an OV 624 capillary column and a flame ionization detector (FID, H2 flow rate: 30 mL/min; air flow rate: 2.23 mL/min).

#### 5.3.4.4 Total nitrogen analysis

Total nitrogen was measured using Kjeldahl method with a Foss Tecator™ digester system and a Kjettec™ 8200 auto distillation unit [44].

## 5.4 Results and discussion

### 5.4.1 Strategies for the detoxification of acid hydrolysates

#### 5.4.1.1 Strategy A: Effect of combining a pre-hydrolysate concentration step with a hydrolysate biodetoxification step by a sequential co-culture of two bacteria

Figure 5-2 shows the inhibitors composition (i.e. phenolic compounds, furfural and 5-HMF) of the hydrolysates at different steps in the strategy A.1. The hydrolysis of the pre-hydrolysate was accompanied with an increase of furfural and 5-HMF contents from 0.53 g/L and 0.08 g/L in the pre-hydrolysate to 0.83 g/L and 0.11 g/L respectively (Figure 5-2A). This increase was due to a further degradation of the pentoses and hexoses under the harsh operating conditions of acid hydrolysis (temperature, duration, acid concentration) [45]. Biodetoxification was applied as the sole detoxification method using a sequential inoculation with 24 h between the inoculation of *U. thermosphaericus* and *C. taiwanensis*. A final degradation of 38% phenolics and 45% furfural (Figure 5-2A) was achieved after 24 h of *U. thermosphaercius* inoculation, with no additional degradation in terms of phenolic compounds. However, the concentration of 5-HMF did not change. This short duration of hydrolysate biodetoxification (before *C. taiwanensis* inoculation) can be explained as follows: First, the residual concentrations of toxic compounds remaining after *U. thermosphaericus* treatment or even their degradation products might be toxic to *C. taiwanensis*

metabolism. In fact, the *C. taiwanensis* affinity level for the phenol degradation was reported by Chen *et al.* [46] to be 900 mg/L in a batch process. Then, it is possible that *U. thermosphaericus* would have depleted a specific nutrient required by *C. taiwanensis* thus preventing its growth.

Considering that the strategy A.1 led to ~ 24 g/L of xylose, a concentration step was added between the pre-hydrolysate and the hydrolysate increase the sugars concentration thus favoring the ABE fermentation of the biodetoxified hydrolysate in the strategy A.2. A nanofiltration step was integrated upstream of the acid hydrolysis allowing an increase of the sugars content to ~ 52 g/L.

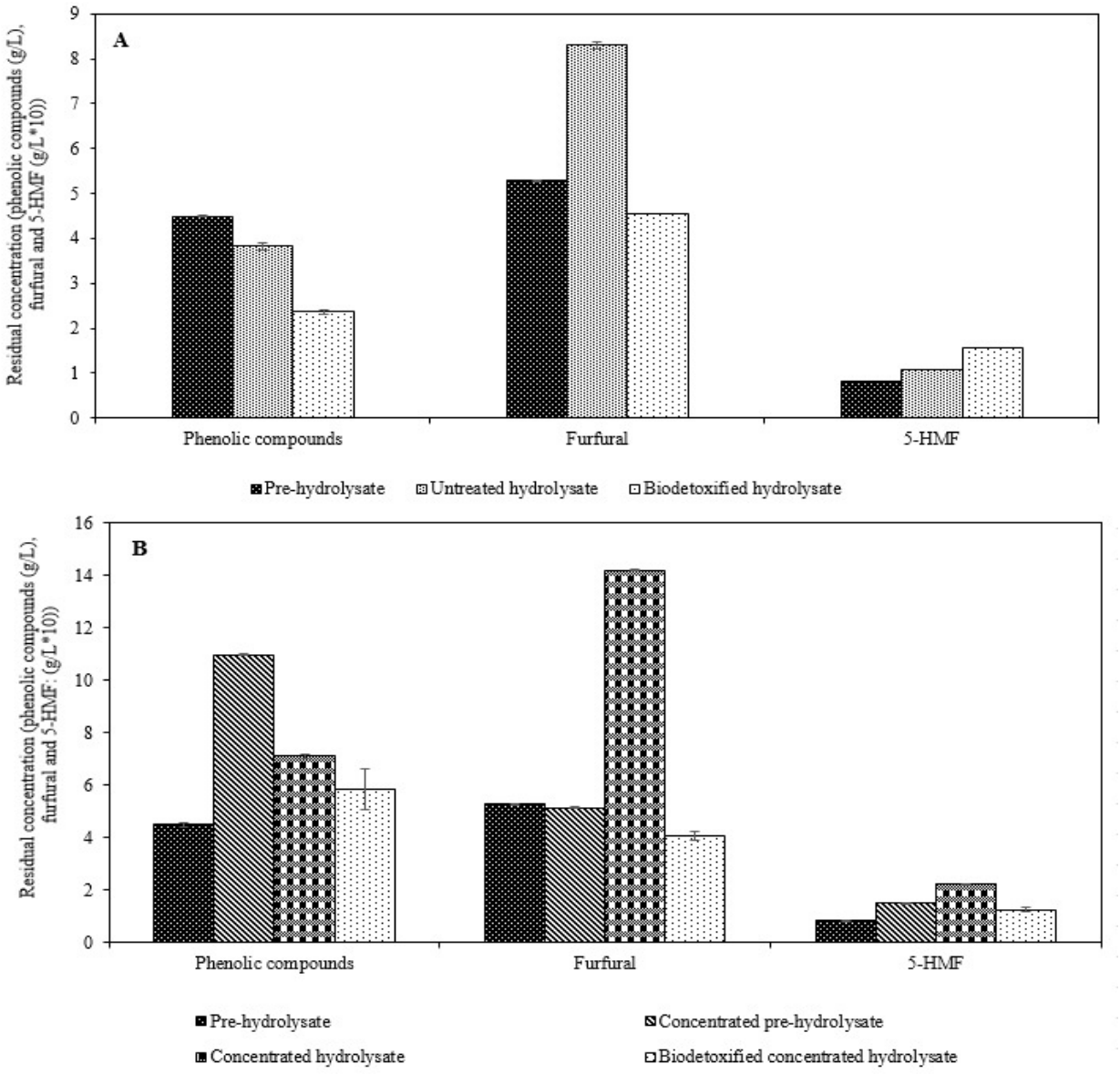



Figure 5-2 : Residual phenolic compounds, furfural and 5- HMF remaining in the hydrolysates produced from strategy A: (A) Strategy A.1, and (B) Strategy A.2. (Experiments were performed in duplicate and results are presented as a mean of two values). The duration of the biodetoxification experiments were 24 h in strategy A.1 and 49 h in strategy A.2.

As shown in Figure 5-2B, the acid hydrolysis of the concentrated pre-hydrolysate contributed to a decrease in the phenolic compounds concentration from 10.9 g/L to 7.1 g/L, corresponding to a degradation level of 35%, more than twice higher than the one obtained without a nanofiltration

step (Figure 5-2A). After applying the sequential co-culture as a biodetoxification step, phenolics, furfural and 5-HMF contents in the concentrated hydrolysate were degraded to 5.9 g/L, 0.4 g/L and 0.13 g/L respectively, corresponding to removal percentages of 18%, 71% and 43% respectively. In comparison to the 24 h required to reach a steady-state of phenolics degradation without nanofiltration, this new strategy required 49 h of bacterial culture before observing a steady-state. This delay can be explained by the higher inhibitors concentration after nanofiltration. It is worthy to note that the best removal rate with the co-culture at these conditions (with integration of a concentration step) was achieved for furfural, with 71%. The higher affinity of the bacteria to furfural justifies these results.

Despite the high efficiency of the co-culture to degrade phenolics (up to 38%) in strategy A.1, the final phenolics content (2.37 g/L) remained above the inhibition threshold levels for *C. acetobutylicum* (0.3 g/L and 1.1 g/L for untreated and flocculated hydrolysate respectively) [9]. The combination of biodetoxification with another detoxification method is thus recommended to enhance the degradation efficiency. Moving forward in this work, a flocculation step using $Fe_2(SO_4)_3$ was applied in addition to the co-culture strategy. The results of the detoxification step are detailed in the following sub-section.

#### 5.4.1.2 Strategy B: Effect of combining a pre-hydrolysate concentration step with a hydrolysate flocculation step followed by a biodetoxification step using a sequential co-culture of two bacteria

The second strategy (strategy B) was based on the detoxification of the acid hydrolysate by flocculation with $Fe_2(SO_4)_3$ followed by bacterial co-culture. The detoxification of the non-concentrated hydrolysate (strategy B.1) was first tested. Then, the performance obtained by including a nanofiltration step prior to the hydrolysis step (strategy B.2) was assessed. The results are shown in Figure 5-3.

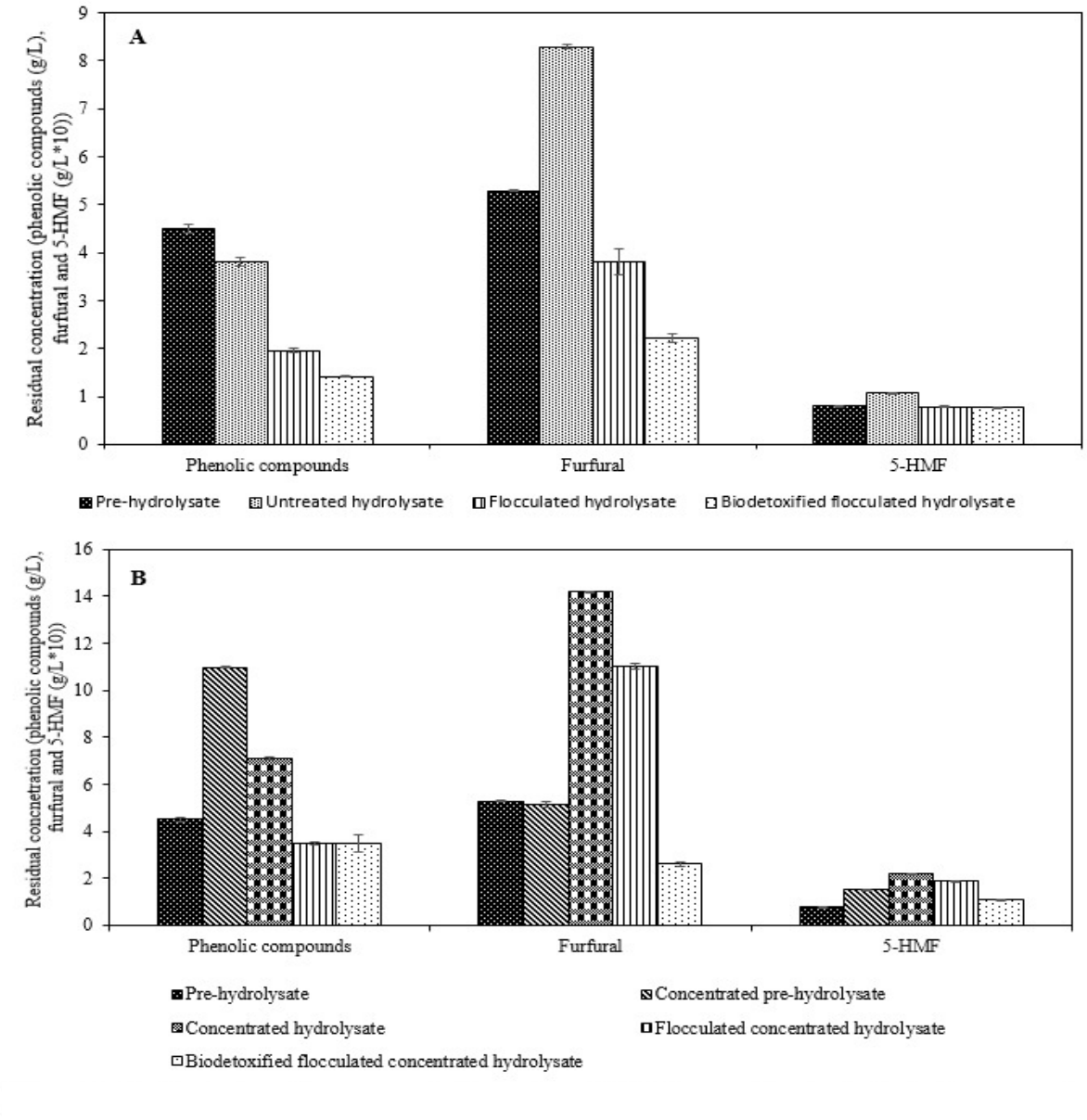



Figure 5-3 : Residual phenolic compounds, furfural and 5- HMF remaining in the hydrolysates produced from strategy B: (A) Strategy B.1, and (B) Strategy B.2. (Experiments were performed in duplicate and results are presented as a mean of two values). The duration of the biodetoxification experiments was 49 h.

The flocculation step in strategy B.1 led to a decrease in the phenolic compounds, furfural and 5-HMF concentration to respectively 1.96 g/L, 0.38 g/L and 0.08 g/L from 4.5 g/L, 0.83 g/L and 0.11

g/L respectively in the hydrolysate. Further biodetoxification with the co-culture eliminated 28% of phenolic compounds after 49 h, with no additional degradation thereafter, 42% of furfural, while 5-HMF concentration remained constant (Figure 5-3A). Although the relative performance of the co-culture was lower for the detoxification of the flocculated hydrolysate than for the treatment of acid hydrolysate (strategy A.1: 38% of phenolics and 45% of furfural removed), the integration of a flocculation step led to lower final concentration of phenolics (1.4 g/L with combination of flocculation with the co-culture versus 2.37 g/L with the co-culture without flocculation step integration). The final concentration of phenolic compounds obtained with strategy B.1 (1.4 g/L) was close to the threshold inhibition concentration of 1.1 g/L determined by Mechmech *et al.* [9].

In strategy B.2 the effect of a nanofiltration step integrated upstream to acid hydrolysis was investigated. The concentration step led to the increase of xylose concentration from ~ 17 g/L obtained from the strategy B.1 to ~ 50 g/L in the hydrolysate from the strategy B.2. Removal of 3.6 g/L, 0.32 g/L and 0.11 g/L of the phenolic compounds, furfural and 5-HMF were respectively achieved after flocculation of the concentrated hydrolysate (Figure 5-3B). The biodetoxification with the sequential co-culture after the flocculation stage led to the degradation of 0.84 g/L and 0.08 g/L of furfural and 5-HMF respectively after 49 h. However, the phenolic compounds concentration was not altered by the co-culture treatment. Two assumptions can be drawn to explain this observation. First, the tested bacteria might exhibit a higher affinity for furan aldehydes at high concentrations. In fact, *U. thermosphaericus* demonstrated a strong degradative capacity for furans aldehydes in waste streams hydrolysate [32]. Secondly, the type and concentration of inhibitors, which were left after flocculation of the concentrated hydrolysate, might affect cells metabolism, resistance and affinity of the detoxifying bacteria.

#### 5.4.1.3 Strategy C: Effect of combining a pre-hydrolysate concentration step with a hydrolysate biodetoxification step using a sequential co-culture of two bacteria followed by a flocculation step

The third strategy (strategy C) involved a detoxification of the concentrated and non-concentrated hydrolysate with the co-culture composed of *U. thermosphaericus* and *C. taiwanensis* followed by a flocculation using $Fe_2(SO_4)_3$. The strains were sequentially inoculated with a 24 h delay in the inoculation of *C. taiwanensis* regarding the inoculation of *U. thermosphaericus* for the first 24 h. The results obtained with this strategy are presented in Figure 5-4.

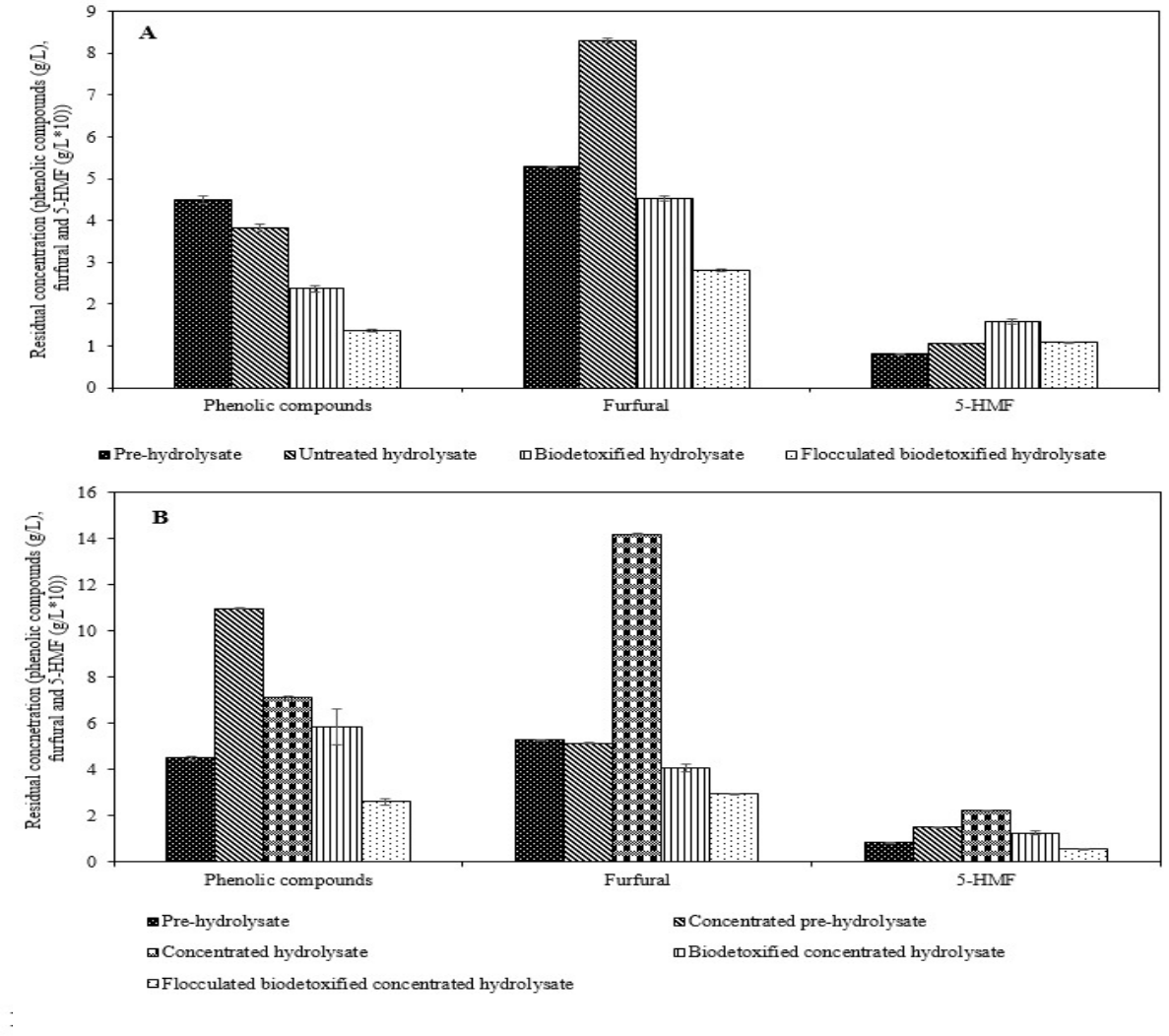



Figure 5-4 : Residual phenolic compounds, furfural and 5- HMF remaining in the hydrolysates produced from strategy C: (A) Strategy C.1, and (B) Strategy C.2. (Experiments were performed in duplicate and results are presented as a mean of two values). The duration of the biodetoxification experiments were 24 h in strategy C.1 and 49 h in strategy C.2.

The biodetoxification of the acid hydrolysate followed by flocculation (strategy C.1) led to the elimination of 64% and 66% of the phenolics and furfural contents respectively. More specifically, averages of 38% and 42% of phenolics were respectively removed after a 24 h-mono-culture of *U. thermosphaericus* and a flocculation, and averages of 45% and 38% of furfural were

correspondingly eliminated. The final flocculation step brought the concentration in phenolic compounds down to 1.38 g/L. Combining flocculation with biodetoxification did not demonstrate a significant effect on the removal efficiency of phenolics. However, furfural and 5-HMF concentrations were reduced from 0.45 g/L and 0.16 g/L in the biodetoxified hydrolysate to respectively 0.28 g/L and 0.11 g/L following this modified strategy C.1 (Figure 5-4A) which were higher than the final concentrations obtained with the strategy B.1.

The integration of a nanofiltration step before the acid hydrolysis, followed by biodetoxification and flocculation (strategy C.2), led to an overall decrease of phenolic compounds, furfural and 5-HMF concentration in the concentrated hydrolysate from respectively 7.1 g/L, 1.42 g/L and 0.22 g/L to 2.61 g/L, 0.29 g/L and 0.06 g/L, corresponding to 63%, 79% and 74% removal efficiency. More specifically, the sequential co-culture step allowed a reduction of phenolics, furfural and 5-HMF contents in the hydrolysate by 18%, 71% and 43% respectively achieved after 49 h, while additional 55%, 27% and 54% of the resulting concentrations were respectively removed by the flocculation step (Figure 5-4B).

The degradation results obtained with the different strategies were summarized in Table 5-2. Overall, the phenolic compounds concentrations always remained higher than the threshold inhibition concentration determined by Mechmech *et al.* [9] Despite this partial degradation, which might be related to the low resistance of the tested co-culture to the inhibitors, residual compounds from combined flocculation and co-culture could have lower inhibitory impacts on ABE fermentation compared to the effects of the diluted flocculated hydrolysate. To confirm the hypothesis that low inhibitory compounds were remained in the detoxified hydrolysate and to validate the efficiency of the proposed detoxification method, *C. acetobutylicum* ATCC 824 was used to perform ABE fermentation on the hydrolysate obtained from flocculation followed by a sequential co-culture detoxification procedure. The selected hydrolysate contained lower residual inhibitors concentrations than those from the other proposed strategies.

Table 5-2 : Comparison of the performance of the different developed strategies in this study

| | Strategy | | Degradation (%) | | |
|---|---|---|---|---|---|
| | | | **Phenolic compounds** | **Furfural** | **5-HMF** |
| **Strategy A: Effect of combining a pre-hydrolysate concentration step to a hydrolysate biodetoxification step by the sequential co-culture of two bacteria** | **A.1:** Biodetoxification of non-concentrated hydrolysate | | 38.02% | 45.36% | - |
| | **A.2:** Biodetoxification of concentrated hydrolysate | | 17.72% | 71.49% | 43.16% |
| **Strategy B: effect of combining a pre-hydrolysate concentration step to a hydrolysate flocculation step followed by a biodetoxification step using sequential co-culture of two bacteria** | **B.1:** Flocculation of non-concentrated hydrolysate followed by the co-culture | 1. Flocculation | 48.88% | 53.97% | 26.16% |
| | | 2. Co-culture | 27.88% | 41.77% | 2.94% |
| | | Overall | 63.13% | 73.2% | 28.33% |
| | **B.2:** Flocculation of concentrated hydrolysate followed by the co-culture | 1. Flocculation | 50.65% | 22.2% | 13.91% |
| | | 2. Co-culture | 0.3% | 76.45% | 41.87% |
| | | Overall | 50.8% | 81.68% | 49.95% |
| **Strategy C: Effect of combining a pre-hydrolysate concentration step to a hydrolysate biodetoxification step using a sequential co-culture of two bacteria followed by a flocculation step** | **C.1:** Co-culture of non-concentrated hydrolysate followed by flocculation | 1. Co-culture | 38.02% | 45.36% | - |
| | | 2. Flocculation | 41.98% | 38.14% | 30.84% |
| | | Overall | 64.04% | 66.2% | - |
| | **C.2:** Co-culture of concentrated hydrolysate followed by flocculation | 1. Co-culture | 17.72% | 71.49% | 43.16% |
| | | 2. Flocculation | 55.43% | 27.31% | 54.21% |
| | | Overall | 63.33% | 79.28% | 73.97% |

### 5.4.2 Validation of the detoxification strategies by ABE fermentation

To validate the efficiency of the selected detoxification strategy, the fermentability of the generated hydrolysate was tested by ABE fermentation with *C. acetobutylicum* ATCC 824. The results are illustrated in Figure 5-5. The mass balances in term of carbon were evaluated for the different ABE fermentation experiments and are shown in Table 5-3.

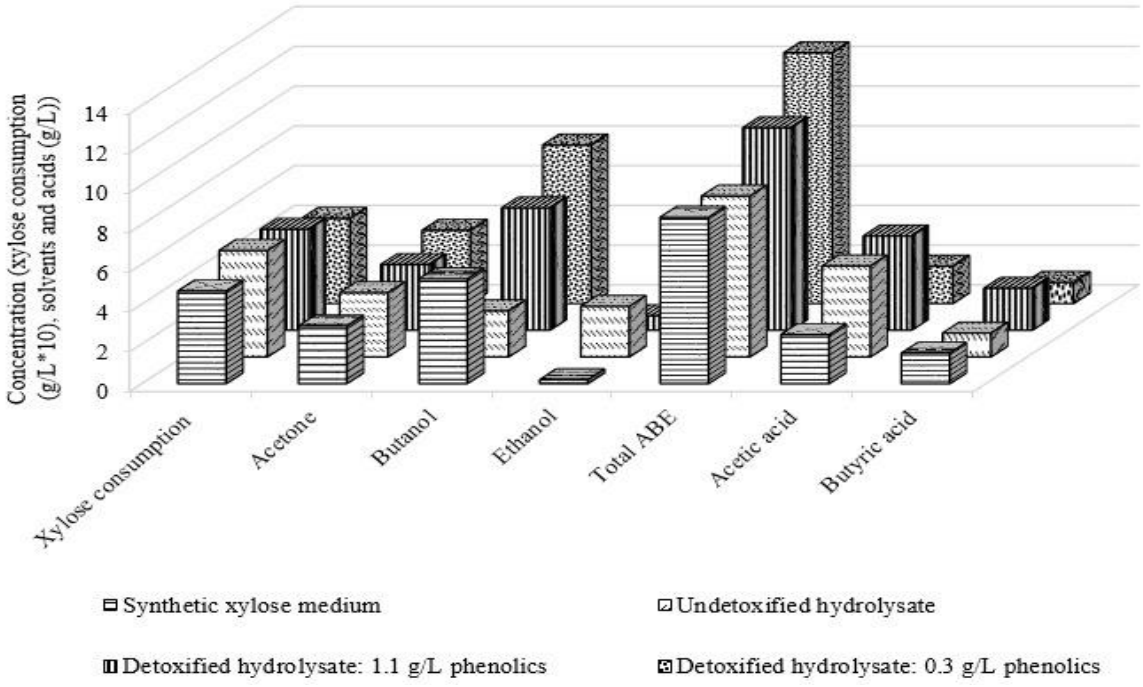



Figure 5-5 : Xylose consumption, butanol and acids profiles of *C. acetobutylicum* ATCC 824 grown in xylose synthetic medium (containing 60 g/L xylose), un-treated and treated hydrolysates. The duration of ABE fermentation was 120 h.

