FÉDÉRATION WALLONIE-BRUXELLES

ACADÉMIE UNIVERSITAIRE WALLONIE-EUROPE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

ÉTUDE DU COMPLEXE ENZYMATIQUE DES SYMBIONTES DU TERMITE *RETICULITERMES FLAVIPES* (EX. *SANTONENSIS*)

Cédric TARAYRE

Dissertation originale présentée en vue de l'obtention du grade de Docteur en Sciences Agronomiques et Ingénierie Biologique

Promoteur : Prof. Philippe THONART

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RÉSUMÉ

Tarayre, Cédric (2014) - Étude du complexe enzymatique des symbiontes du termite *Reticulitermes flavipes* (ex. *santonensis*). Thèse de doctorat. Université de Liège – Gembloux Agro-Bio Tech, Belgique.

Au vu du contexte économique, environnemental et social dans lequel nous nous trouvons actuellement, il est devenu indéniable que l'usage des énergies fossiles n'est pas concevable à long terme. Des alternatives ont donc émergé ces dernières années. Les biocarburants de seconde génération constituent une de ces alternatives et sont basés sur l'exploitation de biomasse végétale, dite lignocellulosique. Cette matière, pour être utilisable, requiert une étape d'hydrolyse réalisable notamment par l'utilisation d'enzymes.

Certains insectes, tels que les termites, abritent au sein de leur système digestif des communautés microbiennes complexes. Celles-ci sont capables de produire des enzymes utilisables dans le domaine de l'hydrolyse de la biomasse végétale. Le présent travail se situe dans cette optique.

La majeure partie du travail accompli s'est focalisée sur la recherche de microorganismes (bactéries, mycètes et protistes) producteurs d'enzymes responsables de la dégradation des hémicelluloses et de la cellulose, dont l'hydrolyse libère des sucres fermentescibles. L'insecte utilisé dans le cadre de ce travail est *Reticulitermes flavipes* (ex. *santonensis*), termite inférieur, possédant une microflore intrinsèque très diversifiée. Cette thèse décrit la caractérisation des souches microbiennes isolées ainsi que des enzymes qu'elles sécrètent.

Une partie complémentaire à cette recherche s'est concentrée sur des termites élevés sur diètes artificielles. L'objectif de cette partie était multiple : isoler des souches microbiennes productrices d'enzymes, non extraites selon la méthode conventionnelle, et caractériser les microflores induites par les diètes artificielles appliquées. Cette analyse pluridisciplinaire s'est basée sur la microscopie, la protéomique, la métagénomique et la caractérisation du métabolisme appliquées aux différents consortia microbiens.

SUMMARY

Tarayre, Cédric (2014) – Study of the enzyme complex of the symbionts of the termite *Reticulitermes flavipes* (ex. *santonensis*). Doctoral thesis. University of Liège – Gembloux Agro-Bio Tech, Belgium.

In the light of the economic, environmental and social context in which we live today, it has become obvious that the use of fossil fuels is not conceivable over the long term. Some alternatives have therefore emerged in recent years. Second-generation biofuels are one of those alternatives and are based on the exploitation of vegetal biomass, also called lignocellulosic biomass. These materials require a hydrolysis step which can notably be achieved by enzymes.

Some insects, such as termites, harbor complex microbial communities inside their digestive tracts. Those communities are able to produce enzymes which can be used in the field of the hydrolysis of vegetal biomass. This is what this thesis deals with.

The main part of the work done focused on the research of enzyme-producing microorganisms (bacteria, mycetes and protists) responsible for the degradation of hemicelluloses and cellulose, the hydrolysis of which releases fermentable sugars. The insect which was used in this work was *Reticulitermes flavipes* (ex. *santonensis*), a lower termite, harboring a highly diversified internal microflora. This thesis describes the characterization of the microbial strains which were isolated and the enzymes they secrete.

A complementary part of this research focussed on termites grown on artificial diets. The objective of this part was multiple : isolating enzyme-producing strains, not extractable according to the standard technique, and characterizing the microflora resulting from the applied artificial diets. This multidisciplinary approach was based on microscopy, proteomics, metagenomics and the assessment of metabolism applied to the different microbial consortia.

REMERCIEMENTS

Tous les doctorants et docteurs le savent : la réalisation d'une thèse de doctorat est loin d'être une tâche facile. Il n'est pas rare d'avoir besoin d'aide lorsqu'on réalise un tel travail, qu'il s'agisse d'une assistance scientifique ou morale. On pourrait résumer le contexte de la thèse de doctorat en une phrase : « Achève ta thèse avant qu'elle ne parvienne à t'achever ».

A travers cette section, je tiens à mettre en avant les différentes personnes sans qui l'accomplissement de ce travail aurait été bien plus difficile.

Tout d'abord, je tiens à remercier M. le professeur Philippe Thonart, promoteur de cette thèse de doctorat et chef de service des Bio-Industries, pour m'avoir accueilli au sein de son unité en tant que doctorant. Je le remercie également de m'avoir si bien soutenu en me fournissant le temps et les moyens nécessaires à l'accomplissement de cette thèse.