Table 5-3 : Carbon mass balance[i] of the initial and final compositions of the different fermentation media in this study.

| Sample | | Initial xylose (g) | Ethanol (g) | Butanol (g) | Acetone (g) | Acetic acid (g) | Butyric acid (g) | Xylose (g) |
|---|---|---|---|---|---|---|---|---|
| **Synthetic xylose medium** | | 4.80 | 0.02 | 0.68 | 0.36 | 0.20 | 0.17 | 1.07 |
| **Undetoxified hydrolysate** | | 4.80 | 0.27 | 0.30 | 0.40 | 0.37 | 0.13 | 0.52 |
| **Detoxified hydrolysate** | **1.1 g/L phenolics** | 4.80 | 0.08 | 0.80 | 0.41 | 0.38 | 0.23 | 0.74 |
| | **0.3 g/L phenolics** | 4.80 | 0.10 | 1.04 | 0.46 | 0.15 | 0.12 | 1.37 |

[i] The carbon mass for each product was calculated following the Equation 2:

$$Carbon\ mass = \frac{Weight\ (g) \times Carbon\ mass\ in\ one\ mole\ (\frac{g}{moL})}{Molecular\ weight\ (\frac{g}{moL})} \quad (2).$$

The carbon mass in one mole of the product was determined by multiplying the molecular weight of the carbon atom by the number of carbon atoms in the product.

A butanol production of ~ 5 g/L was obtained in a synthetic medium containing 60 g/L of xylose (without inhibitors). A total solvents concentration of 8.37 g/L along with 2.47 g/L acetic acid and 1.54 g/L butyric acid was reached after 120 h, while only 46.6 g/L of xylose were consumed. Afterwards, the ABE fermentation was tested for undetoxified hydrolysate, diluted such as to obtain a concentration of 0.3 g/L of phenolic compounds, which corresponds to the limit of inhibition of the undetoxified hydrolysate [9]. This diluted hydrolysate was supplemented with xylose up to a concentration of 60 g/L prior to fermentation experiments. In this case, only 2.32 g/L butanol were produced with *C. acetobutylicum* ATCC 824, barely reaching half of the amount obtained in the synthetic medium. However, although a low butanol production was observed, the total solvents production reached 8.1 g/L (Figure 5-5), a level comparable to the one obtained in synthetic xylose medium. In fact, ethanol and acetone are respectively synthesized from acetyl-CoA and acetoacetyl-CoA, metabolic intermediates that can be further processed through pathways leading to butanol [15]. In this work, the undetoxified hydrolysate contained levels of inhibitors (e.g. phenolic compounds) which could be toxic [47], even after their dilution, and affect the metabolic pathways leading to butanol. In addition, the final acetic acid concentration in the undetoxified hydrolysate was nearly 2-folds higher than that produced in the synthetic medium (Figure 5-5). Indeed, un-detoxified hydrolysate, even diluted, contains toxic compounds which prevent transition from the acidogenic phase to the solventogenic phase due to its contribution to

cell death [48]. In such inhibitory environment, *C. acetobutylicum* ATCC 824 would turn its metabolism towards xylose consumption in order to produce the energy required for its survival and maintenance, which could explain the higher consumption of sugars observed in the hydrolysate: 53.6 g/L, compared to 46.6 g/L in the xylose medium (Figure 5-5 and Table 5-3). In the ABE fermentation, sugars are required both for acid production during the acidogenic phase and for re-assimilation of these acids for solvents production during the solventogenic phase [10].

Treating the hydrolysate with flocculation followed by biodetoxification with sequential co-culture decreased the phenolic compounds concentration to 1.4 g/L. However, it remained higher than the minimum inhibitory level determined by Mechmech *et al.* [9], which was 0.3 g/L in the untreated hydrolysate and 1.1g/L in the flocculated hydrolysate. Hence, the detoxified hydrolysate was diluted to reach 1.1 g/L and 0.3 g/L of phenolic compounds. The results of the ABE fermentation of the obtained hydrolysates are shown in Figure 5-5. The production of solvents (close to 13 g/L) in presence of 0.3 g/L phenolic compounds was more significant than the production observed in the presence of 1.1 g/L of phenolics (10.19 g/L). These observations were related to the prominent butanol production in the hydrolysate containing 0.3 g/L of phenolics, which was close to 8 g/L versus 6.15 g/L from the fermentation of the more concentrated hydrolysate (1.1 g/L of phenolic compounds) (Figure 5-5). In terms of acids concentration in the detoxified hydrolysates, residual acetic and butyric acids were lower in the hydrolysate containing 0.3 g/L phenolic compounds. In the hydrolysate containing 1.1 g/L of phenolics, the production was nearly doubled as illustrated in Figure 5-5. As explained above, the generation of acids by *C. acetobutylicum* is accompanied by the energy production required to survive in the less diluted hydrolysate (1.1 g/L phenolics) leading to a lower production of the solvents. In terms of sugars consumption, only 42.89 g/L of xylose were assimilated by *Clostridium* to produce 8 g/L of butanol from the detoxified hydrolysate containing 0.3 g/L of phenolic compounds. This amount was the lowest observed among all tested hydrolysates. A possible explanation of this observation is the non-inhibitory environment limiting the consumption of monosaccharides to produce energy for their maintenance. Another explanation of the lower xylose consumption could be the presence of inhibitors at concentrations stimulating solvents production. Indeed, Li *et al.* [49] confirmed an improvement of solvents concentration until 12%, 10% and 3% respectively with the presence of 0.5 mM o-vanillin, 5 mM and 7.5 mM benzaldehyde. This finding may explain the higher solvents production in the detoxified hydrolysates compared to the synthetic medium fermentation. Furthermore, the high loads of

degradation products in the treated hydrolysates do increase medium viscosity (not evaluated) which could have limited negative effects of agitation on *C. acetobutylicum* ATCC 824 [50] by a reduction of the shear stress [51].

The ABE production results obtained in this work, particularly for butanol, are more promising than those obtained in several other studies. Indeed, solvents concentrations of 0.6 g/L and 6 g/L were produced from the flocculated hydrolysates (containing respectively 1.1 g/L and 0.3 g/L of phenolic compounds) in the study of Mechmech *et al.* [9], compared to 10.18 g/L and 12.62 g/L of ABE produced in the present work from the same initial concentration of phenolic compounds (i.e. 1.1 g/L and 0.3 g/L). Butanol concentrations after 144 h of fermentation were close to 0.2 g/L and 4 g/L with hydrolysates at 1.1 g/L and 0.3 g/L phenolics respectively [9] compared to 6.15 g/L and 8 g/L obtained in the actual work. Hence, for the same initial concentrations of phenolic compounds, the strategy adopted in this study may lead to the degradation of other strongly inhibitory compounds. In the study of Allard-Massicotte *et al.* [6], only 4.17 g/L of butanol was produced from a hydrolysate detoxified by a combination of laccase treatment and flocculation, while 8 g/L was produced in this study. A concentration of 8.79 g/L of ABE and nearly 5 g/L of butanol were generated from a 4-fold diluted $SO_2$-ethanol-water spent liquor with 35 g/L of glucose using *C. acetobutylicum* DSM 792 [20]. Plaza *et al.* [52]. used Brewer's spent grain for ABE production and 7.4 g/L of total solvents and 6 g/L of butanol were generated from the fermentation of the diluted hydrolysate.

In this study, combining flocculation with biodetoxification by a sequential co-culture was confirmed as a novel, promising detoxification technology thanks to the low-cost of chemicals [47] (The cost of flocculant $Fe_2(SO_4)_3$ used in this study is 82 $ per 500 g, compared to 163.97 $ and 175 $ per 500 g of chitin and alum ($KAl(SO_4)_2 \cdot 12H_2O$) respectively). In addition, the use of bacteria avoids enzymes purification steps, thus reducing the cost of butanol production, while increasing the efficiency of inhibitors removal and the production of butanol from a low-diluted detoxified hydrolysate. Indeed, a low dilution factor was required in the present study to reduce the phenolic compounds concentration from 1.4 g/L in the detoxified hydrolysate to 1.1 g/L and to obtain a high butanol production of 6.15 g/L. Several dilutions to reduce the amount of phenolic compounds could thus be avoided.

## 5.5 Conclusion

Detoxification of a hemicellulosic hydrolysate with flocculation using ferric sulfate, followed by the co-culture of *C. taiwanensis* and *U. thermosphaericus*, with sequential inoculation with a 24 h delay in the inoculation of *C. taiwanensis*, was applied for the first time. The use of this novel technology decreased the concentration of the toxic phenolic compounds, furfural and 5-HMF by 63%, 73% and 28% respectively. The higher inhibitors removal was mainly obtained after the flocculation step. ABE fermentation of the hydrolysate diluted to 1.1 g/L and 0.3 g/L of phenolic compounds resulted in butanol production of 6.15 g/L and 8 g/L respectively. At 0.3 g/L of phenolics, higher solvents (i.e. acetone, butanol and ethanol) concentration and lower residual acids (i.e. acetic and butyric acids) were obtained. These results are of great importance for the production of high butanol concentration. Further improvement of the coculture performance, enhancement of the inhibitors resistance and scale-up of the selected process may significantly improve butanol production.

## 5.6 Acknowledgments

This work was supported by a grant from the College-University I2I Program of the Natural Sciences and Engineering Research Council of Canada (NSERC) (grant number 437803-12) to Prof. M. Marinova and Dr. H. Chadjaa, and NSERC Discovery Grant (# 380070-08) to Prof. M. Jolicoeur. The authors wish to express their gratitude to FPInnovations for supplying the pre-hydrolysate samples and to the Centre National en Électrochimie et Technologies Environnementales (Shawinigan, QC) for hosting the experimental work, in particular to Jean-François Lemay and Patrick Quessy for their help.

## 5.7 Appendix: Optimisation of the inoculation interval in the coculture of *U. thermosphaericus* and *C. taiwanensis*

The bacterial coculture composed of *U. thermosphaericus* and *C. taiwanensis* was evaluated to detoxify hemicelluloses hydrolysate. Before tackling with biodetoxification procedure, we have evaluated the effect of inoculating the strains sequentially in the pre-hydrolysate, with mono-cultures as controls. The negative control was composed of non-inoculated pre-hydrolysate (Figure 5-6 and Figure 5-7). In this section, we only studied the performance of phenolic compounds degradation since they are the strong inhibitors of ABE fermentation with *C. acetobutylicum* ATCC 824 [54].

As illustrated in Figure 5-6, *C. taiwanensis* exhibited the weaker degradation of phenolic compounds along the first 24 h of inoculation which was 8.48% after one-day. This percentage of degradation increased progressively with time until approximately 18.04% after 60 h of culture. This value was higher than that obtained with the mono-culture *U. thermosphaericus* and in the negative control. The higher degradation with *U. thermosphaericus* was obtained just after 12 h (17.53%). Then, this value was slightly decreased with time. A possible explanation of this observation was the generation of by-products inhibitory to *U. thermosphaericus*. In fact, laccase, a multicopper enzyme produced by our bacterium [55], lead to monoelectronic oxidation of phenolic compounds generating phenoxy radicals via redox reaction. Cross-coupling phenomenon may occur between reactive radicals contributing to lignin polymerization parallelly to degradation [56].

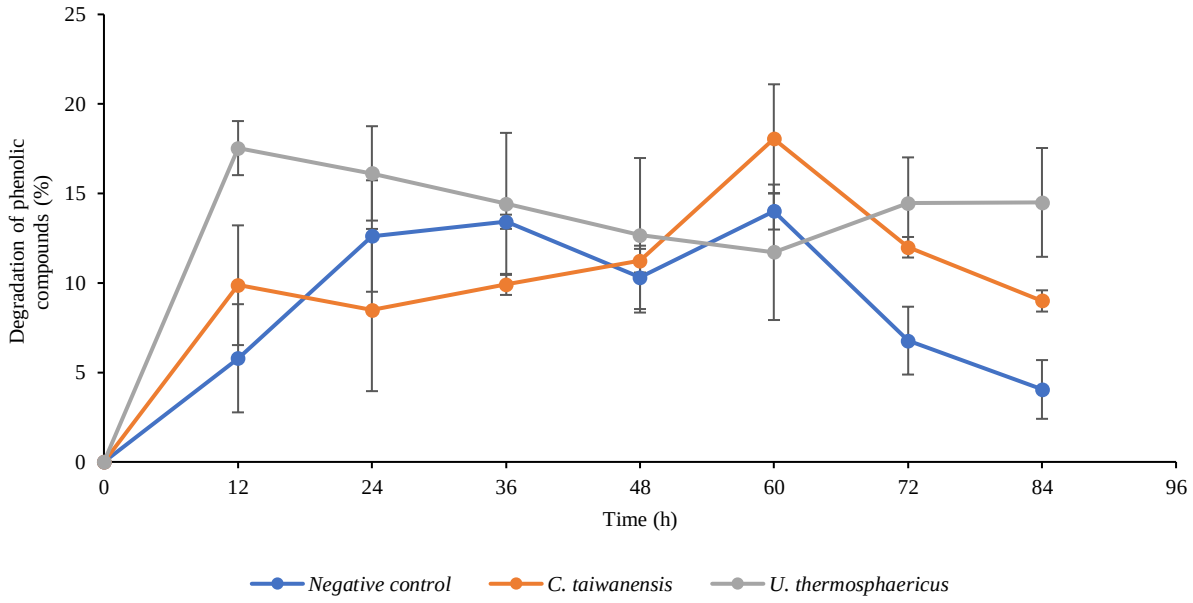



Figure 5-6: Degradation of phenolic compounds initially present in the hemicelluloses pre-hydrolysate with pure cultures of *U. thermosphaericus* and *C. taiwanensis*. The negative control curve represents the results from incubation of an uninoculated pre-hydrolysate at the same operating conditions as the bacterial cultures (41 °C, 200 rpm). Reaction volume: 100 mL. Inoculum doses: 2 mL for *U. thermosphaericus* and 1.33 mL for *C. taiwanensis*. Initial concentration of phenolic compounds in the pre-hydrolysate was 4.5 g/L (Experiments were performed in duplicate and results are mean of two values).

Figure 5-7 shows the results of total phenolic compounds degradation (%) with bacterial cultures inoculated at different intervals (0 h, 12 h and 24 h). The uninoculated pre-hydrolysate represents the negative control. As shown in Figure 5-7A, inoculating bacteria simultaneously gave a tendency of degradation similar to that obtained in the negative control. This observation reveals the weak performance of detoxification with the coculture compared to pure cultures. A possible explanation of this finding was a competition for nutrients and carbon source utilization between the strains. Another possible explanation was an antagonism between bacteria in the coculture leading to bacteria death. The weak performance of detoxification with mixed cultures might equally be attributed to a competition for space. In fact, Nissen *et al.* [57] tested the survival of *Kluyveromyces thermotolerans* (*K. thermotolerans*) and *Torulaspora delbrueckii* (*T. delbrueckii*) mixed separately with *Saccharomyces cerevisae* (*S. cerevisae*) in a grape juice medium. The authors observed early deaths of *K. thermotolerans* and *T. delbrueckii* compared to *S. cerevisae*

and they demonstrated that the early deaths would be attributed to the cell-to-cell mechanism at elevated concentrations of the strain *S. cerevisae* and to the weak performance of non-*Saccharomyces* yeasts for space than *S. cerevisae* [57]. The inoculation of *U. thermosphaericus* after 12 h of *C. taiwanensis* incubation gave a maximum phenolic compounds' degradation of 17.58% after 84 h of incubation (Figure 5-7B). The coculture sequence of *U. thermosphaericus* followed by *C. taiwanensis* with an interval of 12 h induced a maximum phenolic compounds degradation of 15.26% at 36 h which was close to that obtained with the reverse bacteria inoculation sequence. Our results showed a progressive decline of degradation performance of *U. thermosphaericus* followed by *C. taiwanensis* ($\Delta t$=12 h) from 15.26% at 36 h to 6.3% at 84 h (Figure 5-7B). These observations might be associated to several factors as nutrients limitation, antagonism, competition for space, etc. In fact, several studies demonstrated antagonism effects between microorganisms in coculture. Velázquez-Cedeño *et al.* [58] investigated the inhibition of *Pleurotus ostreatus* for mushroom production in presence of *Trichoderma harzianum*. In the study of Mackie *et al.* [59], *Trichoderma viride hampered* laccase production with *Phanaerochaete magnoliae.* Further investigation is recommended to understand interactions between *U. thermosphaericus* and *C. taiwanensis* under different operating conditions. Increasing the inoculation interval to 24 h had no significant effect on the phenolics degradation performance with *C. taiwanensis* followed by *U. thermosphaericus*. However, by inoculating *C. taiwanensis* after 24 h of *U. thermosphaericus* incubation, 25% of phenolic compounds in the non-diluted pre-hydrolysate were eliminated after 84 h of culture (Figure 5-7C). This value was the highest reported between the different cultures. The long duration of the coculture to obtain the highest degradation (84 h) may be reduced if we combine consortium treatment with another detoxification method. In this study, the sequential cultures of *U. thermosphaericus* followed by *C. taiwanensis* with an interval inoculation of 24 h was selected for further experiments of detoxification and integration of flocculation was tested for the performance of inhibitors removal in concentrated and non-concentrated hydrolysates.

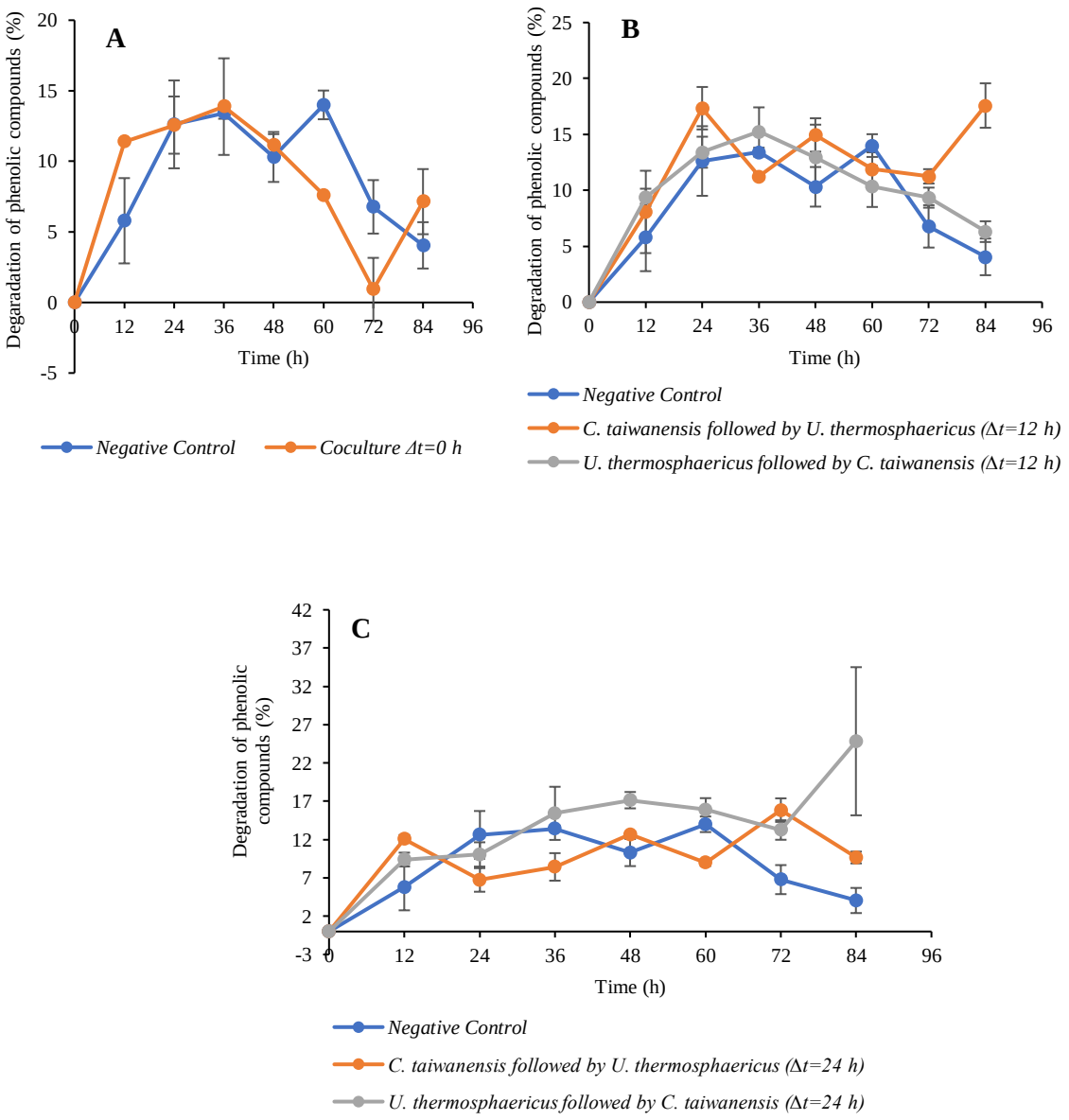



Figure 5-7: Degradation of phenolic compounds in the hemicelluloses pre-hydrolysate with cocultures. The negative control curves represent the results from incubation of an uninoculated pre-hydrolysate at the same operating conditions as the bacterial cultures (41 °C, 200 rpm). *U. thermosphaericus* and *C. taiwanensis* were inoculated at different times: (A) Δt=0 h, (B) Δt=12 h, and (C) Δt=24 h. Reaction volume: 100 mL. Inoculum doses: 1.2% (v/v) for *U. thermosphaericus* and 0.8% (v/v) for *C. taiwanensis*. Initial concentration of phenolic compounds in the pre-hydrolysate was 4.5 g/L (Experiments were performed in duplicate and results are mean of two values.

## 5.8 References

[1] M. Akbari, A. O. Oyedun, S. Jain, and A. Kumar, "Options for the conversion of pulp and paper mill by-products in Western Canada," Sustainable Energy Technologies and Assessments, 2017.

[2] M. Towers, T. Browne, R. Kerekes, J. Paris, and H. Tran, "Biorefinery opportunities for the Canadian pulp and paper industry," Pulp & Paper Canada, vol. 108, no. 6, pp. 26-29, 2007.

[3] N. C. Poudyal, O. Joshi, A. M. Taylor, and D. G. Hodges, "Prospects of wood-based energy alternatives in revitalizing the economy impacted by decline in pulp and paper industry," Forest Products Journal, 2017.

[4] A. Mazar, N. Jemaa, W. W. Al Dajani, M. Marinova, and M. Perrier, "Furfural production from a pre-hydrolysate generated using aspen and maple chips," Biomass and Bioenergy, vol. 104, pp. 8-16, 2017.

[5] K. Özdenkçi et al., "A novel biorefinery integration concept for lignocellulosic biomass," Energy Conversion and Management, vol. 149, pp. 974-987, 2017.

[6] R. Allard-Massicotte, H. Chadjaa, and M. Marinova, "Phenols Removal from Hemicelluloses Pre-Hydrolysate by Laccase to Improve Butanol Production," Fermentation, vol. 3, no. 3, p. 31, 2017.

[7] W. Guan, G. Xu, J. Duan, and S. Shi, "Acetone–Butanol–Ethanol Production from Fermentation of Hot-Water-Extracted Hemicellulose Hydrolysate of Pulping Woods," Industrial & Engineering Chemistry Research, vol. 57, no. 2, pp. 775-783, 2018.

[8] T. Guo et al., "Butanol production from hemicellulosic hydrolysate of corn fiber by a Clostridium beijerinckii mutant with high inhibitor-tolerance," Bioresource technology, vol. 135, pp. 379-385, 2013.

[9] F. Mechmech, H. Chadjaa, M. Rahni, M. Marinova, N. B. Akacha, and M. Gargouri, "Improvement of butanol production from a hardwood hemicelluloses hydrolysate by combined sugar concentration and phenols removal," Bioresource technology, vol. 192, pp. 287-295, 2015.

[10] Z. Sun and S. Liu, "Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by *Clostridia acetobutylicum* ATCC824," Biomass and bioenergy, vol. 39, pp. 39-47, 2012.

[11] X. Zhao, M. Kasbi, J. Chen, S. Peres, and M. Jolicoeur, "A dynamic metabolic flux analysis of ABE (acetone-butanol-ethanol) fermentation by *Clostridium acetobutylicum* ATCC 824, with riboflavin as a by-product," Biotechnology and bioengineering, vol. 114, no. 12, pp. 2907-2919, 2017.

[12] E. M. Green, "Fermentative production of butanol—the industrial perspective," Current opinion in biotechnology, vol. 22, no. 3, pp. 337-343, 2011.

[13] Y. Ni and Z. Sun, "Recent progress on industrial fermentative production of acetone–butanol–ethanol by *Clostridium acetobutylicum* in China," Applied Microbiology and Biotechnology, journal article vol. 83, no. 3, p. 415, May 09 2009.

[14] Y. Dahman, "Sustainable Biobutanol and Working towards the Green Gasoline of the Future," Ferment Technol, vol. 1, p. e111, 2012.

[15] S. Nanda, D. Golemi-Kotra, J. C. McDermott, A. K. Dalai, I. Gökalp, and J. A. Kozinski, "Fermentative production of butanol: perspectives on synthetic biology," New biotechnology, vol. 37, pp. 210-221, 2017.

[16] N. Qureshi and T. C. Ezeji, "Butanol,‘a superior biofuel’production from agricultural residues (renewable biomass): recent progress in technology," Biofuels, Bioproducts and Biorefining, vol. 2, no. 4, pp. 319-330, 2008.

[17] K. R. Szulczyk, "Which is a better transportation fuel--butanol or ethanol?," International Journal of Energy & Environment, no. 3, 2010.

[18] Y.-S. Jang, A. Malaviya, C. Cho, J. Lee, and S. Y. Lee, "Butanol production from renewable biomass by *clostridia*," Bioresource technology, vol. 123, pp. 653-663, 2012.

[19] B. C. Saha, "Hemicellulose bioconversion," Journal of Industrial Microbiology and Biotechnology, vol. 30, no. 5, pp. 279-291, 2003.

[20] S. A. Survase, E. Sklavounos, G. Jurgens, A. Van Heiningen, and T. Granström, "Continuous acetone–butanol–ethanol fermentation using SO2–ethanol–water spent liquor from spruce," Bioresource technology, vol. 102, no. 23, pp. 10996-11002, 2011.

[21] B. L. Magalhães, M. C. B. Grassi, G. A. Pereira, and M. Brocchi, "Improved n-butanol production from lignocellulosic hydrolysate by Clostridium strain screening and culture-medium optimization," Biomass and Bioenergy, vol. 108, pp. 157-166, 2018.

[22] V. Ujor, C. V. Agu, V. Gopalan, and T. C. Ezeji, "Glycerol supplementation of the growth medium enhances in situ detoxification of furfural by *Clostridium beijerinckii* during butanol fermentation," Applied microbiology and biotechnology, vol. 98, no. 14, pp. 6511-6521, 2014.

[23] F. Xin et al., "High biobutanol production integrated with in situ extraction in the presence of Tween 80 by *Clostridium acetobutylicum*," Process Biochemistry, vol. 67, pp. 113-117, 2018.

[24] D. H. Cho, Y. J. Lee, Y. Um, B.-I. Sang, and Y. H. Kim, "Detoxification of model phenolic compounds in lignocellulosic hydrolysates with peroxidase for butanol production from *Clostridium beijerinckii*," Applied microbiology and biotechnology, vol. 83, no. 6, pp. 1035-1043, 2009.

[25] O. Ajao, M. Le Hir, M. Rahni, H. Chadjaa, and M. Marinova, "Comparative biocatalytic degradation of Kraft prehydrolysate phenolic fermentation inhibitors using bacteria-derived laccase," Wood science and technology, vol. 51, no. 3, pp. 585-599, 2017.

[26] A. D. Moreno, D. Ibarra, P. Alvira, E. Tomás-Pejó, and M. Ballesteros, "A review of biological delignification and detoxification methods for lignocellulosic bioethanol production," Critical reviews in biotechnology, vol. 35, no. 3, pp. 342-354, 2015.

[27] S. Larsson, A. Reimann, N.-O. Nilvebrant, and L. J. Jönsson, "Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce," Applied biochemistry and biotechnology, vol. 77, no. 1-3, pp. 91-103, 1999.

[28] Y. He, J. Zhang, and J. Bao, "Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation," Biotechnology for biofuels, vol. 9, no. 1, p. 19, 2016.

[29] A. P. Wulandari, T. Triyana, and P. Andayaningsih, "Delignification of rice straw with ligninase from novel *Penicillium* sp. strain *apw-tt2* for biopulping," International Journal of Bioscience, Biochemistry and Bioinformatics, vol. 3, no. 1, p. 43, 2013.

[30] D. Kim, E. A. Ximenes, N. N. Nichols, G. Cao, S. E. Frazer, and M. R. Ladisch, "Maleic acid treatment of biologically detoxified corn stover liquor," Bioresource technology, vol. 216, pp. 437-445, 2016.

[31] B.-Y. Chen, W.-M. Chen, and J.-S. Chang, "Optimal biostimulation strategy for phenol degradation with indigenous rhizobium *Ralstonia taiwanensis*," Journal of hazardous materials, vol. 139, no. 2, pp. 232-237, 2007.

[32] N. Okuda, M. Soneura, K. Ninomiya, Y. Katakura, and S. Shioya, "Biological detoxification of waste house wood hydrolysate using *Ureibacillus thermosphaericus* for bioethanol production," Journal of bioscience and bioengineering, vol. 106, no. 2, pp. 128-133, 2008.

[33] C. Hernández, A. M. F. Milagres, G. Vázquez-Marrufo, K. M. Muñoz-Páez, J. A. García-Pérez, and E. Alarcón, "An ascomycota coculture in batch bioreactor is better than polycultures for cellulase production," Folia Microbiologica, journal article February 08 2018.

[34] K. Tao, X. Liu, X. Chen, X. Hu, L. Cao, and X. Yuan, "Biodegradation of crude oil by a defined co-culture of indigenous bacterial consortium and exogenous *Bacillus subtilis*," Bioresource Technology, vol. 224, pp. 327-332, 2017/01/01/ 2017.

[35] G. N. Ijoma and M. Tekere, "Potential microbial applications of co-cultures involving ligninolytic fungi in the bioremediation of recalcitrant xenobiotic compounds," International Journal of Environmental Science and Technology, journal article vol. 14, no. 8, pp. 1787-1806, August 01 2017.

[36] E. Franchi et al., "Phytoremediation of a multi contaminated soil: mercury and arsenic phytoextraction assisted by mobilizing agent and plant growth promoting bacteria," vol. 17, no. 5, pp. 1224-1236, 2017.

[37] T. Gao, L. Foulston, Y. Chai, Q. Wang, and R. J. M. Losick, "Alternative modes of biofilm formation by plant-associated *Bacillus cereus*," vol. 4, no. 3, pp. 452-464, 2015.

[38] M. B. Hamed et al., "Large-scale production of a thermostable Rhodothermus marinus cellulase by heterologous secretion from *Streptomyces lividans*," vol. 16, no. 1, p. 232, 2017.

[39] O. Ajao, M. Rahni, M. Marinova, H. Chadjaa, and O. Savadogo, "Retention and flux characteristics of nanofiltration membranes during hemicellulose prehydrolysate concentration," Chemical Engineering Journal, vol. 260, pp. 605-615, 2015.

[40] O. Ajao, M. Le Hir, M. Rahni, M. Marinova, H. Chadjaa, and O. Savadogo, "Concentration and detoxification of Kraft prehydrolysate by combining nanofiltration with flocculation," Industrial & Engineering Chemistry Research, vol. 54, no. 3, pp. 1113-1122, 2015.

[41] T. Ezeji, N. Qureshi, and H. P. Blaschek, "Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation," Biotechnology and bioengineering, vol. 97, no. 6, pp. 1460-1469, 2007.

[42] V. L. Singleton and J. A. Rossi, "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents," American journal of Enology and Viticulture, vol. 16, no. 3, pp. 144-158, 1965.

[43] A. Mittal, G. M. Scott, T. E. Amidon, D. J. Kiemle, and A. J. Stipanovic, "Quantitative analysis of sugars in wood hydrolyzates with 1H NMR during the autohydrolysis of hardwoods," Bioresource technology, vol. 100, no. 24, pp. 6398-6406, 2009.

[44] J. Bremner, "Total Nitrogen 1," Methods of soil analysis. Part 2. Chemical and microbiological properties, no. methodsofsoilanb, pp. 1149-1178, 1965.

[45] P. Sivagurunathan et al., "Fermentative hydrogen production using lignocellulose biomass: an overview of pre-treatment methods, inhibitor effects and detoxification experiences," Renewable and Sustainable Energy Reviews, vol. 77, pp. 28-42, 2017.

[46] W.-M. Chen et al., "*Ralstonia taiwanensis* sp. nov., isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient," International Journal of Systematic and Evolutionary Microbiology, vol. 51, no. 5, pp. 1729-1735, 2001.

[47] N. R. Baral and A. Shah, "Microbial inhibitors: formation and effects on acetone-butanol-ethanol fermentation of lignocellulosic biomass," Applied microbiology and biotechnology, vol. 98, no. 22, pp. 9151-9172, 2014.

[48] S. J. Choi, J. Lee, Y.-S. Jang, J. H. Park, S. Y. Lee, and I. H. Kim, "Effects of nutritional enrichment on the production of acetone-butanol-ethanol (ABE) by *Clostridium acetobutylicum*," Journal of Microbiology, vol. 50, no. 6, pp. 1063-1066, 2012.

[49] J. Li, S. Shi, S. Adhikari, and M. Tu, "Inhibition effect of aromatic aldehydes on butanol fermentation by *Clostridium acetobutylicum*," RSC Advances, vol. 7, no. 3, pp. 1241-1250, 2017.

[50] Y. Wang and H. P. J. B. t. Blaschek, "Optimization of butanol production from tropical maize stalk juice by fermentation with *Clostridium beijerinckii* NCIMB 8052," vol. 102, no. 21, pp. 9985-9990, 2011.

[51] I. Maddox, N. Qureshi, and K. J. P. B. Roberts-Thomson, "Production of acetone-butanol-ethanol from concentrated substrate using clostridium acetobutylicum in an integrated fermentation-product removal process," vol. 30, no. 3, pp. 209-215, 1995.

[52] P. E. Plaza, L. J. Gallego-Morales, M. Peñuela-Vásquez, S. Lucas, M. T. García-Cubero, and M. Coca, "Biobutanol production from brewer's spent grain hydrolysates by *Clostridium beijerinckii*," Bioresource technology, vol. 244, pp. 166-174, 2017.

[53] A. K. Verma, R. R. Dash, and P. Bhunia, "A review on chemical coagulation/flocculation technologies for removal of colour from textile wastewaters," Journal of environmental management, vol. 93, no. 1, pp. 154-168, 2012.

[54] R. Allard-Massicotte, H. Chadjaa, and M. J. F. Marinova, "Phenols Removal from Hemicelluloses Pre-Hydrolysate by Laccase to Improve Butanol Production," vol. 3, no. 3, p. 31, 2017.

[55] K. Glendinning, L. Macaskie, and N. J. B. l. Brown, "Mercury tolerance of thermophilic *Bacillus* sp. and *Ureibacillus* sp," vol. 27, no. 21, pp. 1657-1662, 2005.

[56] A. Dong et al., "Changes on content, structure and surface distribution of lignin in jute fibers after laccase treatment," vol. 15, no. 3, pp. 384-395, 2018.

[57] P. Nissen and N. J. A. o. M. Arneborg, "Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*," vol. 180, no. 4, pp. 257-263, 2003.

[58] M. Velázquez-Cedeño, A. M. Farnet, G. Mata, and J.-M. J. B. t. Savoie, "Role of Bacillus spp. in antagonism between *Pleurotus ostreatus* and *Trichoderma harzianum* in heat-treated wheat-straw substrates," vol. 99, no. 15, pp. 6966-6973, 2008.

# CHAPITRE 6 : ARTICLE 3: HYDROLYSIS AND DETOXIFICATION OF HEMICELLULOSIC HARDWOOD PRE-HYDROLYSATE BY SEQUENTIAL AND SIMULTANEOUS BACTERIAL CULTURES FOR ENHANCEMENT OF BUTANOL PRODUCTION

**Biochemical Engineering Journal**

**Authors: Mariem Theiri [1,2], Mariya Marinova [3], Hassan Chadjaa [2] and Mario Jolicoeur [1,*]**

[1] Research Laboratory in Applied Metabolic Engineering, Department of Chemical Engineering, École Polytechnique de Montréal, J.-A. -Bombardier Pavilion, 2900 Édouard-Montpetit Blvd., Montréal, QC, H3T 1J4 Canada.

[2] Centre National en Électrochimie et en Technologies Environnementales, 5230, Boulevard Royal, Shawinigan, QC G9N 4R6, Canada.

[3] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, 13 General Crerar Crescent, Kingston, ON K7K 7B4, Canada.

***** Corresponding author: mario.jolicoeur@polymtl.ca; Tel.: 514-340-4711 ext. 4525.

## 6.1 Abstract

For an eco-efficient and performant conversion of hemicelluloses to biofuels, the use of microorganisms in the hydrolysis and detoxification steps prior to fermentation is a promising option. In this project, the treatment of hemicellulosic pre-hydrolysate by flocculation, followed by simultaneous or separate detoxification with *Ureibacillus thermosphaericus* and *Cupriavidus taiwanensis* co-culture and hydrolysis with *Paenibacillus campinasensis*, was investigated for the first time. The use of three different strategies did not increase xylose concentration, and in parallel, a decrease of phenolics concentration was obtained. A reduction percentage of phenolic compounds of 56% was reached with separate and simultaneous hydrolysis and detoxification.

This decrease was achieved mainly after flocculation. The fermentation of diluted hydrolysates produced 6.8 g $L^{-1}$ of butanol after 116 h, obtained with hydrolysate from simultaneous hydrolysis and detoxification used as a sole carbon source. In brief, the research deals with the application of a novel consortium composed of detoxifying strains *Ureibacillus thermosphaericus* and *Cupriavidus taiwanensis* and a hydrolysing strain *Paenibacillus campinasensis* for treatment of a hardwood hemicelluloses pre-hydrolysate. Higher butanol production than xylose synthetic medium as a sole carbon source, was obtained via ABE fermentation of hydrolysate from the novel microbial approach which emphasizes the cost-effectiveness of butanol production process. The higher biofuel concentration in the hydrolysate was attributed to the existence of other carbon sources than xylose.

**Keywords:** hemicelluloses; microorganisms; co-culture; flocculation; hydrolysis; butanol.

## 6.2 Introduction

Fossil fuels are major factors affecting ecosystems [1]. In fact, their extensive use led to global warming [2] and contributed to an increase in greenhouse gas (GHG) emissions [3]. Furthermore, the increase in Canadian energy consumption, which raised from 2,710 PJ (Petajoule) in 1990 to 3,321.5 PJ in 2011 and 3,540.5 PJ in 2015 [4], can cause the depletion of fossil fuels sources [5]. A possible way to mitigate these problems is to develop sustainable and eco-friendly alternative energy sources [3, 6]. Biofuels from lignocellulosic biomass are part of these alternatives [7-9].

Lignocellulosic biomass, characterized by its abundance, renewability and low cost [10], can be used for biofuels production, among others butanol [11], characterized by its high-energy content, low vapor pressure and low volatility. Furthermore, this biofuel can be transported in existing pipelines and can replace gasoline. Butanol can be produced biologically by fermentation of monomeric sugars using microorganisms such as species of *Clostridium* [12]. Members of this last genus are used at large scale for versatile feedstocks and show high tolerance to toxic compounds in most used substrates [13]. The ability to metabolize both hexoses and pentoses, the main sugars in hardwood hemicelluloses, is another interesting criterion of *Clostridium* species [14].

To convert efficiently hemicellulosic sugars into biofuels with solventogenic microorganisms, pre-treatment and hydrolysis steps are required in order to release fermentable sugars [15]. Hydrolysis can be accomplished with chemical reagents such as sulfuric acid. Although acid hydrolysis is

known by its high yields of monomeric sugars, this process is accompanied by the formation of inhibitors such as organic acids, phenolic compounds and furan derivatives due to the harsh conditions applied [16]. To alleviate these shortcomings, hemicelluloses can be hydrolysed biologically using specific enzymes. Even though enzymatic hydrolysis is known by its specificity and respect to environment criterion, enzymes production, purification, optimisation and use of high dose, mandatory for an efficient hydrolysis of complex structure biomass, are additional charges to the butanol production process [17].

Direct application of microorganisms producing hydrolysing enzymes is a highly eco-efficient process compared to enzymes utilisation as it does not imply purification steps and uses directly nutrients and polysaccharides naturally present in the lignocellulosic biomass [18]. *Paenibacillus campinasensis* BL11 is a potential microorganism for xylanase production. The activity of the purified enzyme was 2392 IU $mg^{-1}$, higher than those obtained with *Bacillus* spp. and *Alicyclobacillus* sp. A4 [19]. The strain *Paenibacillus campinasensis* DSM 21989 (*P. campinasensis*) was used for the first time in the present work to hydrolyse hemicelluloses pre-hydrolysate for its thermophilic and alkaliphilic properties. The literature on this strain is scarce and, to the best of our knowledge, the only study that has been devoted solely to the characterisation of *P. campinasensis* DSM 21989, also designed *P. campinasensis* 324T, was the study of Yoon *et al.* [20] who demonstrated the potential of cyclodextrin production with this bacterium. Our rationale was based on the ability of *P. campinasensis* DSM 2198 to grow on a pH range of 7.5-10.5, close to those of *C. taiwanensis* (6-8) [21] and *U. thermosphaericus* (7-9) [22] applied in a consortium in this study. Furthermore, the optimal temperature of *P. campinasensis* growth is 41 °C [20] which is similar to that of the co-culture of *U. thermosphaericus* and *C. taiwanensis* (see section 4.4.1.1 in this thesis). *P. campinasenis* can hydrolyse polysaccharides such as casein and starch and is able to grow in the presence of 7% (w/v) NaCl, contrarily to many other species of *P. campinasensis* which do not tolerate concentrations of NaCl of 5% (w/v) [20]. The high tolerance to NaCl and the alkaliphilic criterion of *P. campinasensis* enhance its potential for industrial application. In addition, it is important to note that the hemicelluloses pre-hydrolysates are rich in inhibitors such as phenolic compounds, furan aldehydes, weak acids, salts, etc. that are originally present in the biomass or generated from pre-hydrolysis and neutralisation steps. These compounds are strong inhibitors of bacterial growth, enzymes production and activities [23-25]. De Cassia Pereira *et al.* [24] detailed metal ions inhibitors of hemicellulases and investigated possible

mechanisms of inhibition. In fact, $Hg^{2+}$, $Pb^{2+}$ and $Cu^{2+}$ were studied as strong inhibitors of *Bacillus halodurans* TSEV1 xylanase via catalysis of autoxidation of cysteine thiol group. $Hg^{2+}$ metal ion has an impact on the availability of AFase by interacting with tryptophan and histidine residues [24]. The integration of a detoxification step is thus required for efficient hydrolysis of hemicelluloses and hence for maximising biofuels production [25]. An inexpensive and environmental process of detoxification consisting on direct application of microorganisms was designed for removal of a broad range of toxic compounds contrary to laccase treatment targeting particularly phenolic compounds [26].

In this study, combined hydrolysis and detoxification steps, applied sequentially or simultaneously using specific microorganisms, were tested for the first time; the results in terms of phenolic compounds and sugars concentrations were compared. Flocculation with ferric sulfate as flocculant agent followed by development of mixed cultures of *Ureibacillus thermosphaericus* NCIMB 13819 (*U. thermosphaericus*) and *Cupriavidus taiwanensis* DSM 173434 (*C. taiwanensis*) were tested for detoxification. This procedure was selected based on the results obtained in a previous work from the authors (see Section 5.4.1 in this thesis). The hydrolysis step was conducted with *P. campinasensis* DSM 21989. To validate the proposed strategies, hydrolysates for butanol production using *Clostridium acetobutylicum* ATCC 824 (*C. acetobutylicum*) were used and results were compared to trials with synthetic xylose medium and non-detoxified hydrolysate.

## 6.3 Materials and Methods

### 6.3.1 Bacterial strains and culture conditions

In this study, the microorganisms applied for the detoxification step were *U. thermosphaericus* NCIMB 13819 and *C. taiwanensis* DSM 17343, while *P. campinasensis* DSM 21989 was used for the hydrolysis step and *C. acetobutylicum* ATCC 824 served for the Acetone-Butanol-Ethanol (ABE) fermentation.

*Ureibacillus thermosphaericus* NCIMB 13819 was purchased from NCIMB Ltd. (YA, Scotland). The stock culture was prepared by adding 30% (v/v) glycerol to the pellet of 3% (w/v) Tryptic Soy Broth (TSB) (Quelab Laboratories inc.; QB-39-5626) obtained after incubation with *U. thermosphaericus* at 55 °C and 180 rpm until two deceleration points of Optical Density (OD600). The composition of TSB is as follows (in g $L^{-1}$): dextrose 2.5, soy peptone 3, casein peptone 17,

sodium chloride 5 and dipotassium phosphate 2.5. This medium was equally used for inoculum preparation at 50 °C and orbitally shaked at 180 rpm during 10 h (New Brunswick Scientific Innova 44, USA). For the detoxification experiments, the inoculum 1.2% (v/v) was added to the flocculated pre-hydrolysate.

*Cupriavidus taiwanensis* DSM 17343, obtained from DSMZ (Braunschweig, Germany), was activated in R2A medium composed of (in g $L^{-1}$): glucose 0.5 (Thermo Fisher Scientific D1610), yeast extract 0.5 (BD, catalog number: 212750), soluble starch 0.5 (Fisher Chemical; S516-500), proteose peptone 0.5 (Nutri-Bact; QB-39-1910), casamino acids 0.5 (organotechnie), Na-pyruvate 0.3 (Fisher Bioreagents; catalog number 253 S-15629U), $MgSO_4.7H_2O$ 0.05 (Acro Organics; catalog number 423905000) and $K_2HPO_4$ 0.3 (Fisher Bioreagents; 19 L-15781U). The culture was incubated at 28 °C and 180 rpm until two deceleration points of OD600 after the end of exponential phase for stock glycerol preparation. Then, TSB was inoculated with the glycerol stock at a ratio of 0.1% (v/v) for inoculum preparation at 37 °C and shaked at 180 rpm during 10 h (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA). A volume of inoculum 0.8% (v/v) was inoculated in the flocculated pre-hydrolysate for detoxification.

*Paenibacillus campinasensis* DSM 21989 obtained from DSMZ (Braunschweig, Germany) was activated in 3% (w/v) sterilized TSB adjusted to pH 10 by adding Na-sesquicarbonate solution (composed of (in g $L^{-1}$): $NaHCO_3$ 4.2 and $Na_2CO_3$ anhydrous 5.3) and then incubated at 37 °C and 180 rpm until two deceleration points of OD600. For stock culture preparation, the pellets were supplemented with glycerol 30% (v/v) and were then kept at -80 °C. The inoculum was prepared by adding 0.1% (v/v) of the glycerol stock to TSB at the same operating conditions of reviving. After 24 h of incubation, 2% (v/v) of the culture was inoculated in the hydrolysis medium.

For stock glycerol preparation, the strain *Clostridium acetobutylicum* ATCC 824 (*C. acetobutylicum*) obtained from the American Type Culture Collection (ATCC) was cultivated under anaerobic conditions in sterilized Reinforced Culture Medium (RCM) (Nutri-Bact; QB-39-3724) containing (in g $L^{-1}$): tryptose 10, beef extract 10, glucose 5, yeast extract 3, soluble starch 0.5, sodium chloride 5 and L-cysteine-HCl 0.5 at 37 °C and 110 rpm until an OD600 of 1.9-2. Glycerol 30% (v/v) was then supplemented to the pellet of RCM culture and stored at -80 °C for further use. For inoculum preparation, RCM was inoculated with 0.2% (v/v) of the glycerol stock and incubated at 37 °C under shaking at 110 rpm until an OD600 between 0.6 and 0.9. To suppress

oxygen in the fermentative medium, a gas mixture composed of 80% $N_2$ and 20% $CO_2$ was purged in order to remove the dissolved oxygen before medium sterilisation.

### 6.3.2 Preparation of the hydrolysate

To perform the hydrolysis and detoxification steps of the hemicelluloses pre-hydrolysate, three strategies were developed and are presented in Figure 6-1. In the first strategy (Strategy A), the pre-hydrolysate has undergone a 49 h hydrolysis step with *P. campinasensis* as the sole treatment method in order to test the efficiency of the bacterium to convert hemicelluloses without a previous detoxification step. To study the performance of *P. campinasensis* to hydrolyse a detoxified hydrolysate, a detoxification step was integrated in the second strategy (Strategy B) prior to the hydrolysis. Coagulation-flocculation followed by sequential co-culture of *U. thermosphaericus* and *C. taiwanensis* were applied as a detoxification procedure. The last strategy (Strategy C), combined the microorganisms for hydrolysis and detoxification in the same reactor to treat the flocculated pre-hydrolysate. The biotreatment in Strategy C was initiated with the co-inoculation of *U. thermosphaericus* and *P. campinasensis*, and after 24 h of incubation *C. taiwanensis* was inoculated for another 25 h. The negative control used in these sets of experiments was composed of an uninoculated pre-hydrolysate supplemented with the nutrients that were served for the biotreatments. The control medium was incubated under the same operating conditions of hydrolysis (41 °C, 200 rpm and pH 10). It is worthy to note that the negative control experiments were performed for at least three replicates. The residual concentrations of phenolic compounds and xylose from the different stages were compared.

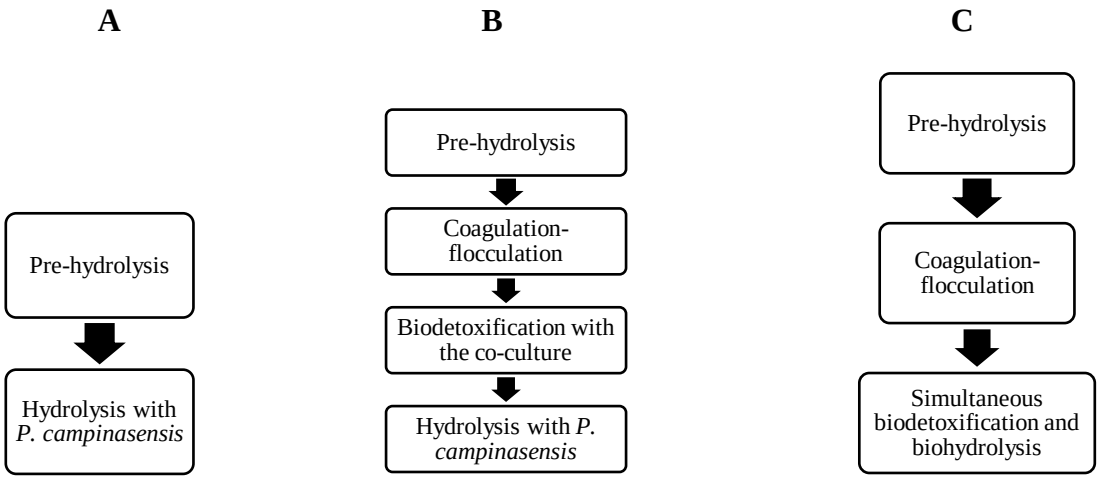



Figure 6-1 : Different strategies (A-C) developed for sequential and simultaneous detoxification and hydrolysis of hemicelluloses pre-hydrolysate.

#### 6.3.2.1 Pre-hydrolysis

The pre-hydrolysate used in this study (composition detailed in Table 6-1) was a blend of maple (40%) and aspen (60%) wood. The pre-hydrolysis step was conducted using steam explosion combined with hot water in a 56 L pilot digester at FPInnovations (Pointe-Claire, Canada). The pre-hydrolysate was delivered to the Centre National en Électrochimie et en Technologies Environnementales (CNETE) where it was kept at 4 °C prior to utilisation. The pre-treatment process was detailed in the work of [27].

Table 6-1 : Composition of the pre-hydrolysate in this study

| Component | Values (g $L^{-1}$) |
|---|---|
| **Total sugars**[2] | 20.3 [2] |
| **Monomeric xylose** | 3.2 |
| **Phenolic compounds (UV/Vis)** | 4.5 |
| **Furfural** | 0.5 |
| **5-HMF** | 0.1 |
| **Acetic acid** | 3.6 |

[2] The oligomeric sugars content was previously quantified by Ajao *et al.* [2] who used the same pre-hydrolysate obtained from FPInnovations and delievered to the Centre National en Électrochimie et en Technologies Environnementales (CNETE). To determine the concentration of oligomeric sugars, Ajao *et al.* [2] added 2.5% (w/v) of sulfuric acid solution and autoclaved the mixture for 120 min.

In order to reduce the concentration of inhibitors in the pre-hydrolysate, a two-steps detoxification procedure was applied: coagulation-flocculation using ferric sulfate solution followed by sequential cultures of *U. thermosphaericus* NCIMB 13819 and *C. taiwanensis* DSM 17343 lasting 49 h. The last strain was inoculated 24 h after incubation with the former bacterium. The detoxification procedure was selected based on results of a previous work (see Section 5.4.1 in this thesis).

#### 6.3.2.2 Coagulation-flocculation

The coagulation-flocculation experiments took place in a jar test equipment, where the pre-hydrolysate was complemented with 200 g $L^{-1}$ of ferric sulfate solution (97%, Sigma-Aldrich, Co., St. Louis, MO, USA) containing the flocculant iron (III) until a final ratio of 1 g iron/1 g phenolic compounds. The mixture was shaken at 150 rpm for 10 min while adjusting the pH to 7 (+/- 2%) before reducing the shaking speed to 50 rpm for 30 min. A sedimentation step lasting 2 h was used to settle the formed flocs. The pre-hydrolysate was then filtered through 1.5 µm membranes prior to the biodetoxification procedure.

#### 6.3.2.3 Biodetoxification procedure

Flocculated pre-hydrolysate was used as a sole carbon source for the co-culture of *U. thermosphaericus* NCIMB 13819 and *C. taiwanensis* DSM 17343 in order to further detoxify the inhibitory medium. The biodetoxification was conducted in 500 mL-baffled flasks (Fisher Scientific) containing 100 mL flocculated pre-hydrolysate and nutrients source (composed of: NaCl 0.5 g $L^{-1}$, $KH_2PO_4$ 3 g $L^{-1}$, $Na_2HPO_4$ 7 g $L^{-1}$, $NH_4Cl$ 1 g $L^{-1}$, $MgSO_4.7H_2O$ 0.58 g $L^{-1}$, $CuSO_4.5H_2O$ 0.043 mg $L^{-1}$, $CaCl_2.2H_2O$ 53.6 mg $L^{-1}$, $CoCl_2.5H_2O$ 0.2 g $L^{-1}$, $MnSO_4.H_2O$ 0.38 mg $L^{-1}$ and $FeSO_4.7H_2O$ 7 mg $L^{-1}$) [28]. The co-culture was applied sequentially with *C. taiwanensis* inoculated 24 h after *U. thermosphaericus* incubation, the overall duration of the biodetoxification was fixed to 49 h. The biodetoxification procedure was conducted in duplicate, at least, at 41 °C and 200 rpm in an orbital shaker (INFORS HT Multitron). The obtained detoxified medium was finally sterilized at 121 °C for 5 min to eliminate living cells.

#### 6.3.2.4 Hydrolysis with *P. campinasensis* DSM 21989

For solvent production with *C. acetobutylicum* ATCC 824, a hydrolysis step was required to produce monomeric sugars. The strain *P. campinasensis*, a potent strain for polysaccharide

hydrolysis [20], was inoculated and then incubated for 49 h at 41 °C and 200 rpm (INFORS HT Multitron). Samples of culture media were taken before and after growth of *P. campinasensis* to determine the concentration of xylose and phenolic compounds. The generated hydrolysates were autoclaved at 121 °C for 5 min to kill cells prior to ABE fermentation.

### 6.3.3 ABE fermentation

Solvent production was achieved via ABE fermentation; autoclaved carbon and nitrogen sources were mixed with nutrient source which were sterile introduced through 0.22 µm Millipore membranes. The mixture was then incubated for 116 h at 37 °C and 110 rpm shaking under anaerobic conditions (Barnstead/Lab-Line, MaxQ 4000 Orbital Shakers, USA). Treated or non-treated hydrolysates were supplemented with commercial xylose to a final concentration of 60 g $L^{-1}$ then assayed as carbon sources for ABE experiments [29]. The content of phenolic compounds in the studied hydrolysates was reduced by dilution to 0.3 g $L^{-1}$. Nitrogen source consisted of 5 g $L^{-1}$ yeast extract and the nutrients source contained (in g $L^{-1}$): $KH_2PO_4$ 0.5, $K_2HPO_4.3H_2O$ 0.5, ammonium acetate 2.2, $MgSO_4.7H_2O$ 0.2, $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, NaCl 0.01, para-aminobenzoic acid $10^{-3}$, thiamin $10^{-3}$ and biotin $10^{-5}$ [30]. The preparation of the different components was achieved separately under anaerobic conditions by boiling in 0.1% resazurin (Sigma; catalog number 1001824725) and purging with a blend of 20% $CO_2$ and 80% $N_2$. Carbon and nitrogen sources were mixed after sterilisation in a 250-mL screw capped Schott bottle with 200 mL reaction volume then incubated during 48 h in anaerobic jars equipped with Gas Pak envelopes (BD Gas Pak ™ EZ Anaerobe Container System, Franklin Lakes, NJ, USA) with indicators (BD BBL ™ Dry Anaerobic Indicator Strips, Franklin Lakes, NJ, USA) to suppress oxygen in the medium. Nutrient source was added to the mixture just prior to inoculation with 5% (v/v) of *C. acetobultylicum* inoculum. The solventogenic bacterium was allowed to grow for 116 h. Fermentation experiments were performed in duplicate.

### 6.3.4 Analytical methods

For monomeric xylose quantification, a High Performance Liquid Chromatography system (HPLC) (Agilent Technologies, Germany) equipped with an EC Nucleodur RP-$NH_2$ column (250 mm x 4.6 mm, 5 µm) and a refractive index detector was used. The elution solvent was a mixture of 75% acetonitrile and 25% deionized water which was fed into the system at 1.5 mL/min at 40

°C. The concentration of phenolic compounds was determined using Folin-cioacalteu reagent method [31] with an UV-visible spectrophotometer (Pharmacia Biotech Novaspec®II, Piscatoway, NJ, USA) at a wave length of 725 nm. The concentration of furan aldehydes was quantified with the same HPLC and a Nucleosil C18 (150 X 4.6 mm) column and a diode array detector (DAD) at 280 nm were used. A mixture of 84% of acetonitrile, 15% of deionized water and 1% of acetic acid served as an elution solvent for furans quantification. For organic acids analysis by HPLC, an Interstil ODS-3 column (150 X 4.6 mm) at 40 °C and a solvent of 99% $KH_2PO_4$ and 1% acetonitrile, fed at 1.25 mL/min, were applied. Solvents concentration was determined using a gas chromatography equipment (GC 7890A, Agilent Technologies) with an OV 624 capillary column and a flame ionization detector (FID, $H_2$ flow rate: 30 mL/min; air flow rate: 2.23 mL/min).

### 6.3.5 Statistical analyses

Statistical analyses were performed using Excel 365 software. Comparisons were done using a one-way analysis of variance (ANOVA) allowing the calculation of p-values to determine if results are significantly different.

### 6.3.6 Mass balances

The mass balances were calculated for carbon in detoxification and hydrolysis strategies and solvents production and the corresponding equations are presented in the Section 6.9. Appendix B.

## 6.4 Results and discussion

### 6.4.1 Treatment of hemicelluloses pre-hydrolysate

In this work, three strategies for the treatment of hemicelluloses pre-hydrolysate (Figure 6-1) were applied in order to study the efficiency of simultaneous and separate detoxification and hydrolysis steps. Details of the different strategies are given in the Section 6.3.2. Briefly, in strategy A, the pre-hydrolysate was solely hydrolysed with *P. campinasensis*. The performance of *P. campinasensis* was evaluated with a detoxified hydrolysate as a carbon source (Strategy B). Simultaneous detoxification and hydrolysis steps were finally investigated by the development of a consortium of the different strains (Strategy C).

#### 6.4.1.1 Strategy A: Hydrolysis with *P. campinasensis* as the sole treatment method

Prior to treatment of the pre-hydrolysate with *P. campinasensis*, the bacterium was tested for its performance to hydrolyse synthetic mediums composed of different concentrations of xylooligosaccharides. The results of conversion rates and xylose production are shown in Figure 6-6. The growth and pH curves in synthetic mediums are given in Figure 6-7. It is worthy to note that the growth curves of *P. campinasensis* in the pre-hydrolysates were not considered in this study as we faced problems of turbidity and presence of toxic compounds in the culture mediums that impeded accurate measurements of cells density by spectrophotometer and quantitative polymerase chain reaction (q-PCR).

The results of xylose and phenolic compounds contents variation in the pre-hydrolysate when using strategy A are presented in Figure 6-2. The experiments results are an average of a minimum of two replicates with standard deviation. Xylose concentration in the negative culture was reduced to 2.8 g $L^{-1}$ from 3.2 g $L^{-1}$ in the pre-hydrolysate (Table 6-1) after 49 h. A possible explanation of this observation is the presence of interactions between polyphenols and carbohydrates. In fact, You *et al.* [32] claimed the formation of lignin-carbohydrates (mostly xylan and glucomannan [33]) interactions in the lignocellulosic biomass by covalent interactions. Ether links may also permit the linkage between the phenolic compounds and ferulate that is ester linked to carbohydrates as detailed by You *et al.* [32]. A high affinity could also exist between the two molecules attributed, according to several authors, to the presence of cavities in carbohydrates, which trap polyphenols [34]. The formation of such a complex could be an explanation of the reduction in phenolics concentration in the control culture as shown in Figure 6-2. In fact, their concentration decreased from 4.5 g $L^{-1}$ to 3.9 g $L^{-1}$. The operating conditions (temperature, speed of agitation and luminosity) could be responsible of this observation [31, 35, 36].

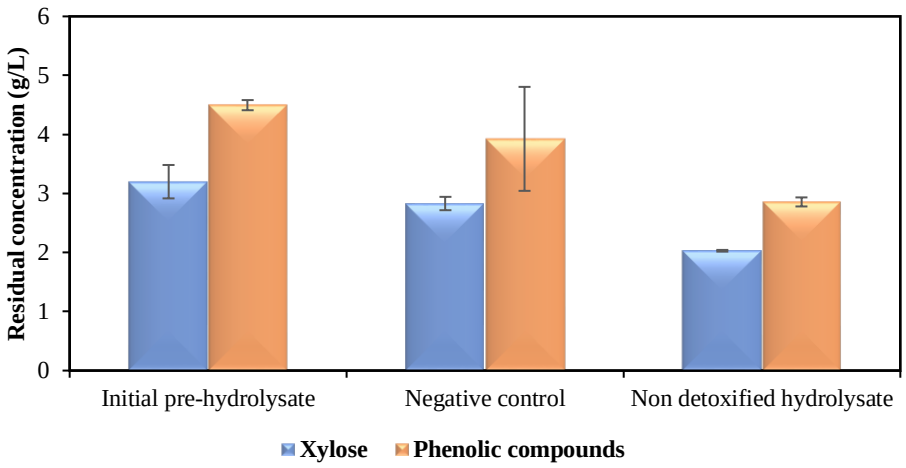



Figure 6-2 : Hydrolysis of the pre-hydrolysate with *P. campinasensis* at 41 °C with agitation at 200 rpm in an orbital shaker. The results were obtained after 49 h of incubation.

The composition of the hydrolysate from the hydrolysis step with *P. campinasensis* is shown in Figure 6-2. Xylose concentration was reduced by 37% from the pre-hydrolysate to a final concentration of 2.0 g $L^{-1}$. A first explanation of these observations is that *P. campinasensis* DSM 21989 grew on xylose. In fact, this bacterium can assimilate the xylose as a carbon source for its respiration [20]. The growth of *P. campinasensis* in the pre-hydrolysate was not measured for the turbidity and heterogenic and complex structure of the pre-hydrolysate. A second plausible explanation was thus suggested which is that toxic compounds present at high concentrations in the pre-hydrolysate inhibit hydrolysing enzymes secretion and activity by *P. campinasensis*. In fact, phenolic compounds are known as major inhibitors of cellulolytic and hemicellulolytic microorganisms and their respective enzymes can be inactivated [23, 24]. In the same way, Ázar *et al.* [23] observed 40% decreases in endoxylanase activity within 30 minutes when 7.4 g $L^{-1}$ of cinnamic acid was added. In another work, the same authors showed that a supplementation of 3.8 g $L^{-1}$ and 4.25 g $L^{-1}$ of vanillin and gallic acid respectively induced deactivation of 60% of xylanase with a co-culture of *Chrysoporthe cubenis* and *Penicillium pinophilum* [23]. González-Bautista *et al.* [24] noticed 77% decrease in the activity of xylanase produced by *Cellulomonas flavigena* PR-22 following the supplementation of 0.1 g $L^{-1}$ of phenolic compounds in the sugar cane bagasse culture medium. Aside from phenolic compounds, metal ions are cited as potent inhibitors of biological hydrolysis. In this study, the heavy metal ions can originate from neutralisation steps [24] or be present in the nutrients source of *P. campinasensis*. Zheng *et al.* [37] found that $Fe^{3+}$,

$Fe^{2+}$ and $Zn^{2+}$ are strong inhibitors of the purified recombinant xylanase secreted by *Bacillus megaterium* MS941. Activity of purified xylanase from *P. campinasensis* BL11 expressed in *Escherchia coli* was totally inhibited by 1 g/L of $Hg^{2+}$ attributed to the catalytic interaction of the metal ion with cysteine contained in xylanase [19]. This decline could also be due to a complex formation between xylose and phenolic compounds. This interaction is favored by the abundance of phenolics with hydroxyl groups and carbohydrates with oxygen atoms allowing hydrogen bonds creation [34].

In terms of variation in phenolic compounds concentration, following hydrolysis with *P. campinasensis*, a decrease from 4.5 g $L^{-1}$ in the pre-hydrolysate to 2.9 g $L^{-1}$ was observed. The decrease in phenolic compounds concentration might be explained by the fact that several other members of the genus *Paenibacillus* are known to degrade and grow on phenolic compounds rich effluents [38-41]. The final concentration was reached after 49 h of incubation and was 27% lower than the value obtained in the negative control (Figure 6-2). This decrease may be related to the production of catalase by *P. campinasensis* [20] which increases the oxidation of phenolic compounds. Another possible explanation of the phenolic compounds decrease might be related to the adsorption of xylanases by lignin and low molecular weight phenolic compounds [42]. Phenolic compounds might thus not be quantified.

#### 6.4.1.2 Strategy B: Separate biodetoxification and biohydrolysis

In the strategy B, detoxification and hydrolysis steps with the corresponding microorganisms were conducted separately. The composition of biomasses in terms of phenolic compounds and xylose concentration through the different steps is detailed in Figure 6-3. It is noteworthy that the biodetoxification experiments with the bacterial co-culture in strategy B were conducted in quadruplicate and results are an average of the four replicates with standard deviation. Regarding the hydrolysis experiments with *P. campinasensis*, the results are an average of three replicates with standard deviation.

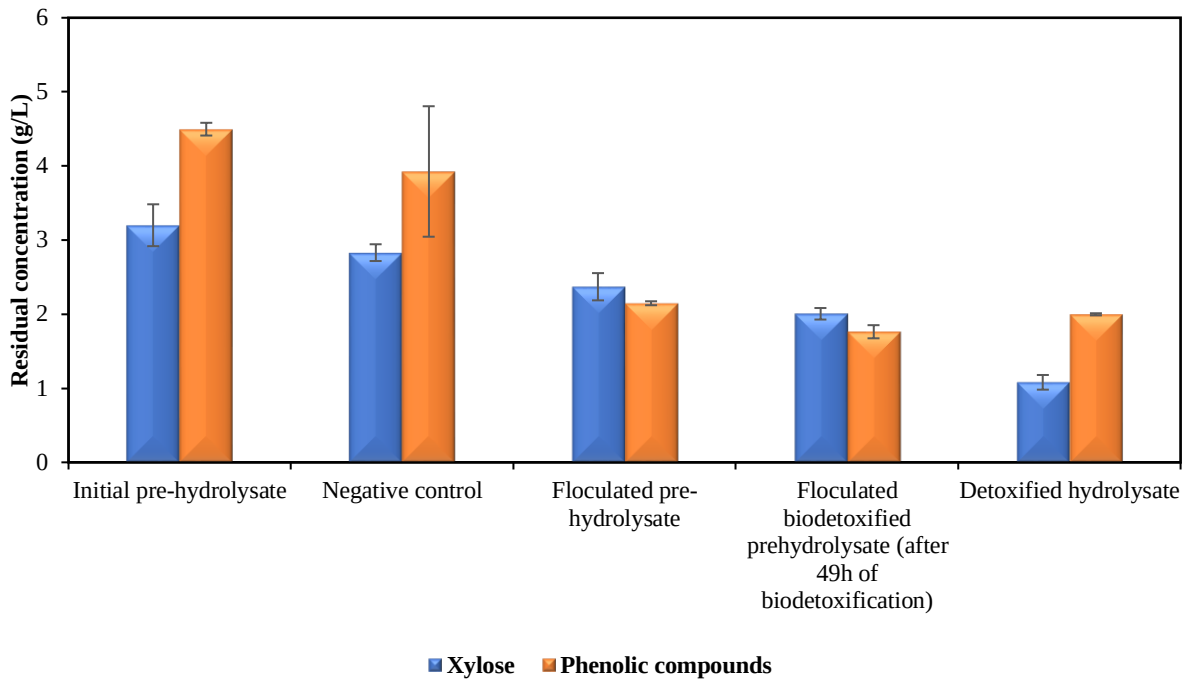



Figure 6-3 : Pre-hydrolysate detoxification with flocculation, incubation with the co-culture of *U. thermosphaericus* NCIMB 13819 and *C. taiwanensis* DSM 17343 at 41 °C and 200 rpm and hydrolysis with *P. campinasensis* DSM 21989 at 41 °C with agitation at 200 rpm in an orbital shaker.

The obtained results show a decrease in xylose concentration in the pre-hydrolysate following the detoxification procedure. In fact, xylose concentration was reduced from 3.2 g $L^{-1}$ in the pre-hydrolysate to 2.4 g $L^{-1}$ and 2.0 g $L^{-1}$ after flocculation and biodetoxification respectively. The decrease of 26% was close to that obtained by Ajao *et al.* [27], who detoxified hemicelluloses pre-hydrolysate with a similar composition, by flocculation with ferric sulfate. Sugar losses might be due to the xylose richness in hydroxyl groups which increase their ionic removal with the flocculant agent [43]. After biodetoxification of the flocculated pre-hydrolysate, xylose concentration was decreased by 15%. Although *U. thermosphaericus*, present in the co-culture, is known by its inability to consume xylose, a decrease in carbohydrates concentration in the culture has been attributed, according to Okuda *et al.* [44], to a possible oxidation by oxidase enzymes secreted by the bacterium. Xylose contents was further decreased after hydrolysis with *P. campinasensis* to 1.1 g $L^{-1}$ which was 46% lower than that reported after detoxification. This finding might be attributed to the consumption of xylose as a carbon and energy source by *P. campinasensis*. It is worthy to

remind that the autoclave of the pre-hydrolysate at 121 °C was conducted just after biodetoxification to kill cells before proceeding to hydrolysis. This step of sterilization, even short in duration (5 min), caused an increase in phenolic compounds concentration (from 1.8 g/L after the biodetoxification step to 2.5 g/L after autoclave) which could be released from lignin oxidation in the pre-hydrolysate. Furthermore, the autoclave could participate in the destruction of carbohydrate-phenolic compound complexes, thus releasing phenolics in the medium. This can cause strong inhibition of the hydrolysing bacterium *P. campinasensis* leading to the cell lysis. Cell inhibition might also be caused by the high organic load of the medium (cells debris, proteins, etc.) due to the sterilization of biodetoxified pre-hydrolysate. Therefore, formation of a large number of complexes might occur between the different constituents in the hydrolysing medium among other carbohydrates containing complexes (carbohydrates-phenolic compounds, carbohydrates-proteins, carbohydrates-lipids, carbohydrates-metal ions, etc.). Such associations could explain the significant drop in xylose concentration after the hydrolysis step. A thorough analysis of the composition of the generated mediums are required to validate these hypotheses. Formation of carbohydrates containing complexes could also occur, among other carbohydrates-proteins, carbohydrates-lipids and carbohydrates-metal ions. Such associations could explain the significant drop in xylose concentration after the hydrolysis step. A thorough analysis of the composition of the generated mediums are required to validate these hypotheses.

In terms of phenolic compounds variation through the different stages in the strategy B (separate detoxification and hydrolysis with bioagents), a decrease in concentration of 52% and 18% respectively by flocculation and bacterial detoxification of the pre-hydrolysate was obtained. At the end of hydrolysing step, the concentration of phenolics in the detoxified pre-hydrolysate raised to 2.0 g $L^{-1}$ from 1.8 g $L^{-1}$. The weak extent of degradation with the 49 h co-culture might be related to the presence of toxic compounds in the medium at inhibitory levels else after flocculation. The increase of phenolic compounds at the end of hydrolysis might be due, as mentioned above, to the probable degradation of lignin and release of low molecular weight phenolic compounds in the medium during autoclaving at 121 °C and 5 min after biodetoxification. In addition, during hydrolysis step, polysaccharides could be degraded by *P. campinasensis* liberating hence phenolic compounds trapped into the carbohydrates structure. Although most species of the *Paenibacillus* genus are known by their ability to use phenolic compounds, the composition of the detoxified

hydrolysate in this study could be inhibitory to the detoxification performance of *P. campinasensis* DSM 21989.

#### 6.4.1.3 Strategy C: Simultaneous detoxification and hydrolysis with a co-culture of three bacteria

In the strategy C, the microorganisms for detoxification and hydrolysis were applied simultaneously. The results obtained in terms of phenolic compounds and xylose concentration variation are shown in Figure 6-4.

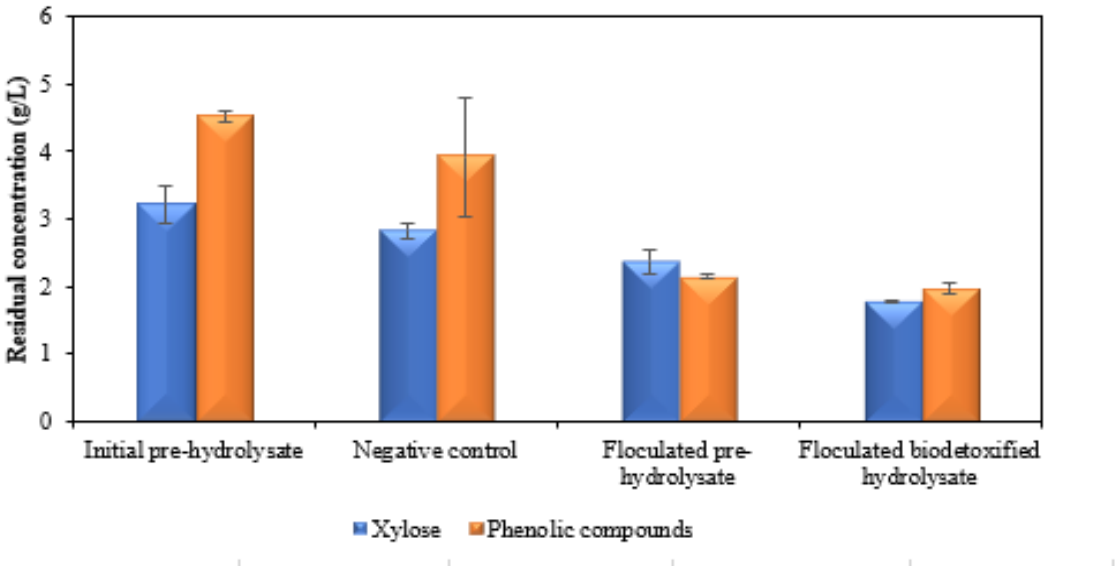



Figure 6-4 : Pre-hydrolysate detoxification with flocculation, followed by simultaneous detoxification with the co-culture of *U. thermosphaericus* and *C. taiwanensis* and hydrolysis with *P. campinasensis* at 41 °C and 200 in an orbital shaker. The duration of the biotreatments was fixed to 49 h.

The results demonstrate a decrease of 25% of xylose concentration from 2.4 g $L^{-1}$ in the flocculated pre-hydrolysate to 1.8 g $L^{-1}$ in the flocculated biodetoxified hydrolysate. This final concentration was lower than that reported in strategy A (2.0 g $L^{-1}$). A plausible explanation of this observation is that toxic compounds are still present in the medium at inhibitory levels even after flocculation, *P. campinasensis* may thus consume more xylose for its maintenance [20]. It is important to note that the final xylose concentration of 1.8 g $L^{-1}$, obtained with simultaneous detoxification and hydrolysis (strategy C), was higher than that obtained from separate detoxification and hydrolysis (strategy B) which was 1.1 g $L^{-1}$. This could be due to the action of ligninolytic enzymes, secreted

by the bacterial mixture, degrading phenolic compounds associated with carbohydrates [45] and liberating thus sugars in the medium.

In terms of phenolic compounds variation with strategy C, a decrease of phenolic compounds concentration by 52% and 8% ($p \leq 0.05$) respectively following flocculation and simultaneous microbial detoxification and hydrolysis was observed. The overall reduction from the pre-hydrolysate was 56%. A first plausible explanation of this observation might be due to pH parameter. In fact, the optimal pH for hydrolysis with *P. campinasensis* was reported to be 10 [20] and that for detoxification with *U. thermosphaericus* [46] and *C. taiwanensis* [47] to be 7, while the pH used in this study for simultaneous hydrolysis and detoxification was 8 which could be responsible for the lower performance in terms of phenolic compounds degradation. The pH at the end of the experiment was not measured in this study. A second possible hypothesis could be associated to xylanase adsorption on lignin fractions [48]. Formation of such complex limits the accessibility of ligninolytic bacteria to lignin, in addition to competition for nutrients, carbon source and space, which could be a third possible explanation of the observed low performance of phenolic compounds biodegradation in strategy C. The variation in concentration of the different types of phenolic compounds and of furfural and 5-HMF through the different process strategies were determined and presented in Section 6.8. Appendix A.

### 6.4.2 Validation of bio-based treatment methods of the pre-hydrolysate by ABE fermentation with *C. acetobutylicum* ATCC 824

The hydrolysates generated from the separate and simultaneous biodetoxification and hydrolysis strategies were assessed, for the first time, for their fermentability by *C. acetobutylicum* ATCC 824 for solvents production. The hydrolysates were diluted to 0.3 g $L^{-1}$ of phenolic compounds, a value corresponding to the threshold inhibition of non-detoxified acid hydrolysate determined previously by Mechmech *et al.* [29]. To investigate the fermentability of the detoxified hydrolysates, solvents and acids production were evaluated with synthetic xylose medium (composed of 60 g $L^{-1}$ of xylose) and undetoxified hydrolysate used as carbon sources (Figure 6-5). The mass balance in term of carbon was evaluated for the ABE fermentation of the different carbon sources (see Section 6.9. Appendix B).

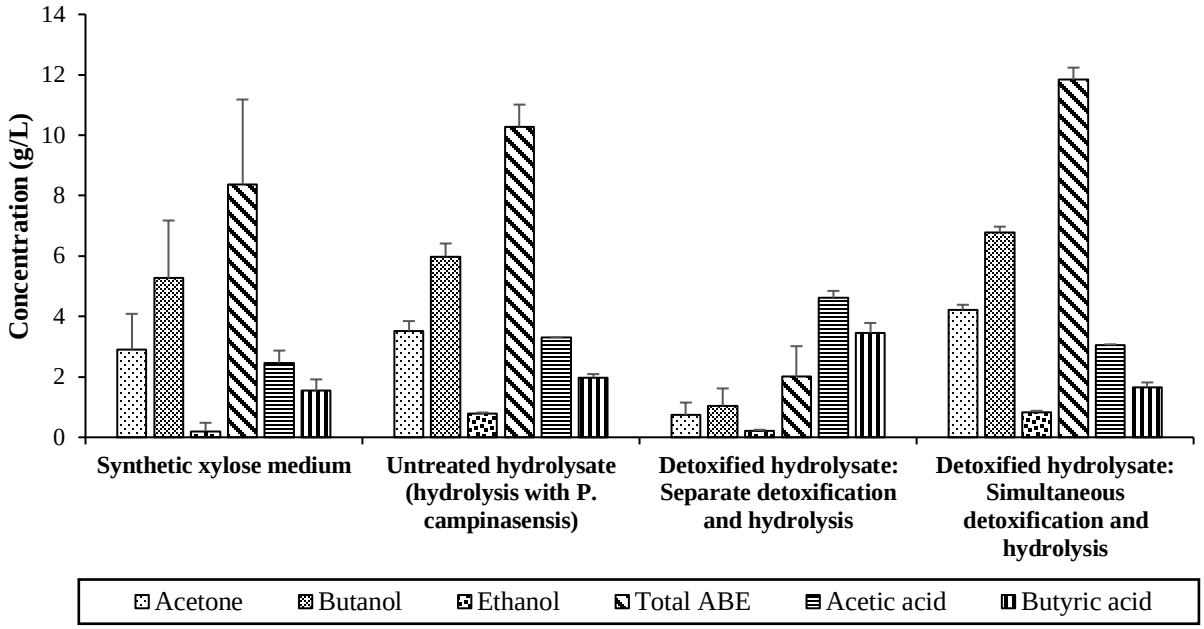



Figure 6-5 : Butanol and acids profiles of *C. acetobutylicum* ATCC 824 grown in xylose synthetic medium (containing 60 g $L^{-1}$ xylose), un-detoxified and detoxified hydrolysates. ABE mediums were incubated at 37 °C with agitation of 110 rpm in an orbital shaker during 116 h in the case of treated and untreated hydrolysates and 120 h for the synthetic xylose medium.

As shown in Figure 6-5, when synthetic xylose medium was used as a carbon source, 5.3 g $L^{-1}$ of butanol was produced after 116 h of incubation with a total ABE production of 8.4 g $L^{-1}$. In terms of acids production, 2.5 g $L^{-1}$ of acetic acid and 1.5 g $L^{-1}$ of butyric acid were obtained.

Results of ABE fermentation of undetoxified hydrolysate obtained after 49 h with *P. campinasensis* DSM 21989 incubated in the pre-hydrolysate demonstrated higher production of solvents and acids than those obtained from synthetic xylose medium fermentation. As illustrated on Figure 6-5, the concentrations of total solvents and acids at the end of undetoxified hydrolysate fermentation were around 1.2-fold higher than those obtained with synthetic medium culture. Indeed, the ABE production reached 10.3 g $L^{-1}$ from which around 6.0 g $L^{-1}$ of butanol were produced, which is 3-fold higher than results reported with acid hydrolysate generated from the same initial pre-hydrolysate (paper submitted but not published). This finding could be due to the lack of inhibitors generation with microbial hydrolysis when compared to acid hydrolysis [16]. The higher performance of solvents production compared to synthetic mediums reveals the existence of ABE stimulators in the undetoxified hydrolysate. In fact, as detailed above (Section 6.4.1.1), hydrolysis

with *P. campinasensis* was accompanied with a decrease of 36% in phenolic compounds. Phenolics and other inhibitors which might be eliminated after the hydrolysis step might be potential inhibitors of butanol production with *C. acetobutylicum* ATCC 824. Residual inhibitors in the medium among other furan aldehydes, acetic acid and phenolic compounds, may play the role of stimulators of ABE fermentation. In fact, Zhang *et al.* [48] demonstrated that *C. acetobutylicum* ATCC 824 is able to convert furan aldehydes to less toxic compounds, thus enhancing butanol production.

The fermentability of the hydrolysate obtained with strategy B (separate detoxification and hydrolysis) was tested. The results showed the lowest production of solvents and the highest generation of acids. Butanol production from this hydrolysate was 1.1 g $L^{-1}$ about 80% lower than results obtained in control experiments (synthetic xylose medium and non-detoxified hydrolysate). These findings could be attributed to the presence of highly toxic compounds in the treated hydrolysate even after dilution to 0.3 g $L^{-1}$ of phenolic compounds. In fact, as investigated above in Section 6.4.1.2, phenolic compounds concentration in the detoxified pre-hydrolysate was increased by 12% at the end of the hydrolysis step. This increase statistically significant ($p \leq 0.05$) was attributed to a probable release of low molecular weight phenolic compounds during autoclave or to the liberation of phenolics, which were trapped into polysaccharides structures, after the hydrolysis step.

The fermentation of the hydrolysate obtained with strategy C (simultaneous biodetoxification and hydrolysis) produced 4.2 g $L^{-1}$ of acetone, 6.8 g $L^{-1}$ of butanol and 0.8 g $L^{-1}$ of ethanol. The total ABE production was 11.8 g $L^{-1}$, which was 29%, 13% and 83% higher than concentrations reported with synthetic xylose medium, non-detoxified hydrolysate and hydrolysate from strategy B respectively. Acetic and butyric acids production were respectively 3.1 g $L^{-1}$ and 1.7 g $L^{-1}$. In fact, even after adjusting the initial concentration of phenolic compounds to the same value for all tested mediums, the greater performance of ABE production when using hydrolysate from simultaneous detoxification and hydrolysis could be due to their destruction with this strategy. Residual toxic compounds such as furan aldehydes could be rather stimulatory to solvents production with the solventogenic strain [30], which may explain the higher performance of the hydrolysate than that of the synthetic xylose medium devoid of inhibitors. The ABE production from the hydrolysate generated by simultaneous detoxification and hydrolysis was around 3-fold higher than that from dilute acid hydrolysate of pea pod waste, detoxified with activated charcoal until 0.03 g $L^{-1}$ of

phenolic compounds [50]. It was also higher than that from enzymatic hydrolysate of *Coleus forskohlii*, treated by resin and over liming with a final phenolic compounds concentration of 0.78 g $L^{-1}$ [51]. Hence, this new procedure for hemicelluloses pre-hydrolysate treatment by flocculation with ferric sulfate solution followed by simultaneous detoxification and hydrolysis with co-cultivated *U. thermosphaericus*, *C. taiwanensis* and *P. campinasensis* has a great potential for solvents production by *C. acetobutylicum* ATCC 824. However, further improvements are required to maximize xylose yield from hydrolysis and to minimize inhibitors content in detoxified hydrolysate to avoid dilution prior to ABE fermentation. Fed batch process with optimisation of the inoculation interval of hydrolysing and detoxifying bacteria could boost the performance of simultaneous detoxification and hydrolysis.

## 6.5 Conclusion

This study investigated for the first time the performance of simultaneous and separate detoxification and hydrolysis strategies for ABE production. The detoxification procedure consisted of flocculation followed by co-culture of *U. thermosphaericus* and *C. taiwanensis* and the hydrolysis was conducted with *P. campinasensis*. The results obtained showed higher performance of phenolic compounds removal up to 56% with the strategies combining hydrolysis and detoxification steps. While, a decrease in xylose concentration rather than an increase was observed with the different strategies. The ABE fermentation showed higher butanol production of 6.8 g L-1 with the hydrolysate from the simultaneous detoxification and hydrolysis steps. Higher titers of butanol using the studied hydrolysate as a sole carbon source will increase the cost-effectiveness of butanol production process as the hydrolysate was produced via biological methods. In parallel, improvements of xylose release from the pre-hydrolysate will also maximize ABE production.

## 6.6 Conflicts of interest

Conflicts of interest: none.

## 6.7 Acknowledgments

This work was supported by a grant from the College-University I2I Program of the Natural Sciences and Engineering Research Council of Canada (NSERC) (grant number 437803-12) of Dr.

M. Marinova, the NSERC Industrial Research Chair for Colleges in Bioprocess and Fermentation Technology of Dr. H. Chadjaa, and the NSERC Discovery Grant (# 380070-08) of Prof. M. Jolicoeur. The authors wish to express their gratitude to FPInnovations for supplying the pre-hydrolysate samples and to the Centre National en Électrochimie et Technologies Environnementales (Shawinigan, QC) for hosting the experimental work.

## 6.8 Appendix A. Hydrolysis using *P. campinasensis* DSM 21989

### 6.8.1 Hydrolysis of synthetic xylooligosaccahrides

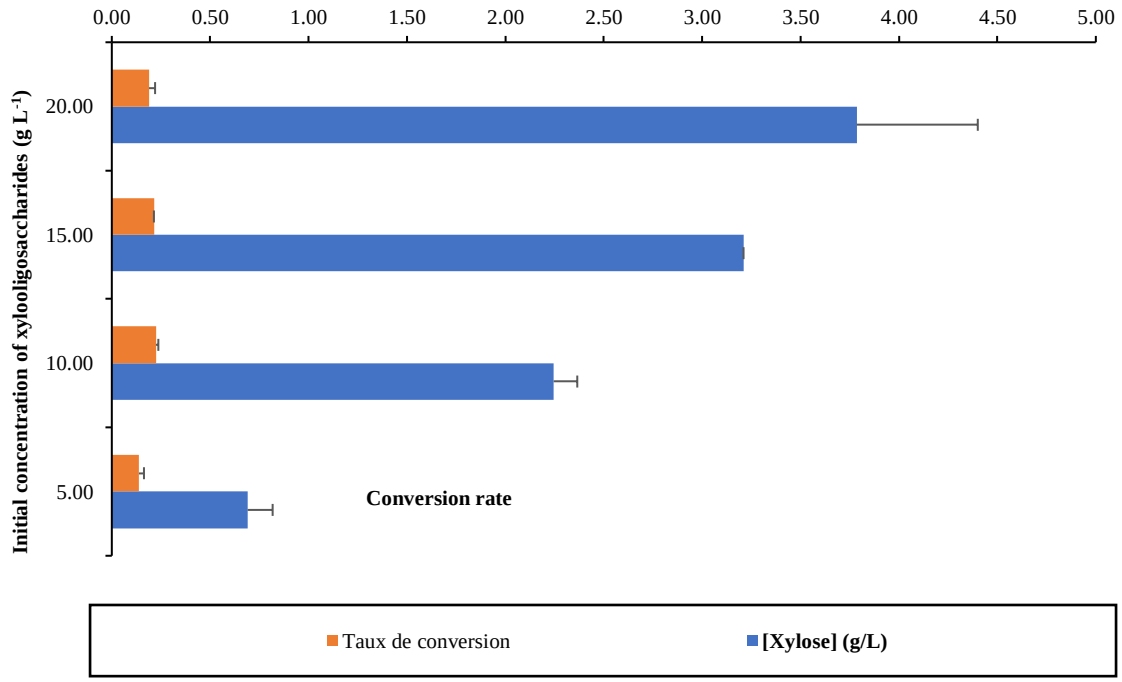



Figure 6-6 : Hydrolysis of synthetic xylooligosaccharides to xylose using *P. campinasensis* DSM 21989 at different initial concentrations of the xylooligosaccharides. The growth medium was composed of xylooligosaccharides, nitrogen source (yeast extract: 5 g/L, peptone: 5 g/L; $MgSO_4.7H_2O$: 0.2 g/L; $K_2HPO_4$: 1 g/L) and inoculum (2% (v/v)). The hydrolysis trials were conducted at 40 °C and 200 rpm for 24 h. The pH was adjusted to 10 using Na-sesquicarbonate solution (10% (v/v)). The experiments were performed in duplicate.

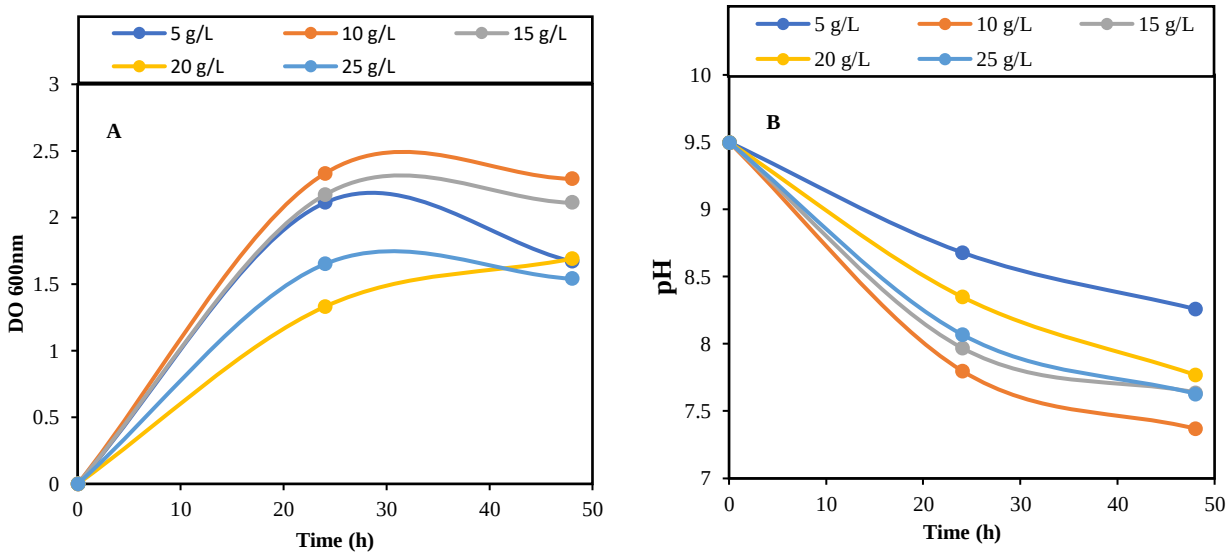



Figure 6-7 : Growth and pH curves of *P. campinasensis* DSM 21989 in synthetic xylooligosaccharides mediums at different initial concentrations of the xylooligosaccharides. (A) Growth curves. (B) pH curves.

### 6.8.2 Tretatment of hemicellulosic pre-hydrolysate

Table 6-2 : Phenolic compounds composition of different hydrolysates.

| Sample | Gallic acid (mg $L^{-1}$) | Catechol (mg $L^{-1}$) | Vanillin (mg $L^{-1}$) | Syringaldehyde (mg $L^{-1}$) | Total phenolics (mg $L^{-1}$) |
|---|---|---|---|---|---|
| **Pre-hydrolysate** | 0 | 0 | 48.1 | 109.5 | 157.6 |
| **Acid hydrolysate** | 88.4 | 0 | 38.7 | 109.1 | 236.2 |
| **Neutralized acid hydrolysate (pH 7)** | 48.1 | 51.7 | 36 | 93.5 | 229.3 |
| **Bio-hydrolysate[i]** | 0 | 173.7 | 22.55 | 42.85 | 239.1 |
| **Floculated pre-hydrolysate** | 12.5 | 337 | 21 | 57.1 | 427.6 |
| **Floculated acid hydrolysate** | 22 | 389,4 | 34,3 | 68,8 | 514,5 |

[i] The bio-hydrolysate was obtained from strategy A which consists on the hydrolysis of the pre-hydrolysate with *P. campinasensis* DSM 21989 for 49 h as a sole treatment method.

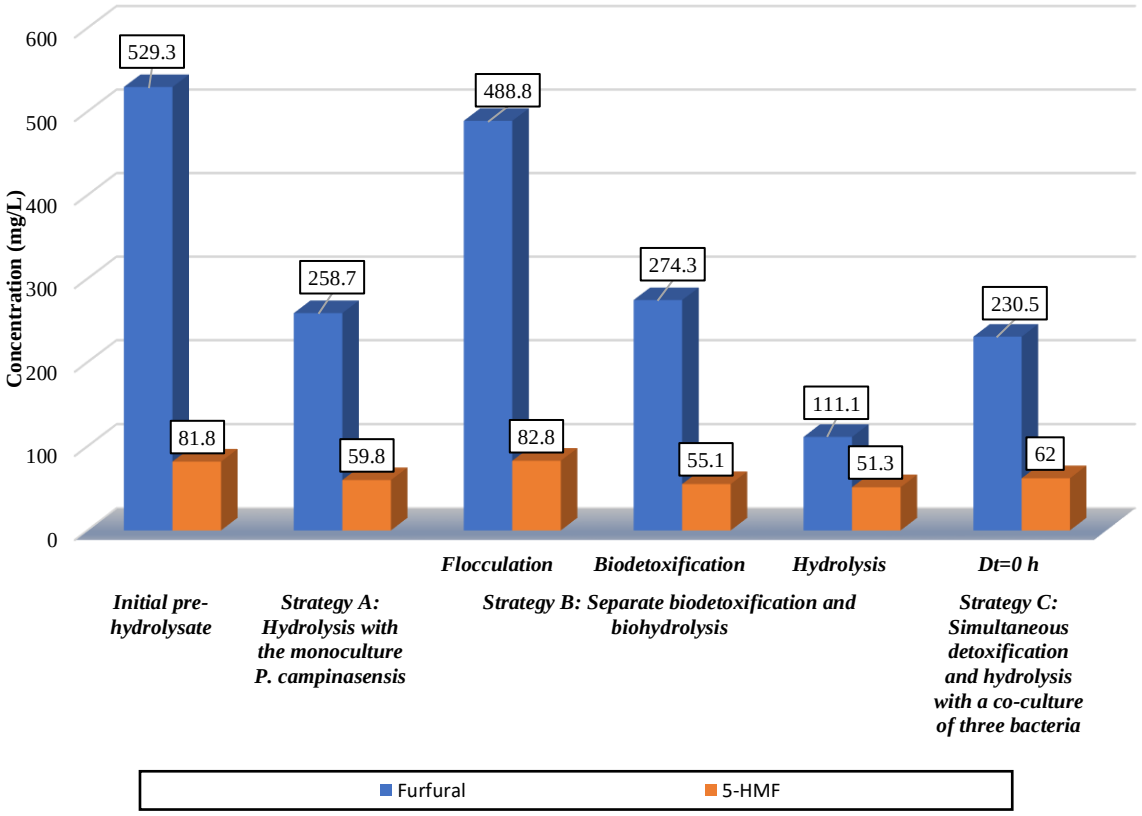



Figure 6-8 : Furfural and 5-HMF variation throughout the process strategies.

## 6.9 Appendix B. Carbon mass balance calculations

Table 6-3 : Calculations of CR (%)[i] through the different steps of strategies in this study.

| Strategy | | From initial composition (%) | From composition of last step (%) |
|---|---|---|---|
| **Hydrolysis with the monoculture *P. campinasensis*** | | 63,53 | 63,53 |
| **Separate biodetoxification and biohydrolysis** | Floculation | 52,89 | 52,89 |
| | Co-culture | 43,69 | 82,61 |
| | Hydrolysis | 42,36 | 96,97 |
| **Simultaneous detoxification and hydrolysis** | Floculation | 52,89 | 52,89 |
| | Simultaneous biodetoxification and biohydrolysis | 46,15 | 87,25 |

[i] $CR[\%] = \frac{No.of\ carbon\ of\ Xyl\left(C_{Xyl}/M_w(Xyl)\right)+No.of\ carbon\ of\ PhOH\left(C_{PhOH}/M_w(PhOH)\right)}{No.of\ carbon\ of\ Xyl\left(C_{Xyli}/M_w(Xyl)\right)+No.of\ carbon\ of\ PhOH\left(C_{PhOHi}/M_w(PhOH)\right)}$ (B1); Where: $C_{Xyl}$ represents the concentration of xylose at the end of stage; $C_{Xyli}$ represents the initial concentration of xylose; $C_{PhOH}$ represents the concentration of total phenolic compounds; $C_{PhOHi}$ represents the initial concentration of phenolic compounds; $M_w(Xyl)$ represents the molecular weight of xylose, i.e. 150 g mol$^{-1}$; $M_w(PhOH)$ represents the molecular weight of phenolic compounds and it was calculated following Eq. (B2): $M_w(PhOH) = 0.3 \times M_w(vanillin) + 0.7 \times M_w(syringaldehyde)$ $(B2)$

First, the carbon yields transferred from hemicellulose hydrolysates into valuable products (i.e. butanol, acetone and ethanol) were determined. The results are presented in Table 6-4.

Table 6-4 : Carbon mass balance[i] of the initial and final compositions of the different fermentation media in this study.

| Sample | Initial xylose (g) | Ethanol (g) | Butanol (g) | Acetone (g) | Acetic acid (g) | Butyric acid (g) |
|---|---|---|---|---|---|---|
| **Synthetic xylose medium** | 4.80 | 0.02 | 0.68 | 0.36 | 0.20 | 0.17 |
| **Untreated hydrolysate (hydrolysis with *P. campinasensis*)** | 4.80 | 0.08 | 0.78 | 0.44 | 0.26 | 0.22 |
| **Detoxified hydrolysate: Separate detoxification and hydrolysis** | 4.80 | 0.02 | 0.14 | 0.09 | 0.37 | 0.38 |
| **Detoxified hydrolysate: Simultaneous detoxification and hydrolysis** | 4.80 | 0.09 | 0.88 | 0.52 | 0.24 | 0.18 |

[i] The carbon mass for each product was calculated following the Eq. 3:

$$Carbon\ mass = \frac{Weight\ (g) \times Carbon\ mass\ in\ one\ mole\ (\frac{g}{moL})}{Molecular\ weight\ (\frac{g}{moL})} \quad \text{(B3).}$$

The carbon mass in one mole of the product was determined by multiplying the molecular weight of the carbon atom by the number of carbon atoms in the product.

Secondly, the productivities of the solvents and acids were determined and results are given in Table 6-5.

Table 6-5 : Solvents and acids productivities from ABE fermentation of hydrolysates from different strategies of treatment. The duration of ABE fermentation experiments was 120 h for synthetic xylose medium and 116 h for treated and untreated hydrolysates. The results are given in g $L^{-1}$ $h^{-1}$.

| Sample | Ethanol | Butanol | Acetone | Total ABE | Acetic acid | Butyric acid |
|---|---|---|---|---|---|---|
| **Synthetic xylose medium** | 0.0017 | 0.0440 | 0.0242 | 0.0698 | 0.0206 | 0.0128 |
| **Untreated hydrolysate (hydrolysis with *P. campinasensis*)** | 0.0068 | 0.0516 | 0.0303 | 0.0887 | 0.0284 | 0.0171 |
| **Detoxified hydrolysate: Separate detoxification and hydrolysis** | 0.0019 | 0.0090 | 0.0064 | 0.0173 | 0.0398 | 0.0298 |
| **Detoxified hydrolysate: Simultaneous detoxification and hydrolysis** | 0.0071 | 0.0585 | 0.0364 | 0.1020 | 0.0263 | 0.0143 |

The maximum rates of detoxification of phenolic compounds with the different strategies were also calculated and results are given in Table 6-6.

Table 6-6 : Maximum rates of detoxification with the different developed strategies.

| Strategy | | Maximum rates of phenolic compounds removal (g $L^{-1}$ $h^{-1}$) |
|---|---|---|
| **Hydrolysis with *P. campinasensis*** | | 0.0584 |
| **Separate detoxification and hydrolysis** | After biodetoxification | 0.0359 |
| | After hydrolysis | 0.0408 |
| **Simultaneous detoxification and hydrolysis** | | 0.0404 |

## 6.10 References

[1] O. Petrov, X. Bi, A. Lau, Impact assessment of biomass-based district heating systems in densely populated communities. Part II: Would the replacement of fossil fuels improve ambient air quality and human health?, Atmospheric environment. 161 (2017) pp. 191-199.

[2] S. K. Khanal, W.-H. Chen, L. Li, S. Sung, Biological hydrogen production: effects of pH and intermediate products, International journal of hydrogen energy. 29 (2004) pp. 1123-1131.

[3] T. Covert, M. Greenstone, C. R. Knittel, Will we ever stop using fossil fuels?, Journal of Economic Perspectives. 30 (2016) pp. 117-38.

[4] Natural Resources Canada. Office of energy efficiency. http://oee.nrcan.gc.ca/corporate/statistics/neud/dpa/menus/trends/comprehensive/trends_agg_ca.cfm, 2015.

[5] J. Cuddihy, C. Kennedy, P. Byer, Energy use in Canada: environmental impacts and opportunities in relationship to infrastructure systems, Canadian Journal of Civil Engineering. 32 (2005) pp. 1-15.

[6] J. P. Maity, J. Bundschuh, C.-Y. Chen, P. Bhattacharya, Microalgae for third generation biofuel production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future perspectives–A mini review, Energy. 78 (2014) pp. 104-113.

[7] N. Gaurav, S. Sivasankari, G. Kiran, A. Ninawe, J. Selvin, Utilization of bioresources for sustainable biofuels: A Review, Renewable and Sustainable Energy Reviews. 73 (2017) pp. 205-214.

[8] B. Ravindran, M. B. Kurade, A. N. Kabra, B.-H. Jeon, S. K. Gupta, Recent Advances and Future Prospects of Microalgal Lipid Biotechnology, in: Algal Biofuels, Springer, 2017, pp. 1-37.

[9] M. Riazi, D. Chiaramonti, Biofuels Production and Processing Technology, 2017.

[10] J. A. Quintero, J. Moncada, C. A. Cardona, Techno-economic analysis of bioethanol production from lignocellulosic residues in Colombia: a process simulation approach, Bioresource technology. 139 (2013) pp. 300-307.

[11] X. Zhao, M. Kasbi, J. Chen, S. Peres, M. Jolicoeur, A dynamic metabolic flux analysis of ABE (acetone-butanol-ethanol) fermentation by *Clostridium acetobutylicum* ATCC 824, with riboflavin as a by-product, Biotechnology and bioengineering. 114 (2017) pp. 2907-2919.

[12] C. Thirmal, Y. Dahman, Comparisons of existing pretreatment, saccharification, and fermentation processes for butanol production from agricultural residues, The Canadian Journal of Chemical Engineering. 90 (2012) pp. 745-761.

[13] P. M. Mordaka, J. T. Heap, Stringency of synthetic promoter sequences in *Clostridium* revealed and circumvented by tuning promoter library mutation rates, ACS synthetic biology. 7 (2018) pp. 672-681.

[14] W. Guan, G. Xu, J. Duan, S. Shi, Acetone–Butanol–Ethanol Production from Fermentation of Hot-Water-Extracted Hemicellulose Hydrolysate of Pulping Woods, Industrial & Engineering Chemistry Research. 57 (2018) pp. 775-783.

[15] S. A. Survase, E. Sklavounos, G. Jurgens, A. Van Heiningen, T. Granström, Continuous acetone–butanol–ethanol fermentation using SO2–ethanol–water spent liquor from spruce, Bioresource technology. 102 (2011) pp. 10996-11002.

[16] W. Liao, Y. Liu, C. Liu, S. Chen, Optimizing dilute acid hydrolysis of hemicellulose in a nitrogen-rich cellulosic material—dairy manure, Bioresource Technology. 94 (2004) pp. 33-41.

[17] S. Maiti et al., Hydrolytic pre-treatment methods for enhanced biobutanol production from agro-industrial wastes, Bioresource technology. 249 (2018) pp. 673-683.

[18] V. B. Barua, V. V. Goud, A. S. Kalamdhad, Microbial pretreatment of water hyacinth for enhanced hydrolysis followed by biogas production, Renewable Energy. 126 (2018) pp. 21-29.

[19] C.-H. Ko et al., Molecular cloning and characterization of a novel thermostable xylanase from *Paenibacillus campinasensis* BL11, Process Biochemistry. 45 (2010) pp. 1638-1644.

[20] J.-H. Yoon et al., *Paenibacillus campinasensis* sp. nov., a cyclodextrin-producing bacterium isolated in Brazil, International Journal of Systematic and Evolutionary Microbiology. 48 (1998) pp. 833-837.

[21] Y.-H. Wei et al., Screening and evaluation of polyhydroxybutyrate-producing strains from indigenous isolate *Cupriavidus taiwanensis* strains, International journal of molecular sciences. 12 (2011) pp. 252-265.

[22] M. G. Fortina et al., *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Andersson et al. 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov, International journal of systematic and evolutionary microbiology. 51 (2001) pp. 447-455.

[23] R. I. L. Ázar, T. Morgan, A. C. F. dos Santos, E. de Aquino Ximenes, M. R. Ladisch, V. M. Guimarães, Deactivation and activation of lignocellulose degrading enzymes in the presence of laccase, Enzyme and microbial technology. 109 (2018) pp. 25-30.

[24] J. de Cassia Pereira et al., Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities, in: Enzyme Inhibitors and Activators, InTech, 2017.

[25] M. P. García-Aparicio, I. Ballesteros, A. González, J. M. Oliva, M. Ballesteros, M. J. Negro, Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis, Applied biochemistry and biotechnology. 129 (2006) pp. 278-288.

[26] S. Larsson, A. Reimann, N.-O. Nilvebrant, L. J. Jönsson, Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce, Applied biochemistry and biotechnology. 77 (1999) pp. 91-103.

[27] O. Ajao, M. Le Hir, M. Rahni, M. Marinova, H. Chadjaa, O. Savadogo, Concentration and detoxification of Kraft prehydrolysate by combining nanofiltration with flocculation, Industrial & Engineering Chemistry Research. 54 (2015) pp. 1113-1122.

[28] B.-Y. Chen, J.-W. You, Y.-T. Hsieh, J.-S. Chang, Feasibility study of exponential feeding strategy in fed-batch cultures for phenol degradation using *Cupriavidus taiwanensis*, Biochemical Engineering Journal. 41 (2008) pp. 175-180.

[29] F. Mechmech, H. Chadjaa, M. Rahni, M. Marinova, N. B. Akacha, M. Gargouri, Improvement of butanol production from a hardwood hemicelluloses hydrolysate by combined sugar concentration and phenols removal, Bioresource technology. 192 (2015) pp. 287-295.

[30] T. Ezeji, N. Qureshi, H. P. Blaschek, Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation, Biotechnology and bioengineering. 97 (2007) pp. 1460-1469.

[31] O. Ajao, M. Le Hir, M. Rahni, H. Chadjaa, M. Marinova, Comparative biocatalytic degradation of Kraft prehydrolysate phenolic fermentation inhibitors using bacteria-derived laccase, Wood science and technology. 51 (2017) pp. 585-599.

[32] T.-T. You, L.-M. Zhang, S.-K. Zhou, F. Xu, Structural elucidation of lignin–carbohydrate complex (LCC) preparations and lignin from Arundo donax Linn, Industrial Crops and Products. 71 (2015) pp. 65-74.

[33] R. Deshpande et al., The reactivity of lignin carbohydrate complex (LCC) during manufacture of dissolving sulfite pulp from softwood, Industrial Crops and Products. 115 (2018) pp. 315-322.

[34] L. Jakobek, Interactions of polyphenols with carbohydrates, lipids and proteins, Food Chemistry. 175 (2015) pp. 556-567.

[35] R. Alnaizy, A. Akgerman, Advanced oxidation of phenolic compounds, Advances in Environmental Research. 4 (2000) pp. 233-244.

[36] R. Sekar, H. D. Shin, T. J. DiChristina, Direct conversion of cellulose and hemicellulose to fermentable sugars by a microbially-driven Fenton reaction, Bioresource technology. 218 (2016) pp. 1133-1139.

[37] H. Zheng, X. Liu, Y. Han, J. Wang, F. Lu, Overexpression of a *Paenibacillus campinasensis* xylanase in Bacillus megaterium and its applications to biobleaching of cotton stalk pulp and saccharification of recycled paper sludge, Bioresource technology. 125 (2012) pp. 182-187.

[38] M. Aguilera et al., Characterisation of *Paenibacillus jamilae* strains that produce exopolysaccharide during growth on and detoxification of olive mill wastewaters, Bioresource technology. 99 (2008) pp. 5640-5644.

[39] R. Chandra, S. Yadav, R. N. Bharagava, V. Rai, Phenol degradation by *Paenibacillus thiaminolyticus* and *Bacillus cereus* in axenic and mixed conditions, World Journal of Microbiology and Biotechnology. 27 (2011) pp. 2939-2947.

[40] A. Raj, S. Kumar, I. Haq, S. K. Singh, Bioremediation and toxicity reduction in pulp and paper mill effluent by newly isolated ligninolytic *Paenibacillus* sp, Ecological engineering. 71 (2014) pp. 355-362.

[41] A. Raj, M. K. Reddy, R. Chandra, Decolourisation and treatment of pulp and paper mill effluent by lignin-degrading *Bacillus* sp, Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology. 82 (2007) pp. 399-406.

[42] G. Zeder-Lutz et al., Novel surface-based methodologies for investigating GH11 xylanase–lignin derivative interactions, Analyst. 138 (2013) pp. 6889-6899.

[43] Q. Wang, M. S. Jahan, S. Liu, Q. Miao, Y. Ni, Lignin removal enhancement from prehydrolysis liquor of kraft-based dissolving pulp production by laccase-induced polymerization, Bioresource technology. 164 (2014) pp. 380-385.

[44] N. Okuda, M. Soneura, K. Ninomiya, Y. Katakura, S. Shioya, Biological detoxification of waste house wood hydrolysate using *Ureibacillus thermosphaericus* for bioethanol production, Journal of bioscience and bioengineering. 106 (2008) pp. 128-133.

[45] L. P. Christopher, B. Yao, Y. Ji, Lignin biodegradation with laccase-mediator systems, Frontiers in Energy Research. 2 (2014) p. 12.

[46] C. Asada, C. Sasaki, T. Takamatsu, Y. Nakamura, Conversion of steam-exploded cedar into ethanol using simultaneous saccharification, fermentation and detoxification process, Bioresource technology. 176 (2015) pp. 203-209.

[47] B. Y. Chen, J. S. Chang, Phenol degradation and toxicity assessment upon biostimulation to an indigenous rhizobium *Ralstonia taiwanensis*, Biotechnology progress. 21 (2005) pp. 1085-1092.

[48] E. González-Bautista et al., Phenolic compounds inhibit cellulase and xylanase activities of *Cellulomonas flavigena* PR-22 during saccharification of sugarcane bagasse, Fuel. 196 (2017) pp. 32-35.

[49] Y. Zhang, B. Han, T. C. Ezeji, Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by *Clostridium acetobutylicum* ATCC 824 during butanol fermentation, New biotechnology. 29 (2012) pp. 345-351.

[50] P. R. Nimbalkar, M. A. Khedkar, P. V. Chavan, S. B. Bankar, Biobutanol production using pea pod waste as substrate: Impact of drying on saccharification and fermentation, Renewable Energy. 117 (2018) pp. 520-529.

[51] S. M. Harde et al., Acetone-butanol-ethanol (ABE) fermentation using the root hydrolysate after extraction of forskolin from *Coleus forskohlii*, Renewable energy. 86 (2016) pp. 594-601.

# CHAPITRE 7 : DISCUSSION GÉNÉRALE

L'industrie des pâtes et papiers au Canada passe par une période difficile caractérisée par une crise économique aigüe. L'intégration de bioraffinerie industrielle pour l'extraction et la conversion des hémicelluloses représente une alternative très prometteuse pour limiter ces difficultés et générer des produits à haute valeur ajoutée (e.g. butanol). L'extraction dans le procédé Kraft est assurée par une étape de préhydrolyse. Puis une étape d'hydrolyse permet de libérer des glucides fermentescibles. Les méthodes conventionnelles de prétraitements et d'hydrolyse utilisent souvent des conditions opératoires extrêmes (température, dose des réactifs chimiques, pression, durée, etc.) qui génèrent en plus des sucres des inhibiteurs, qui empêchent une conversion efficace des hémicelluloses. L'utilisation des bioagents permet d'empêcher la formation des inhibiteurs en plus de leurs caractéristiques environnementale et économique. Néanmoins, l'intégration d'une étape de détoxification est incontournable et l'application directe des microorganismes pour la détoxification est très prometteuse pour une conversion plus rentable en bioproduits.

L'objectif de cette thèse est de développer une approche novatrice de détoxification de préhydrolysat de bois par voies biotechnologiques. Le développement d'une co-culture bactérienne composée de deux bactéries : *U. thermosphaericus* et *C. taiwanensis* était investiguée pour la première fois dans cette recherche dans le but de détoxifier les hydrolysats de bois. Les souches étudiées ne requièrent pas de glucides pour leur métabolisme et utilisent plutôt divers inhibiteurs comme sources de carbone. À cela s'ajoute les durées de traitement courtes qui ne dépassent pas 24 h. Ces atouts sont recherchés dans les procédés industriels pour une conversion efficace et économique de la biomasse lignocellulosique. Les deux souches ont été appliquées dans cette thèse en cultures mixtes dans le but d'élargir la gamme des inhibiteurs dégradés et de réduire davantage la durée de détoxification. Ces faits permettraient de rentabiliser le procédé de production des biocarburants à partir des hémicelluloses sur les plans économique, environnementale et rendement de production ce qui favoriserait sa mise à l'échelle industrielle. Mais il est important de noter que dans les expériences de détoxification par la co-culture, on a fixé la fraction initiale de chaque souche à moitié en termes de concentration (UFC/mL). Pour ce faire, on a procédé au dénombrement cellulaire de chaque inoculum et on a représenté les résultats en fonction des valeurs de $DO_{600nm}$ comme le montre la figure A-1 (a et b) dans l'Annexe A. Deux méthodes de

dénombrement (par hémacytomètre et par ensemencement sur gélose) ont été évaluées et la décompte par hémacytomètre a été démontrée plus précise et rapide. L'approche de Q-PCR a été utilisée dans la présente étude pour valider les résultats de décompte trouvés et un ratio volumique initiale de 1x unité de *C. taiwanensis* pour 1.5x unités de *U. thermosphaericus* était retenue pour établir la coculture de détoxification.

Rappelant l'objectif principal de la thèse qui consiste à détoxifier les hémicelluloses par voies biotechnologiques novatrices. Cependant, la composition très hétérogène de préhydrolysat de bois qui est à l'origine de sa complexité pourrait entraver une étude poussée de la performance de la nouvelle co-culture développée. Par conséquent, on a investigué dans l'Article 1 (Chapitre 4) la détoxification avec des microorganismes des milieux synthétiques, composés des inhibiteurs et formulés de manière à simuler la composition des préhydrolysats souvent générés dans les procédés industriels. Dans l'Article 1, les mono- et co-cultures ont été étudiées d'abord sur un milieu TSB comme système modèle à différentes températures d'incubation (37 °C, 41 °C et 45 °C). Des cinétiques réactionnelles similaires des mono- et des co-cultures ont été rapportées à 41 °C. Dans une deuxième étape, on a étudié la performance de dégradation des composés phénoliques par les cultures pures et mixtes dans des milieux synthétiques composés de concentrations variables des inhibiteurs (composés phénoliques (acide gallique, vanilline, catéchol et syringaldéhyde), furfural, 5-HMF et sels) et de source des nutriments. Cette dernière est différente pour les deux souches en cultures pures. En effet, des milieux enrichis en extrait de levure et en peptone ont été utilisés pour la détoxification par *U. thermosphaericus* [160], alors que des milieux à base des sels et des éléments traces ont permis la croissance et le métabolisme de *C. taiwanensis* [151]. Une étude comparative de la supplémentation des deux sources de nutriments sur la performance de détoxification des composés inhibiteurs de type phénoliques avec les mono- et co-cultures a été effectuée. Une efficacité supérieure de dégradation a été observée avec un milieu à base des sels et des éléments traces. On a par la suite évalué la performance de détoxification des milieux synthétiques par des co-cultures simultanées et séquentielles. Puis, la séquence d'inoculation des bactéries maximisant la dégradation spécifique de chaque type de composés phénoliques était testée avec les cultures pures dans des solutions des phénoliques individuellement préparées. Les résultats obtenus ont montré des performances de détoxification par les co-cultures plus élevées en comparaison avec les mono-cultures à 8 g/L des composés phénoliques et en coprésence des autres inhibiteurs et des composés phénoliques (furfural, 5-HMF et sels). En effet, 87% ont été dégradés

avec les cultures séquentielles de *U. thermosphaericus* suivie de *C. taiwanensis* par rapport à 53% et 49% obtenus avec *U. thermosphaercius* et *C. taiwanensis* respectivement. Pour valider la nouvelle méthode de détoxification par la co-culture composée de *U. thermosphaercius* et *C. taiwanensis*, un préhydrolysat de bois réel obtenu par explosion à la vapeur couplée à l'eau chaude était traité avec les mono- et co-cultures. L'inoculation décalée de *C. taiwanensis* par rapport à *U. thermosphaericus* (intervalle d'inoculation de 12 h) a donné des meilleurs résultats de dégradation des composés phénoliques de 14%. Ces valeurs ont été plus faibles que celles obtenues avec les milieux synthétiques. En effet, le préhydrolysat de bois, dont la composition est très hétérogène et complexe, pourrait contenir d'autres produits toxiques qui sont plus inhibiteurs aux bactéries de détoxification.

Certaines limitations ont été associées à ces études entre autres la méthode de décompte cellulaire par hémacytomètre utilisée. Il est vrai que cette technique est plus précise et rapide en comparaison avec d'autres méthodes conventionnelles de dénombrement (e.g. par ensemencement sur gélose). Mais, cette méthode reste imparfaite et on doit passer à la validation par Q-PCR pour avoir plus de précision. La limitation de cette technique pourrait être attribuée au type du colorant cellulaire utilisée. En effet, le bleu de trypan, utilisé dans cette recherche, permet de distinguer les cellules vivantes et les cellules mortes. Ces dernières se colorent en bleu à cause de type des membranes poreuses de ces cellules alors que les cellules vivantes, ayant la membrane intacte, n'absorbent pas le colorant. Mais, il est important de noter que la perméabilité membranaire n'implique pas nécessairement la mort cellulaire [184]. De plus, des débris cellulaires peuvent être à l'origine des imprécisions dans les observations microscopiques. L'utilisation d'autres types de colorants est ainsi recommandée. Une autre limite de la présente recherche est que la détermination des concentrations de catéchol par HPLC a renvoyé dans certains cas des concentrations supérieures aux valeurs initiales ce qui a été expliqué par la génération des intermédiaires ayant des temps de rétention similaires. Pour une détermination plus exacte de la concentration du catéchol, les aires d'absorption des contaminants ont été retranchées de l'aire totale observée permettant ainsi de déterminer l'aire exacte pour le catéchol seul. Les contaminants doivent ainsi être identifiés et leurs standards correspondants doivent être testés par HPLC. Le benzoquinone représente un intermédiaire de dégradation de catéchol et un temps de rétention du standard de ce composé déterminé par HPLC était similaire à celui de catéchol. En plus de benzoquinone, d'autres sous-produits peuvent être issus de la dégradation de catéchol et qui peuvent être à l'origine des

concentrations augmentées de ce phénolique. Mais, dans les laboratoires du Centre National en Électrochimie et en Technologies, où les expériences et les analyses de cette thèse ont été effectuées, les standards de ces produits n'étaient pas disponibles. Pour une exactitude rigoureuse des analyses, il est recommandé de commander des standards de tous les intermédiaires possibles du métabolisme de catéchol. Une autre limite de la méthode dans cette étude consiste aux faibles résultats de détoxification de préhydrolysat de bois avec les co-cultures. Cette limite peut être justifiée par la faible résistance des microorganismes étudiés aux inhibiteurs contenus dans les hydrolysats d'hémicelluloses. Des optimisations de la méthode de détoxification (durée, doses d'inoculum, etc.) sont fortement recommandées.

Dans l'Article 2 (Chapitre 5), on a étudié les effets de la combinaison de la floculation qui utilise $Fe_2(SO_4)_3$ avec la co-culture composée de *U. thermosphaericus* et *C. taiwanensis* sur la performance de détoxification des hydrolysats de bois et la production subséquente de butanol. Dans un premier temps, nous avons proposé et développé différentes combinaisons de méthodes de détoxification (i.e. floculation et co-culture) en intégrant dans certains cas une étape de nanofiltration. Cette dernière assure une concentration des sucres qui sont présents à faibles teneurs dans le préhydrolysat de bois étudié. Le choix de la floculation comme une deuxième méthode de détoxification était justifié par la simplicité dans la manipulation, l'efficacité élevée de dégradation des polluants, les faibles coûts opérationnelles et les moindres dommages à l'environnement. Les tests d'optimisation de la méthode de floculation, en termes de dose du floculant, du pH et de l'agent d'ajustement du pH, ont été réalisés et publiés dans le papier de Ajao et al. (2015) pour maximiser à la fois l'élimination des composés phénoliques et la rétention des sucres dans un préhydrolysat de bois partageant la même composition que celle dans notre étude. Ainsi, le choix de la floculation comme une deuxième méthode de détoxification a été opté également pour établir des solides références de comparaison entre les résultats. Dans ce travail, les pourcentages de dégradation les plus élevés des composés phénoliques ont été obtenus par un procédé séquentiel qui implique une étape de floculation avec $Fe_2(SO_4)_3$ suivie de la biodétoxification avec la co-culture. Cette nouvelle approche de détoxification à deux étapes a permis de réduire la concentration des composés phénoliques de 4.5 g/L dans le préhydrolysat de bois à 1.4 g/L qui est proche du seuil d'inhibition de la production de butanol (1.1 g/L). Mais quelques limites caractérisent la nouvelle stratégie de détoxification comme les coûts élevés de floculant ($Fe_2(SO_4)_3$) et de l'agent de neutralisation ($Ca(OH)_2$) utilisés pour la floculation et l'ajustement de pH

respectivement. L'utilisation d'autres types de floculant qui soient moins couteux et plus efficaces à faibles doses peut être une solution recommandée. D'autre part, des volumes importants des flocs ont été observés durant l'étape de sédimentation dans le procédé de floculation. Par conséquent, on était face au colmatage rapide des filtres durant la récupération de l'hydrolysat traité et donc au changement de ces filtres à maintes reprises. Et parfois une deuxième filtration était nécessaire dans notre étude pour récupérer un hydrolysat plus clair dont le volume final était beaucoup plus petit que le volume initial avant floculation. L'optimisation du système de floculation en termes des conditions opératoires et du type du procédé (en batch ou en continu) pourrait limiter ces difficultés. La deuxième partie de l'Article 2 (Chapitre 5) consiste en une validation du procédé de détoxification le plus performant, en termes de dégradation des inhibiteurs, par la production de butanol via fermentation ABE par *C. acetobutylicum* ATCC 824. Une production maximale de 8 g/L a été obtenue après 120 h de culture en utilisant un hydrolysat détoxifié, par couplage de la floculation à la co-culture, et dilué à 0.3 g/L des composés phénoliques comme source de carbone.

Une deuxième validation de l'efficacité de la nouvelle technologie de détoxification a été effectuée dans cette thèse par hydrolyse biologique de préhydrolysat détoxifiée avec la souche *P. campinasensis* DSM 21989. Les résultats de cette étude sont présentés dans l'Article 3 (Chapitre 6). Il s'agit d'élaborer une étude comparative, en termes de variation des concentrations de xylose et des composés phénoliques, des technologies d'hydrolyse et de détoxification séparées ou simultanées, en co-culture des trois souches bactériennes, et précédées par une étape de floculation. Une diminution de la concentration de xylose dans le préhydrolysat plutôt que son augmentation a été observée avec les différentes stratégies développées. Alors qu'une efficacité similaire de dégradation des composés phénoliques (56%) a été obtenue en jumelant l'hydrolyse et la détoxification comparé à 13% et 36% obtenus dans le contrôle négatif (non inoculé) et dans l'hydrolysat non détoxifié respectivement. Pour la validation des stratégies d'hydrolyse et de détoxification développées dans le cadre de cette thèse, les hydrolysats générés ont été dilués jusqu'à 0.3 g/L des composés phénoliques puis utilisés comme sources de carbone dans la fermentation ABE. La concentration la plus élevée en butanol de 7 g/L a été produite après 116 h de culture avec l'hydrolysat détoxifié et hydrolysé simultanément. La deuxième production de butanol de 6 g/L a été obtenue avec l'hydrolysat microbiologique comme traitement unique de préhydrolysat et cette concentration était plus élevée que celle obtenue dans le milieu synthétique (5.27 g/L) et dans l'hydrolysat issus des étapes de détoxification et hydrolyse séparées (1.05 g/L).

Le recours aux cultures simultanées des souches spécifiques d'hydrolyse et de détoxification dans cette étude représente une originalité. En fait, les études qui utilisent des co-cultures bactériennes pour une détoxification et hydrolyse simultanées d'hydrolysats de bois sont très rares. Récemment, Tsegaye et al. ont testé une co-culture composée de *Bacillus* sp. BMP01 pour l'hydrolyse des polysaccharides et *Ochrobactrum oryzae* BMP03 pour la délignification. Les auteurs Tsegaye et al. ont montré que la biodetoxification de préhydrolysat durant 16 h a dégradé 44.47% de lignine et a favorisé l'Augmentation de la performance de biohydrolyse grâce à une accessibilité plus élevée aux glucides. La biohydrolyse subséquente a produit 439 mg/g des sucres réducteurs après 16 jours d'incubation avec *Bacillus* sp. BMP01. La performance d'hydrolyse était plus faible de 9.45% avec les cultures simultanées des souches. Il est vrai que le traitement biologique proposé par Tsegaye et al. a permis des meilleurs rendements d'hydrolyse et de détoxification, mais la durée de traitement séquentielle qui était de 32 jours est plus longue que l'approche appliquée dans notre projet (la durée de biotraitement ne dépasse pas 98 h). La méthode de traitement proposée dans cette thèse pourrait être améliorée par la variation des conditions opératoires de culture. De plus, les observations dans ce projet sont très prometteuses puisque nous avons démontré pour la première fois la performance de détoxification de préhydrolysat par la souche d'hydrolyse *P. campinasensis*. Le problème associé à l'application de cette technologie pour l'hydrolyse de préhydrolysat se présente au niveau des faibles concentrations finales de xylose. Pour maximiser les rendements d'hydrolyse et de détoxification microbiologiques, il est recommandé d'optimiser les conditions opératoires des mono- et co-cultures. L'originalité dans ce travail se présente tout d'abord dans l'utilisation de *P. campinasensis* DSM 21989 pour la première fois aux meilleures de nos connaissances pour l'hydrolyse de préhydrolysat de bois généré par explosion à la vapeur couplée à l'eau chaude. De plus, le couplage d'hydrolyse biologique (i.e. *P. campinasensis*) et de détoxification biologique (i.e. co-culture de *C. taiwanensis* et *U. thermosphaericus*) et chimique (i.e. floculation) a été testée pour la première fois dans cette thèse.

Les contributions majeures de cette thèse sont résumées comme suit :

- Des milieux synthétiques composés de différents inhibiteurs (i.e. composés phénoliques, furfural, 5-HMF et sels), introduits à des concentrations définis pour simuler la composition des préhydrolysats de bois conventionnels, étaient utilisés comme source de carbone de *Ureibacillus thermosphaericus* et *Cupriavidus taiwanensis* en cultures pures et mixte. Cette étude était

effectuée pour la première fois avec les bactéries d'étude et permet de comprendre leurs voies de métabolisme dans un milieu contrôlé.

- La détoxification de l'hydrolysat de bois avec la nouvelle co-culture composée de *Ureibacillus thermosphaericu*s et *Cupriavidus taiwanensis* a été réalisée dans cette thèse.
- Le jumelage de la co-culture à la floculation était appliquée pour améliorer la détoxification de l'hydrolysat de bois.
- L'utilisation de l'hydrolysat détoxifié par la nouvelle stratégie développée comme source de carbone dans la fermentation ABE a permis de maximiser la production de butanol avec *C. acetobutylicum* ATCC 824 par rapport au milieu synthétique et à l'hydrolysat non détoxifié.
- L'hydrolyse de préhydrolysat de bois par la souche *P. campinasensis* DSM 21989 et sa détoxification par la co-culture bactérienne composée de *U. thermosphaericus* NCIMB 13819 et *C. taiwanensis* DSM 17343 réalisées simultanément ou séquentiellement étaient testées premièrement dans cette thèse.
- Une capacité de dégradation des composés phénoliques dans le préhydrolysat de bois avec la souche d'hydrolyse *P. campinasensis* DSM 21989 a été démontrée pour la première fois dans cette étude conférant ainsi à cette thèse un caractère innovant.

# CHAPITRE 8 : CONCLUSION ET RECOMMANDATIONS

## 8.1 Conclusions

L'objectif principal de cette thèse est de développer des technologies novatrices de détoxification à base de microorganismes pour une hydrolyse bactérienne performante de préhydrolysat hémicellulosique et pour une conversion subséquente efficace en butanol par la souche *Clostridium acetobutylicum* ATCC 824.

Dans cette étude nous avons proposé une nouvelle co-culture bactérienne composée de *U. thermosphaericus* et *C. taiwanensis* pour la réalisation du procédé de détoxification. Des milieux synthétiques des inhibiteurs (i.e. composés phénoliques, furfural, acide acétique et sels) ont été d'abord détoxifiés pour évaluer la performance de la co-culture proposée dans des milieux plus simples et de composition similaire que les préhydrolysats de bois. Ces expériences ont été conduites pour la première fois avec les bactéries *U. thermosphaericus* et *C. taiwanensis* dont les travaux de recherche antérieurs étaient limités à la détoxification d'une gamme plus restreinte des inhibiteurs. Les résultats de la co-culture étaient comparés à ceux obtenus avec les monocultures et une performance supérieure de dégradation des composés phénoliques a été observée dans le cas des cultures mixtes, avec 87% obtenus avec la séquence de *U. thermosphaericus* suivie de *C. taiwanensis*, en présence des concentrations initiales élevées des phénoliques (8 g/L). Le remplacement des milieux synthétiques par le préhydrolysat de bois comme source unique de carbone a donné de plus faibles résultats de dégradation des composés phénoliques (14% ($p \leq 0.05$) avec inoculation décalée de *C. taiwanensis* par rapport à *U. thermosphaericus*) par rapport aux résultats obtenus dans les milieux synthétiques. La floculation avec $Fe_2(SO_4)_3$ a été suggérée comme une deuxième méthode de détoxification dans le but d'améliorer les résultats d'élimination des inhibiteurs. Aux meilleures de nos connaissances, cette combinaison était étudiée pour la première fois dans cette thèse pour la détoxification des hydrolysats hémicellulosiques. Ce procédé de détoxification à deux étapes, avec la floculation intégrée en amont de la biodétoxification, a permis d'éliminer jusqu'à 63% des composés phénoliques, 73% de furfural et 28% de 5-HMF dans le préhydrolysat. La validation de la nouvelle technologie par la fermentation ABE de l'hydrolysat détoxifié et dilué jusqu'à 0.3 g/L des composés phénoliques a montré une production de 8 g/L de

butanol après 120 h de culture. Cette stratégie de détoxification a été également validée par hydrolyse microbiologique avec *P. campinasensis* DSM 21989. Dans cette section, les étapes de détoxification et d'hydrolyse étaient testées en série ou simultanément. Une dégradation maximale de 56% des composés phénoliques a été obtenue alors qu'une incapacité d'hydrolyse des glucides dans le préhydrolysat en xylose a été observée à travers les différentes stratégies dans ce travail. L'utilisation des différents hydrolysats générés comme sources de carbone dans la fermentation ABE a donné des concentrations maximales de butanol de 6.8 g/L et des solvants totaux de 11.8 g/L après 116 h de culture avec *C. acetobutylicum* ATCC 824.

## 8.2 Recommandations

- Investiguer les intermédiaires de dégradation des différents inhibiteurs testés pour établir leurs schémas de dégradation avec les différentes cultures de détoxification (*U. thermosphaericus*, *C. taiwanensis* et leurs co-cultures).
- Appliquer l'approche de Q-PCR pour le suivi temporel de la dynamique des souches de détoxification présentes en co-culture. Ces études vont permettre une meilleure interprétation des résultats de détoxification grâce à une quantification précise de l'ADN de chaque souche en consortium.
- Adapter les souches *U. thermosphaericus* et *C. taiwanensis* dans des précultures contenant des inhibiteurs avant leurs inoculations dans les milieux de détoxification dans le but d'augmenter leurs performances de dégradation.
- Tester d'autres types de floculant et optimiser les paramètres de la floculation pour améliorer les rendements de détoxification.
- Automatiser le système de filtration dans le procédé de floculation pour assurer une plus grande surface de filtration et pour éviter le colmatage des filtres par un système d'évacuation en continu.
- Évaluer une nouvelle approche de traitement de préhydrolysat de bois qui consiste tout d'abord en une unité d'hydrolyse par la souche *P. campinasensis* puis une unité de détoxification à deux étapes (i.e. floculation suivie de co-culture séquentielle de *U. thermosphaericus* et *C. taiwanensis*). Cette approche serait d'un grand intérêt puisqu'on a démontré dans l'Article 3 (Chapitre 6) une réduction des composés phénoliques de 4.5 g/L dans le préhydrolysat à 2.8 g/L

dans l'hydrolysat par traitement biologique unique avec *P. campinasensis*. Une concentration initiale en composés phénoliques de 2.8 g/L dans les milieux synthétiques (Article 1 (Chapitre 4)) a favorisé une performance de dégradation la plus élevée, 90%, en comparaison aux résultats obtenus en présence des concentrations initiales de 0.3 g/L et 8 g/L en phénoliques. Mais pour améliorer les rendements d'hydrolyse microbiologique, il est nécessaire de développer des méthodes d'optimisation des conditions de culture bactérienne (e.g. ajout des enzymes d'hydrolyse dans la culture de *P. campinasensis*, optimiser la température, le pH et la durée de culture, etc.).

- Recourir à une méthode mathématique de simulation (modélisation) du nouveau procédé de détoxification en se basant sur les résultats obtenus avec les classes de composés phénoliques, furfural et sels minéraux étudiés, et autres conditions de culture et pour les différentes stratégies adoptées. Ceci permettra notamment d'analyser tous les paramètres en jeu en même temps pour optimiser la détoxification des inhibiteurs et en visant un rendement maximal des produits cibles en fin de réaction.

# BIBLIOGRAPHIE


[1] B. E. Bogdanski, "The rise and fall of the Canadian pulp and paper sector," *The Forestry Chronicle,* vol. 90, no. 6, pp. 785-793, 2014.

[2] J.-T. Bernard, M. J. Hussain, and M. M. Sinha, *Survival of the Cleanest? Evidence from a Plant Level Analysis of Pollutant Emissions in Canadian Pulp and Paper Industry, 2005-2013*. University of Ottawa Department of Economics, 2016.

[3] H. Garza Villarreal, "Assessment and Enhancement of the Performance of the Pulp Washing Operation in Kraft Mills," École Polytechnique de Montréal, 2011.

[4] O. A. Ajao, "Development of Hemicelluloses Biorefineries for Integration Into Kraft Pulp Mills," École polytechnique de Montréal, 2014.

[5] A. Mazar, N. Jemaa, W. W. Al Dajani, M. Marinova, and M. Perrier, "Comparative study: Furfural production from two types of pre-hydrolysates produced using aspen and maple chips," *Biomass and Bioenergy,* vol. 111, pp. 103-113, 2018.

[6] C. Thirmal and Y. Dahman, "Comparisons of existing pretreatment, saccharification, and fermentation processes for butanol production from agricultural residues," *The Canadian Journal of Chemical Engineering,* vol. 90, no. 3, pp. 745-761, 2012.

[7] W. Guan, G. Xu, J. Duan, and S. Shi, "Acetone–Butanol–Ethanol Production from Fermentation of Hot-Water-Extracted Hemicellulose Hydrolysate of Pulping Woods," *Industrial & Engineering Chemistry Research,* vol. 57, no. 2, pp. 775-783, 2018.

[8] S. Larsson, A. Reimann, N.-O. Nilvebrant, and L. J. Jönsson, "Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce," *Applied biochemistry and biotechnology,* vol. 77, no. 1-3, pp. 91-103, 1999.

[9] H. Ran, J. Zhang, Q. Gao, Z. Lin, and J. Bao, "Analysis of biodegradation performance of furfural and 5-hydroxymethylfurfural by Amorphotheca resinae ZN1," *Biotechnology for biofuels,* vol. 7, no. 1, p. 51, 2014.

[10] D. Zheng, J. Bao, J. Lu, and C. Gao, "Isolation and characterization of a furfural-degrading bacterium Bacillus cereus sp. strain DS1," *Current microbiology,* vol. 70, no. 2, pp. 199-205, 2015.

[11] M. S. Kannangara, "Development and integration of acid precipitation based lignin biorefineries in Kraft pulping mills," École Polytechnique de Montréal, 2015.

[12] W. A. Laftah and W. A. Wan Abdul Rahman, "Pulping process and the potential of using non-wood pineapple leaves fiber for pulp and paper production: A review," *Journal of Natural Fibers,* vol. 13, no. 1, pp. 85-102, 2016.

[13] K. J. Kramer, E. Masanet, E. Worrell, and T. Xu, "Energy Efficiency Improvement and Cost Saving Opportunities for the Pulp and Paper Industry: An ENERGY STAR® Guide for Energy and Plant Managers," 2009.

[14] R. Deshpande, "The initial phase of sodium sulfite pulping of softwood: a comparison of different pulping options," Karlstads universitet, 2016.

[15] J. Hu, Q. Zhang, and D.-J. Lee, "Kraft lignin biorefinery: A perspective," *Bioresource technology,* vol. 247, pp. 1181-1183, 2018.

[16] G. Thompson, J. Swain, M. Kay, and C. Forster, "The treatment of pulp and paper mill effluent: a review," *Bioresource technology,* vol. 77, no. 3, pp. 275-286, 2001.

[17] M. Haddad, "Towards sustainable electrochemical acidification of kraft black liquor for lignin extraction: Proof of concept, control of membrane fouling and yield enhancement," Ecole Polytechnique, Montreal (Canada), 2016.

[18] W. Soetaert, "Defining biorefineries and different concepts," in *Presentation in BioreFuture 2009 conference. Brussels, Belgium*, 2009.

[19] C. Chen *et al.*, "Cellulose (dissolving pulp) manufacturing processes and properties: A mini-review," *BioResources,* vol. 11, no. 2, pp. 5553-5564, 2016.

[20] C. Sawatdeenarunat, K. Surendra, D. Takara, H. Oechsner, and S. K. Khanal, "Anaerobic digestion of lignocellulosic biomass: challenges and opportunities," *Bioresource technology,* vol. 178, pp. 178-186, 2015.

[21] B. C. Saha, "Hemicellulose bioconversion," *Journal of Industrial Microbiology and Biotechnology,* vol. 30, no. 5, pp. 279-291, 2003.

[22] S. K. Dutta and S. Chakraborty, "Kinetic analysis of two-phase enzymatic hydrolysis of hemicellulose of xylan type," *Bioresource technology,* vol. 198, pp. 642-650, 2015.

[23] A. Le Floch, M. Jourdes, and P.-L. Teissedre, "Polysaccharides and lignin from oak wood used in cooperage: Composition, interest, assays: A review," *Carbohydrate research,* vol. 417, pp. 94-102, 2015.

[24] P. Peng and D. She, "Isolation, structural characterization, and potential applications of hemicelluloses from bamboo: A review," *Carbohydrate polymers,* vol. 112, pp. 701-720, 2014.

[25] F. M. Yedro, H. Grénman, J. V. Rissanen, T. Salmi, J. Garcia-Serna, and M. J. Cocero, "Chemical composition and extraction kinetics of Holm oak (Quercus ilex) hemicelluloses using subcritical water," *The Journal of Supercritical Fluids,* vol. 129, pp. 56-62, 2017.

[26] X. Zhou, W. Li, R. Mabon, and L. J. Broadbelt, "A critical review on hemicellulose pyrolysis," *Energy Technology,* vol. 5, no. 1, pp. 52-79, 2017.

[27] A. Ebringerová, "Structural diversity and application potential of hemicelluloses," in *Macromolecular symposia*, 2005, vol. 232, no. 1, pp. 1-12: Wiley Online Library.

[28] A. Ebringerova and T. Heinze, "Xylan and xylan derivatives–biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties," *Macromolecular rapid communications,* vol. 21, no. 9, pp. 542-556, 2000.

[29] S. K. Khanal, W.-H. Chen, L. Li, and S. Sung, "Biological hydrogen production: effects of pH and intermediate products," *International journal of hydrogen energy,* vol. 29, no. 11, pp. 1123-1131, 2004.

[30] T. Covert, M. Greenstone, and C. R. Knittel, "Will we ever stop using fossil fuels?," *Journal of Economic Perspectives,* vol. 30, no. 1, pp. 117-38, 2016.

[31] H. Cai *et al.*, "Well-to-wheels greenhouse gas emissions of Canadian oil sands products: Implications for US petroleum fuels," *Environmental science & technology,* vol. 49, no. 13, pp. 8219-8227, 2015.

[32] J. Cuddihy, C. Kennedy, and P. Byer, "Energy use in Canada: environmental impacts and opportunities in relationship to infrastructure systems," *Canadian Journal of Civil Engineering,* vol. 32, no. 1, pp. 1-15, 2005.

[33] J. P. Maity, J. Bundschuh, C.-Y. Chen, and P. Bhattacharya, "Microalgae for third generation biofuel production, mitigation of greenhouse gas emissions and wastewater treatment: Present and future perspectives–A mini review," *Energy,* vol. 78, pp. 104-113, 2014.

[34] N. Gaurav, S. Sivasankari, G. Kiran, A. Ninawe, and J. Selvin, "Utilization of bioresources for sustainable biofuels: A Review," *Renewable and Sustainable Energy Reviews,* vol. 73, pp. 205-214, 2017.

[35] B. Ravindran, M. B. Kurade, A. N. Kabra, B.-H. Jeon, and S. K. Gupta, "Recent Advances and Future Prospects of Microalgal Lipid Biotechnology," in *Algal Biofuels*: Springer, 2017, pp. 1-37.

[36] M. Riazi and D. Chiaramonti, *Biofuels Production and Processing Technology*. CRC Press, 2017.

[37] S. N. Naik, V. V. Goud, P. K. Rout, and A. K. Dalai, "Production of first and second generation biofuels: a comprehensive review," *Renewable and sustainable energy reviews,* vol. 14, no. 2, pp. 578-597, 2010.

[38] M. F. Demirbas, "Biofuels from algae for sustainable development," *Applied energy,* vol. 88, no. 10, pp. 3473-3480, 2011.

[39] S. Singh, I. Chakravarty, K. D. Pandey, and S. Kundu, "Development of a process model for simultaneous saccharification and fermentation (SSF) of algal starch to third-generation bioethanol," *Biofuels,* pp. 1-9, 2018.

[40] S. Y. Lee, J. H. Park, S. H. Jang, L. K. Nielsen, J. Kim, and K. S. Jung, "Fermentative butanol production by Clostridia," *Biotechnology and bioengineering,* vol. 101, no. 2, pp. 209-228, 2008.

[41] E. M. Green, "Fermentative production of butanol—the industrial perspective," *Current opinion in biotechnology,* vol. 22, no. 3, pp. 337-343, 2011.

[42] N. Qureshi and T. C. Ezeji, "Butanol,'a superior biofuel'production from agricultural residues (renewable biomass): recent progress in technology," *Biofuels, Bioproducts and Biorefining: Innovation for a sustainable economy,* vol. 2, no. 4, pp. 319-330, 2008.

[43] K. R. Szulczyk, "Which is a better transportation fuel--butanol or ethanol?," *International Journal of Energy & Environment,* no. 3, 2010.

[44] Y. Dahman, "Sustainable Biobutanol and Working towards the Green Gasoline of the Future," *Ferment Technol,* vol. 1, p. e111, 2012.

[45] Y.-S. Jang, A. Malaviya, C. Cho, J. Lee, and S. Y. Lee, "Butanol production from renewable biomass by clostridia," *Bioresource technology,* vol. 123, pp. 653-663, 2012.

[46] M. Uyttebroek, W. Van Hecke, and K. Vanbroekhoven, "Sustainability metrics of 1-butanol," *Catalysis today,* vol. 239, pp. 7-10, 2015.

[47] B. Ndaba, I. Chiyanzu, and S. Marx, "n-Butanol derived from biochemical and chemical routes: a review," *Biotechnology Reports,* vol. 8, pp. 1-9, 2015.

[48] D. R. Nielsen and K. J. Prather, "In situ product recovery of n-butanol using polymeric resins," *Biotechnology and bioengineering,* vol. 102, no. 3, pp. 811-821, 2009.

[49] W.-L. Chang, "Acetone-butanol-ethanol fermentation by engineered Clostridium beijerinckii and Clostridium tyrobutyricum," The Ohio State University, 2010.

[50] K. Sivagnanam, "Shotgun proteomic analysis of Clostridium acetobutylicum during butanol fermentation," McGill University, 2012.

[51] V. Zverlov, O. Berezina, G. Velikodvorskaya, and W. Schwarz, "Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery," *Applied microbiology and biotechnology,* vol. 71, no. 5, pp. 587-597, 2006.

[52] M. Lyristis, Z. L. Boynton, D. Petersen, Z. Kan, G. N. Bennett, and F. B. Rudolph, "Cloning, sequencing, and characterization of the gene encoding flagellin, flaC, and the post-translational modification of flagellin, FlaC, from Clostridium acetobutylicum ATCC824," *Anaerobe,* vol. 6, no. 2, pp. 69-79, 2000.

[53] T. C. Ezeji and H. P. Blaschek, "Butanol production from lignocellulosic biomass," *Biofuels from Agricultural Wastes and Byproducts,* pp. 19-37, 2010.

[54] P. Harmsen, W. Huijgen, L. Bermudez, and R. Bakker, "Literature review of physical and chemical pretreatment processes for lignocellulosic biomass," Wageningen UR-Food & Biobased Research9085857570, 2010.

[55] L. J. Jönsson, B. Alriksson, and N.-O. Nilvebrant, "Bioconversion of lignocellulose: inhibitors and detoxification," *Biotechnology for biofuels,* vol. 6, no. 1, p. 16, 2013.

[56] P. Kumar, D. M. Barrett, M. J. Delwiche, and P. Stroeve, "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Industrial & engineering chemistry research,* vol. 48, no. 8, pp. 3713-3729, 2009.

[57] A. Converti, J. M. Domínguez, P. Perego, S. Da Silva, and M. Zilli, "Wood hydrolysis and hydrolyzate detoxification for subsequent xylitol production," *Chemical Engineering & Technology: Industrial Chemistry-Plant Equipment-Process Engineering-Biotechnology,* vol. 23, no. 11, pp. 1013-1020, 2000.

[58] Z. Anwar, M. Gulfraz, and M. Irshad, "Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review," *Journal of radiation research and applied sciences,* vol. 7, no. 2, pp. 163-173, 2014.

[59] E. C. Bensah and M. Mensah, "Chemical pretreatment methods for the production of cellulosic ethanol: technologies and innovations," *International Journal of Chemical Engineering,* vol. 2013, 2013.

[60] Y.-L. Loow, T. Y. Wu, J. M. Jahim, A. W. Mohammad, and W. H. Teoh, "Typical conversion of lignocellulosic biomass into reducing sugars using dilute acid hydrolysis and alkaline pretreatment," *Cellulose,* vol. 23, no. 3, pp. 1491-1520, 2016.

[61] J. S. Kim, Y. Lee, and T. H. Kim, "A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass," *Bioresource Technology,* vol. 199, pp. 42-48, 2016.

[62] Y. Kim *et al.*, "Enzyme hydrolysis and ethanol fermentation of liquid hot water and AFEX pretreated distillers' grains at high-solids loadings," *Bioresource Technology,* vol. 99, no. 12, pp. 5206-5215, 2008.

[63] D. P. Maurya, A. Singla, and S. Negi, "An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol," *3 Biotech,* vol. 5, no. 5, pp. 597-609, 2015.

[64] V. Balan, B. Bals, S. P. Chundawat, D. Marshall, and B. E. Dale, "Lignocellulosic biomass pretreatment using AFEX," in *Biofuels*: Springer, 2009, pp. 61-77.

[65] I. J. Bonner *et al.*, "Impact of ammonia fiber expansion (AFEX) pretreatment on energy consumption during drying, grinding, and pelletization of corn stover," *Drying technology,* vol. 34, no. 11, pp. 1319-1329, 2016.

[66] W. R. Kenealy, C. J. Houtman, J. Laplaza, T. W. Jeffries, and E. G. Horn, "Pretreatments for converting wood into paper and chemicals," in *Materials, chemicals, and energy from biomass energy. Washington, DC: American Chemical Society: Distributed by Oxford University Press, c2007. ACS symposium series; 954: ISBN: 0841239819: 9780841239814: pages 392-408.*, 2007.

[67] D. P. Singh and R. K. Trivedi, "Acid and alkaline pretreatment of lignocellulosic biomass to produce ethanol as biofuel," *International Journal of ChemTech Research,* vol. 5, no. 2, pp. 727-734, 2013.

[68] T. E. Amidon and S. Liu, "Water-based woody biorefinery," *Biotechnology advances,* vol. 27, no. 5, pp. 542-550, 2009.

[69] G. P. Maitan-Alfenas, E. M. Visser, and V. M. Guimarães, "Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products," *Current Opinion in Food Science,* vol. 1, pp. 44-49, 2015.

[70] R. Aguilar, J. Ramırez, G. Garrote, and M. Vázquez, "Kinetic study of the acid hydrolysis of sugar cane bagasse," *Journal of food engineering,* vol. 55, no. 4, pp. 309-318, 2002.

[71] Y.-S. Wang, X. Zhu, L.-Q. Jin, Y. Zheng, C.-J. Liao, and Y.-G. Zheng, "Post-hydrolysis of the prehydrolysate from eucalyptus pulping with xylanase," *Journal of cleaner production,* vol. 142, pp. 2865-2871, 2017.

[72] S. I. Mussatto and I. C. Roberto, "Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review," *Bioresource technology,* vol. 93, no. 1, pp. 1-10, 2004.

[73] Y. Wang, B. Yuan, Y. Ji, and H. Li, "Hydrolysis of hemicellulose to produce fermentable monosaccharides by plasma acid," *Carbohydrate polymers,* vol. 97, no. 2, pp. 518-522, 2013.

[74] L. Moreira, "Insights into the mechanism of enzymatic hydrolysis of xylan," *Applied microbiology and biotechnology,* vol. 100, no. 12, pp. 5205-5214, 2016.

[75] S. K. Dutta and S. Chakraborty, "Mixing effects on the kinetics and the dynamics of two-phase enzymatic hydrolysis of hemicellulose for biofuel production," *Bioresource technology,* vol. 259, pp. 276-285, 2018.

[76] A. Raj, S. Kumar, S. K. Singh, and J. Prakash, "Production and purification of xylanase from alkaliphilic Bacillus licheniformis and its pretreatment of eucalyptus kraft pulp," *Biocatalysis and Agricultural Biotechnology,* 2018.

[77] V. Juturu and J. C. Wu, "Microbial exo-xylanases: a mini review," *Applied biochemistry and biotechnology,* vol. 174, no. 1, pp. 81-92, 2014.

[78] J. Hu and J. N. Saddler, "Why does GH10 xylanase have better performance than GH11 xylanase for the deconstruction of pretreated biomass?," *Biomass and Bioenergy,* vol. 110, pp. 13-16, 2018.

[79] C. Niderhaus, M. Garrido, M. Insani, E. Campos, and S. Wirth, "Heterologous production and characterization of a thermostable GH10 family endo-xylanase from Pycnoporus sanguineus BAFC 2126," *Process Biochemistry,* vol. 67, pp. 92-98, 2018.

[80] V. Juturu, T. M. Teh, and J. C. Wu, "Expression of Aeromonas punctata ME-1 exo-Xylanase X in E. coli for Efficient Hydrolysis of Xylan to Xylose," *Applied biochemistry and biotechnology,* vol. 174, no. 8, pp. 2653-2662, 2014.

[81] H. K. Lim, K. I. Lee, and I. T. Hwang, "Identification of a New 1, 4-beta-D-xylosidase Pae1263 from the Whole Genome Sequence of Paenibacillus terrae HPL-003," *Biotechnology and Bioprocess Engineering,* vol. 23, no. 2, pp. 168-175, 2018.

[82] T. Xu, "Functional Genomics Studies of Clostridium Cellulolyticum for Lignocellulose Bioconversion," 2016.

[83] P. Bajpai, "Chapter 3—Microbial xylanolytic systems and their properties," *Xylanolytic Enzymes. Acad. Press, Amsterdam,* pp. 19-36, 2014.

[84] A. Kunamneni, F. J. Plou, M. Alcalde, and A. Ballesteros, "Trichoderma enzymes for food industries," in *Biotechnology and biology of Trichoderma*: Elsevier, 2014, pp. 339-344.

[85] F. J. Contesini *et al.,* "Structural and functional characterization of a highly secreted α-l-arabinofuranosidase (GH62) from Aspergillus nidulans grown on sugarcane bagasse," *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,* vol. 1865, no. 12, pp. 1758-1769, 2017.

[86] R. Ullrich, F. Moritz, V. D. Giap, M. Hofrichter, and C. Liers, "The ascomycete Xylaria polymorpha produces an acetyl esterase that solubilises beech wood material to release water-soluble lignin fragments," *Journal of the Korean Society for Applied Biological Chemistry,* vol. 58, no. 3, pp. 415-421, 2015.

[87] P. M. A. Pawar *et al.,* "Expression of fungal acetyl xylan esterase in Arabidopsis thaliana improves saccharification of stem lignocellulose," *Plant biotechnology journal,* vol. 14, no. 1, pp. 387-397, 2016.

[88] P. Biely *et al.,* "Trichoderma reesei CE16 acetyl esterase and its role in enzymatic degradation of acetylated hemicellulose," *Biochimica et Biophysica Acta (BBA)-General Subjects,* vol. 1840, no. 1, pp. 516-525, 2014.

[89] S. S. Paul, D. N. Kamra, and V. R. Sastry, "Fermentative characteristics and fibrolytic activities of anaerobic gut fungi isolated from wild and domestic ruminants," *Archives of animal nutrition,* vol. 64, no. 4, pp. 279-292, 2010.

[90] R. Sindhu, P. Binod, and A. Pandey, "Biological pretreatment of lignocellulosic biomass–An overview," *Bioresource technology,* vol. 199, pp. 76-82, 2016.

[91] S. Paul, D. Kamra, V. Sastry, N. Sahu, and A. Kumar, "Effect of phenolic monomers on biomass and hydrolytic enzyme activities of an anaerobic fungus isolated from wild nil gai (Baselophus tragocamelus)," *Letters in applied microbiology,* vol. 36, no. 6, pp. 377-381, 2003.

[92] T. Manavalan, R. Liu, Z. Zhou, and G. Zou, "Optimization of acetyl xylan esterase gene expression in Trichoderma reesei and its application to improve the saccharification efficiency on different biomasses," *Process Biochemistry,* vol. 58, pp. 160-166, 2017.

[93] T. Kogo *et al.*, "Production of rice straw hydrolysis enzymes by the fungi Trichoderma reesei and Humicola insolens using rice straw as a carbon source," *Bioresource technology,* vol. 233, pp. 67-73, 2017.

[94] F. Tang *et al.*, "Improving the thermostability of Trichoderma reesei xylanase 2 by introducing disulfide bonds," *Electronic Journal of Biotechnology,* vol. 26, pp. 52-59, 2017.

[95] J. He *et al.*, "Cost-effective lignocellulolytic enzyme production by Trichoderma reesei on a cane molasses medium," *Biotechnology for biofuels,* vol. 7, no. 1, p. 43, 2014.

[96] G. P. Borin *et al.*, "Comparative secretome analysis of Trichoderma reesei and Aspergillus niger during growth on sugarcane biomass," *PLoS One,* vol. 10, no. 6, p. e0129275, 2015.

[97] M. Häkkinen, D. Sivasiddarthan, N. Aro, M. Saloheimo, and T. M. Pakula, "The effects of extracellular pH and of the transcriptional regulator PACI on the transcriptome of Trichoderma reesei," *Microbial cell factories,* vol. 14, no. 1, p. 63, 2015.

[98] H. Hirasawa *et al.*, "Engineering of the Trichoderma reesei xylanase3 promoter for efficient enzyme expression," *Applied microbiology and biotechnology,* vol. 102, no. 6, pp. 2737-2752, 2018.

[99] T. Matsuzawa, S. Kaneko, and K. Yaoi, "Improvement of thermostability and activity of Trichoderma reesei endo-xylanase Xyn III on insoluble substrates," *Applied microbiology and biotechnology,* vol. 100, no. 18, pp. 8043-8051, 2016.

[100] H. Nakazawa *et al.*, "A high performance Trichoderma reesei strain that reveals the importance of xylanase III in cellulosic biomass conversion," *Enzyme and microbial technology,* vol. 82, pp. 89-95, 2016.

[101] S. Kang, Y. Park, J. Lee, S. Hong, and S. Kim, "Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass," *Bioresource technology,* vol. 91, no. 2, pp. 153-156, 2004.

[102] C.-H. Ko *et al.*, "Molecular cloning and characterization of a novel thermostable xylanase from Paenibacillus campinasensis BL11," *Process Biochemistry,* vol. 45, no. 10, pp. 1638-1644, 2010.

[103] J.-H. Yoon *et al.*, "Paenibacillus campinasensis sp. nov., a cyclodextrin-producing bacterium isolated in Brazil," *International Journal of Systematic and Evolutionary Microbiology*, vol. 48, no. 3, pp. 833-837, 1998.

[104] C.-H. Ko, W.-L. Chen, C.-H. Tsai, W.-N. Jane, C.-C. Liu, and J. Tu, "Paenibacillus campinasensis BL11: a wood material-utilizing bacterial strain isolated from black liquor," *Bioresource technology,* vol. 98, no. 14, pp. 2727-2733, 2007.

[105] C.-H. Ko *et al.*, "Xylanase production by Paenibacillus campinasensis BL11 and its pretreatment of hardwood kraft pulp bleaching," *International Biodeterioration & Biodegradation,* vol. 64, no. 1, pp. 13-19, 2010.

[106] M. H. M. Zainudin, M. A. Hassan, M. Tokura, and Y. Shirai, "Indigenous cellulolytic and hemicellulolytic bacteria enhanced rapid co-composting of lignocellulose oil palm empty fruit bunch with palm oil mill effluent anaerobic sludge," *Bioresource technology,* vol. 147, pp. 632-635, 2013.

[107] T. Ezeji, N. Qureshi, and H. P. Blaschek, "Butanol production from agricultural residues: impact of degradation products on Clostridium beijerinckii growth and butanol fermentation," *Biotechnology and bioengineering,* vol. 97, no. 6, pp. 1460-1469, 2007.

[108] N. R. Baral and A. Shah, "Microbial inhibitors: formation and effects on acetone-butanol-ethanol fermentation of lignocellulosic biomass," *Applied microbiology and biotechnology,* vol. 98, no. 22, pp. 9151-9172, 2014.

[109] Y. Zhang, C. Xia, M. Lu, and M. Tu, "Effect of overliming and activated carbon detoxification on inhibitors removal and butanol fermentation of poplar prehydrolysates," *Biotechnology for Biofuels,* vol. 11, no. 1, p. 178, 2018.

[110] R. Zhai, "Can we enhance cellulose hydrolysis by minimizing enzyme inhibition resulting from pretreatment-derived inhibitors?," University of British Columbia, 2017.

[111] M. Michelin, E. Ximenes, M. d. L. T. de Moraes, and M. R. Ladisch, "Effect of phenolic compounds from pretreated sugarcane bagasse on cellulolytic and hemicellulolytic activities," *Bioresource technology,* vol. 199, pp. 275-278, 2016.

[112] R. Zhai, J. Hu, and J. N. Saddler, "Extent of Enzyme Inhibition by Phenolics Derived from Pretreated Biomass Is Significantly Influenced by the Size and Carbonyl Group Content of the Phenolics," *ACS Sustainable Chemistry & Engineering,* vol. 6, no. 3, pp. 3823-3829, 2018.

[113] E. González-Bautista *et al.*, "Phenolic compounds inhibit cellulase and xylanase activities of Cellulomonas flavigena PR-22 during saccharification of sugarcane bagasse," *Fuel,* vol. 196, pp. 32-35, 2017.

[114] K. M. Lee *et al.*, "Electrochemical detoxification of phenolic compounds in lignocellulosic hydrolysate for Clostridium fermentation," *Bioresource technology,* vol. 187, pp. 228-234, 2015.

[115] O. Ibraheem and B. K. Ndimba, "Molecular adaptation mechanisms employed by ethanologenic bacteria in response to lignocellulose-derived inhibitory compounds," *International journal of biological sciences,* vol. 9, no. 6, p. 598, 2013.

[116] L. J. Jönsson and C. Martín, "Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects," *Bioresource technology,* vol. 199, pp. 103-112, 2016.

[117] T. Y. Mills, N. R. Sandoval, and R. T. Gill, "Cellulosic hydrolysate toxicity and tolerance mechanisms in Escherichia coli," *Biotechnology for biofuels,* vol. 2, no. 1, p. 26, 2009.

[118] J. Liu, Z. Liu, X. Chai, Y. Luo, T. Guo, and H. Ying, "Regulation of ρ-coumaric acid tolerance in Clostridium beijerinckii by disturbing the intracellular electron transport chain," *Process Biochemistry,* vol. 68, pp. 43-52, 2018.

[119] M. H. Thomsen, A. Thygesen, and A. B. Thomsen, "Identification and characterization of fermentation inhibitors formed during hydrothermal treatment and following SSF of wheat straw," *Applied Microbiology and Biotechnology,* vol. 83, no. 3, p. 447, 2009.

[120] G. Qi *et al.*, "CaCO 3 supplementation alleviates the inhibition of formic acid on acetone/butanol/ethanol fermentation by Clostridium acetobutylicum," *Biotechnology letters,* vol. 39, no. 1, pp. 97-104, 2017.

[121] H. B. Klinke, A. Thomsen, and B. K. Ahring, "Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass," *Applied microbiology and biotechnology,* vol. 66, no. 1, pp. 10-26, 2004.

[122] H. Zheng, X. Liu, Y. Han, J. Wang, and F. Lu, "Overexpression of a Paenibacillus campinasensis xylanase in Bacillus megaterium and its applications to biobleaching of cotton stalk pulp and saccharification of recycled paper sludge," *Bioresource technology,* vol. 125, pp. 182-187, 2012.

[123] J. de Cassia Pereira *et al.*, "Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities," in *Enzyme Inhibitors and Activators*: InTech, 2017.

[124] X. Zhao, S. Condruz, J. Chen, and M. Jolicoeur, "A quantitative metabolomics study of high sodium response in Clostridium acetobutylicum ATCC 824 acetone-butanol-ethanol (ABE) fermentation," *Scientific reports,* vol. 6, p. 28307, 2016.

[125] N. Qureshi, A. Lolas, and H. Blaschek, "Soy molasses as fermentation substrate for production of butanol using Clostridium beijerinckii BA101," *Journal of industrial Microbiology and Biotechnology,* vol. 26, no. 5, pp. 290-295, 2001.

[126] Y. He, J. Zhang, and J. Bao, "Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation," *Biotechnology for biofuels,* vol. 9, no. 1, p. 19, 2016.

[127] L. T. P. Trinh, C. Kundu, J.-W. Lee, and H.-J. Lee, "An integrated detoxification process with electrodialysis and adsorption from the hemicellulose hydrolysates of yellow poplars," *Bioresource technology,* vol. 161, pp. 280-287, 2014.

[128] Z. Sun and S. Liu, "Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by Clostridia acetobutylicum ATCC824," *Biomass and bioenergy,* vol. 39, pp. 39-47, 2012.

[129] N. Okuda, M. Soneura, K. Ninomiya, Y. Katakura, and S. Shioya, "Biological detoxification of waste house wood hydrolysate using Ureibacillus thermosphaericus for

bioethanol production," *Journal of bioscience and bioengineering,* vol. 106, no. 2, pp. 128-133, 2008.

[130] A. Martinez, M. E. Rodriguez, M. L. Wells, S. W. York, J. F. Preston, and L. O. Ingram, "Detoxification of dilute acid hydrolysates of lignocellulose with lime," *Biotechnology progress,* vol. 17, no. 2, pp. 287-293, 2001.

[131] E. W. Jennings and D. J. Schell, "Conditioning of dilute-acid pretreated corn stover hydrolysate liquors by treatment with lime or ammonium hydroxide to improve conversion of sugars to ethanol," *Bioresource technology,* vol. 102, no. 2, pp. 1240-1245, 2011.

[132] Y.-J. Kim, K. Shibata, H. Uyama, and S. Kobayashi, "Synthesis of ultrahigh molecular weight phenolic polymers by enzymatic polymerization in the presence of amphiphilic triblock copolymer in water," *Polymer,* vol. 49, no. 22, pp. 4791-4795, 2008.

[133] O. Ajao, M. Le Hir, M. Rahni, M. Marinova, H. Chadjaa, and O. Savadogo, "Concentration and detoxification of Kraft prehydrolysate by combining nanofiltration with flocculation," *Industrial & Engineering Chemistry Research,* vol. 54, no. 3, pp. 1113-1122, 2015.

[134] F. Deng and G. M. Aita, "Detoxification of dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate by soluble polyelectrolyte flocculants," *Industrial Crops and Products,* vol. 112, pp. 681-690, 2018.

[135] F. Mechmech, H. Chadjaa, M. Rahni, M. Marinova, N. B. Akacha, and M. Gargouri, "Improvement of butanol production from a hardwood hemicelluloses hydrolysate by combined sugar concentration and phenols removal," *Bioresource technology,* vol. 192, pp. 287-295, 2015.

[136] H. Shi, P. Fatehi, H. Xiao, and Y. Ni, "A combined acidification/PEO flocculation process to improve the lignin removal from the pre-hydrolysis liquor of kraft-based dissolving pulp production process," *Bioresource technology,* vol. 102, no. 8, pp. 5177-5182, 2011.

[137] N. Nguyen, C. Fargues, W. Guiga, and M.-L. Lameloise, "Assessing nanofiltration and reverse osmosis for the detoxification of lignocellulosic hydrolysates," *Journal of Membrane Science,* vol. 487, pp. 40-50, 2015.

[138] M. Malmali, J. J. Stickel, and S. R. Wickramasinghe, "Sugar concentration and detoxification of clarified biomass hydrolysate by nanofiltration," *Separation and Purification Technology,* vol. 132, pp. 655-665, 2014.

[139] B. Qi, J. Luo, X. Chen, X. Hang, and Y. Wan, "Separation of furfural from monosaccharides by nanofiltration," *Bioresource technology,* vol. 102, no. 14, pp. 7111-7118, 2011.

[140] E. Koivula, M. Kallioinen, S. Preis, L. Testova, H. Sixta, and M. Mänttäri, "Evaluation of various pretreatment methods to manage fouling in ultrafiltration of wood hydrolysates," *Separation and purification technology,* vol. 83, pp. 50-56, 2011.

[141] M. G. Rajulu, L. B. Lai, T. Murali, V. Gopalan, and T. Suryanarayanan, "Several fungi from fire-prone forests of southern India can utilize furaldehydes," *Mycological progress,* vol. 13, no. 4, p. 992, 2014.

[142] C. V. Agu, "Bioabatement to remove microbial inhibitors from Miscanthus giganteus hydrolysates for enhanced butanol fermentation," 2015.

[143] J. Jung and L. Wicker, "Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability," *Food Hydrocolloids,* vol. 28, no. 1, pp. 168-173, 2012.

[144] H. Chen, A. Ji, S. Qiu, Y. Liu, Q. Zhu, and L. Yin, "Covalent conjugation of bovine serum album and sugar beet pectin through Maillard reaction/laccase catalysis to improve the emulsifying properties," *Food Hydrocolloids,* vol. 76, pp. 173-183, 2018.

[145] Z. Abang, N. Dayang, and A. Meyer, "Oxidative enzymatic gelation of sugar beet pectin for emulsion stabilization," *Res. J. BioTechnol,* vol. 8, pp. 81-86, 2013.

[146] Q. Wang, M. S. Jahan, S. Liu, Q. Miao, and Y. Ni, "Lignin removal enhancement from prehydrolysis liquor of kraft-based dissolving pulp production by laccase-induced polymerization," *Bioresource technology,* vol. 164, pp. 380-385, 2014.

[147] E. Jahangiri, B. Seiwert, T. Reemtsma, and D. Schlosser, "Laccase-and electrochemically mediated conversion of triclosan: Metabolite formation and influence on antibacterial activity," *Chemosphere,* vol. 168, pp. 549-558, 2017.

[148] A. D. Moreno, D. Ibarra, P. Alvira, E. Tomás-Pejó, and M. Ballesteros, "A review of biological delignification and detoxification methods for lignocellulosic bioethanol production," *Critical reviews in biotechnology,* vol. 35, no. 3, pp. 342-354, 2015.

[149] G. Guerriero, J. F. Hausman, J. Strauss, H. Ertan, and K. S. Siddiqui, "Lignocellulosic biomass: biosynthesis, degradation, and industrial utilization," *Engineering in Life Sciences,* vol. 16, no. 1, pp. 1-16, 2016.

[150] W.-M. Chen *et al.*, "Ralstonia taiwanensis sp. nov., isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient," *International Journal of Systematic and Evolutionary Microbiology,* vol. 51, no. 5, pp. 1729-1735, 2001.

[151] B.-Y. Chen, J.-W. You, Y.-T. Hsieh, and J.-S. Chang, "Feasibility study of exponential feeding strategy in fed-batch cultures for phenol degradation using Cupriavidus taiwanensis," *Biochemical Engineering Journal,* vol. 41, no. 2, pp. 175-180, 2008.

[152] W.-M. Chen, C.-H. Wu, E. K. James, and J.-S. Chang, "Metal biosorption capability of Cupriavidus taiwanensis and its effects on heavy metal removal by nodulated Mimosa pudica," *Journal of Hazardous Materials,* vol. 151, no. 2-3, pp. 364-371, 2008.

[153] B.-Y. Chen, W.-M. Chen, and J.-S. Chang, "Optimal biostimulation strategy for phenol degradation with indigenous rhizobium Ralstonia taiwanensis," *Journal of hazardous materials,* vol. 139, no. 2, pp. 232-237, 2007.

[154] B.-Y. Chen, C.-Y. Lin, and S.-Y. Hsu, "Toxicity assessment upon augmented biostimulation source to indigenous rhizobium Cupriavidus taiwanensis," *Journal of hazardous materials,* vol. 163, no. 1, pp. 143-151, 2009.

[155] D. Kalyani, P. Patil, J. Jadhav, and S. Govindwar, "Biodegradation of reactive textile dye Red BLI by an isolated bacterium Pseudomonas sp. SUK1," *Bioresource Technology,* vol. 99, no. 11, pp. 4635-4641, 2008.

[156] A. P. Wulandari, T. Triyana, and P. Andayaningsih, "Delignification of rice straw with ligninase from novel Penicillium sp. strain apw-tt2 for biopulping," *International Journal of Bioscience, Biochemistry and Bioinformatics,* vol. 3, no. 1, p. 43, 2013.

[157] J. Wu, Y.-Z. Xiao, and H.-Q. Yu, "Degradation of lignin in pulp mill wastewaters by white-rot fungi on biofilm," *Bioresource Technology,* vol. 96, no. 12, pp. 1357-1363, 2005.

[158] A. F. Billings *et al.*, "Genome sequence and description of the anaerobic lignin-degrading bacterium Tolumonas lignolytica sp. nov," *Standards in genomic sciences,* vol. 10, no. 1, p. 106, 2015.

[159] S. Abbasalizadeh *et al.*, "Draft genome sequence of Ureibacillus thermosphaericus strain thermo-BF, isolated from Ramsar hot springs in Iran," *Journal of bacteriology,* vol. 194, no. 16, pp. 4431-4431, 2012.

[160] C. Asada, C. Sasaki, T. Takamatsu, and Y. Nakamura, "Conversion of steam-exploded cedar into ethanol using simultaneous saccharification, fermentation and detoxification process," *Bioresource technology,* vol. 176, pp. 203-209, 2015.

[161] H. Shi, P. Fatehi, H. Xiao, and Y. Ni, "A process for isolating lignin of pre-hydrolysis liquor of kraft pulping process based on surfactant and calcium oxide treatments," *Biochemical engineering journal,* vol. 68, pp. 19-24, 2012.

[162] M. G. Fortina *et al.*, "Ureibacillus gen. nov., a new genus to accommodate Bacillus thermosphaericus (Andersson et al. 1995), emendation of Ureibacillus thermosphaericus and description of Ureibacillus terrenus sp. nov," *International journal of systematic and evolutionary microbiology,* vol. 51, no. 2, pp. 447-455, 2001.

[163] A. Gagné, M. Chicoine, A. Morin, and A. Houde, "Phenotypic and genotypic characterization of esterase-producing Ureibacillus thermosphaericus isolated from an aerobic digestor of swine waste," *Canadian journal of microbiology,* vol. 47, no. 10, pp. 908-915, 2001.

[164] B.-Y. Kim *et al.*, "Ureibacillus suwonensis sp. nov., isolated from cotton waste composts," *International journal of systematic and evolutionary microbiology,* vol. 56, no. 3, pp. 663-666, 2006.

[165] K. Glendinning, L. Macaskie, and N. Brown, "Mercury tolerance of thermophilic Bacillus sp. and Ureibacillus sp," *Biotechnology letters,* vol. 27, no. 21, pp. 1657-1662, 2005.

[166] X. Jia, X. Lin, Y. Tian, J. Chen, and M. You, "High production, purification, biochemical characterization and gene analysis of a novel catalase from the thermophilic bacterium Ureibacillus thermosphaericus FZSF03," *International journal of biological macromolecules,* vol. 103, pp. 89-98, 2017.

[167] P. Wei, L.-H. Cheng, L. Zhang, X.-H. Xu, H.-l. Chen, and C.-j. Gao, "A review of membrane technology for bioethanol production," *Renewable and Sustainable Energy Reviews,* vol. 30, pp. 388-400, 2014.

[168] M. del Carmen Vargas-García, F. F. Suárez-Estrella, M. J. López, and J. Moreno, "Influence of microbial inoculation and co-composting material on the evolution of humic-like substances during composting of horticultural wastes," *Process Biochemistry,* vol. 41, no. 6, pp. 1438-1443, 2006.

[169] M. Vargas-Garcı, F. Suárez-Estrella, M. López, and J. Moreno, "Effect of inoculation in composting processes: modifications in lignocellulosic fraction," *Waste Management,* vol. 27, no. 9, pp. 1099-1107, 2007.

[170] M. M. Juibari, S. Abbasalizadeh, G. S. Jouzani, and M. Noruzi, "Intensified biosynthesis of silver nanoparticles using a native extremophilic Ureibacillus thermosphaericus strain," *Materials Letters,* vol. 65, no. 6, pp. 1014-1017, 2011.

[171] H. Akita, Y. Fujino, K. Doi, and T. Ohshima, "Highly stable meso-diaminopimelate dehydrogenase from an Ureibacillus thermosphaericus strain A1 isolated from a Japanese compost: purification, characterization and sequencing," *AMB express,* vol. 1, no. 1, p. 43, 2011.

[172] J. Shen, I. Kaur, M. M. Baktash, Z. He, and Y. Ni, "A combined process of activated carbon adsorption, ion exchange resin treatment and membrane concentration for recovery of dissolved organics in pre-hydrolysis liquor of the kraft-based dissolving pulp production process," *Bioresource technology,* vol. 127, pp. 59-65, 2013.

[173] D. B. Hodge, C. Andersson, K. A. Berglund, U. J. E. Rova, and M. Technology, "Detoxification requirements for bioconversion of softwood dilute acid hydrolyzates to succinic acid," vol. 44, no. 5, pp. 309-316, 2009.

[174] J. M. Lee, R. A. Venditti, H. Jameel, W. R. J. B. Kenealy, and bioenergy, "Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium Thermoanaerobacterium saccharolyticum," vol. 35, no. 1, pp. 626-636, 2011.

[175] B. Alriksson, A. Sjöde, N.-O. Nilvebrant, and L. J. Jönsson, "Optimal conditions for alkaline detoxification of dilute-acid lignocellulose hydrolysates," *Applied Biochemistry and Biotechnology,* vol. 130, no. 1-3, pp. 599-611, 2006.

[176] B. Alriksson, I. S. Horváth, A. Sjöde, N.-O. Nilvebrant, and L. J. Jönsson, "Ammonium hydroxide detoxification of spruce acid hydrolysates," in *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*, 2005, pp. 911-922: Springer.

[177] U. Youngsoon, K. Yunje, S. Dongjin, K. M. Lee, and J. M. Ha, "Electrochemical detoxification method of wood-based hydrolysate for producing biochemicals or biofuels," ed: Google Patents, 2017.

[178] M. Jurado, A. Prieto, Á. Martínez-Alcalá, Á. T. Martínez, and M. J. Martínez, "Laccase detoxification of steam-exploded wheat straw for second generation bioethanol," *Bioresource Technology,* vol. 100, no. 24, pp. 6378-6384, 2009.

[179] Z. Fang *et al.*, "Identification of a laccase Glac15 from Ganoderma lucidum 77002 and its application in bioethanol production," *Biotechnology for biofuels,* vol. 8, no. 1, p. 54, 2015.

[180] J. Rocha-Martín, C. Martínez-Bernal, L. S. Zamorano, F. M. Reyes-Sosa, and B. D. García, "Inhibition of enzymatic hydrolysis of pretreated corn stover and sugar cane straw by laccases," *Process Biochemistry,* vol. 67, pp. 88-91, 2018.

[181] K. Rahmani *et al.*, "Elimination and detoxification of sulfathiazole and sulfamethoxazole assisted by laccase immobilized on porous silica beads," *International Biodeterioration & Biodegradation,* vol. 97, pp. 107-114, 2015.

[182] R. Allard-Massicotte, H. Chadjaa, and M. Marinova, "Phenols Removal from Hemicelluloses Pre-Hydrolysate by Laccase to Improve Butanol Production," *Fermentation,* vol. 3, no. 3, p. 31, 2017.

[183] M. Garcia-Torreiro *et al.*, "Simultaneous valorization and detoxification of the hemicellulose rich liquor from the organosolv fractionation," *International Biodeterioration & Biodegradation*, vol. 126, pp. 112-118, 2018.

[184] J. Schroeter, A. Ruggeri, H. Thieme, and C. Meltendorf, "Impact of temporary hyperthermia on corneal endothelial cell survival during organ culture preservation," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 253, no. 5, pp. 753-758, 2015.