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J'adresse un sincère remerciement à mes collègues de projet Julien Bauwens, Catherine Brasseur, Christel Mattéotti et Catherine Millet pour m'avoir permis de mettre en place avec eux un partenariat à la fois efficace et agréable. Nous avons pu, sur base de cette collaboration, aboutir à un travail de qualité, autant sur les plans professionnel que relationnel.

Je tiens à exprimer toute ma gratitude à mes collègues de l'Unité de Bio-Industries pour la précieuse aide qu'ils m'ont apportée à de multiples occasions, ainsi que pour la bonne humeur dont ils ont fait preuve durant toute la durée d'accomplissement de ce travail.

Sur un plan plus personnel, je voudrais remercier ma sœur Caroline ainsi que mon père pour avoir relu et corrigé les parties de mon travail rédigées en anglais. La rédaction d'un

article de résultats n’est en soi pas une chose facile, il l’est encore moins de le faire accepter lorsqu’on n’est pas un anglophone natif. Je tenais donc à mettre en avant leurs compétences à ce niveau.

LISTE DES PUBLICATIONS

Publications acceptées en tant que premier auteur

Cédric Tarayre, Alison Brognaux, Catherine Brasseur, Julien Bauwens, Catherine Millet, Christel Mattéotti, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart (2013) *Isolation and Cultivation of a Xylanolytic Bacillus subtilis Extracted from the Gut of the Termite Reticulitermes santonensis*. **Applied Biochemistry and Biotechnology** 171 (1): 225-245.

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Publications acceptées en tant que co-auteur

Christel Mattéotti, Julien Bauwens, Catherine Brasseur, Cédric Tarayre, Philippe Thonart, Jacqueline Destain, Frédéric Francis, Éric Haubruge, Edwin De Pauw, Daniel Portetelle, Micheline Vandenbol (2012) *Identification and characterization of a new xylanase from Gram-positive bacteria isolated from termite gut (Reticulitermes santonensis)*. **Protein Expression and Purification** 83 (2): 117-127.

Julien Bauwens, Cédric Tarayre, Catherine Brasseur, Christel Mattéotti, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Philippe Thonart, Edwin De Pauw, Éric Haubruge, Frédéric Francis (2012) *Influence of lignin in Reticulitermes santonensis: symbiotic interactions investigated through proteomics*. **Symbiosis** 58 (1-3): 119-125.

Julien Bauwens, Catherine Millet, Cédric Tarayre, Catherine Brasseur, Jacqueline Destain, Micheline Vandenbol, Philippe Thonart, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis (2013) *Symbiont Diversity in Reticulitermes santonensis (Isoptera: Rhinotermitidae): Investigation Strategy Through Proteomics*. **Environmental Entomology** 42 (5): 882-887.

Catherine Brasseur, Julien Bauwens, Cédric Tarayre, Christel Mattéotti, Philippe Thonart, Jacqueline Destain, Frédéric Francis, Éric Haubruge, Daniel Portetelle, Micheline Vandenbol, Jean-François Focant, Edwin De Pauw (2014) *MALDI-TOF MS analysis of cellodextrins and xylo-oligosaccharides produced by hindgut homogenates of Reticulitermes santonensis*. **Molecules** 19 (4): 4578-4594.

Publications actuellement soumises en tant que premier auteur ou co-premier auteur

Julien Bauwens*, Cédric Tarayre*, Frédéric Francis, Éric Haubruge, Jacqueline Destain, Philippe Thonart, Edwin De Pauw, Micheline Vandenbol, Daniel Portetelle (2014) *Review – The termite gut as a source of new enzymes: an approach by microbiological and "omics" techniques*. Soumis à la revue **World Journal of Microbiology and Biotechnology**.

* Premiers co-auteurs

Cédric Tarayre, Julien Bauwens, Catherine Brasseur, Christel Mattéotti, Catherine Millet, Pierre Alexandre Guiot, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart (2014) *Isolation and cultivation of xylanolytic and cellulolytic Sarocladium kiliense and Trichoderma virens from the gut of the termite Reticulitermes santonensis*. Soumis à la revue **Environmental Science and Pollution Research**.

Cédric Tarayre*, Julien Bauwens*, Christel Mattéotti, Catherine Brasseur, Catherine Millet, Jacqueline Destain, Micheline Vandenbol, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart, Daniel Portetelle (2014) *Multiple analyses of microbial communities applied to the gut of the wood-feeding termite Reticulitermes flavipes fed on artificial diets*. Soumis à la revue **Symbiosis**.

* Premiers co-auteurs

TABLE DES MATIÈRES

CHAPITRE I.

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Introduction générale et méthodologie du travail

I.1. Introduction générale

Au cours du 20ème siècle, l'Homme a tenté d'exploiter au mieux les réserves de pétrole, de charbon et de gaz naturel. Ces matières premières, anciennement abondantes, ont été transformées selon divers procédés afin d'en retirer des produits hautement diversifiés : carburants, produits chimiques raffinés, produits pharmaceutiques, détergents, fibres synthétiques, plastiques, pesticides, produits fertilisants, lubrifiants, solvants, cire, coke, asphalte, etc. [1].

A l'heure actuelle, les matières premières d'origine fossile voient leurs réserves diminuer de plus en plus. Cette tendance a pour effet inévitable une hausse progressive du coût de ces matières mais aussi des divers produits qui en proviennent. L'un des aspects principaux de la problématique est situé dans le domaine du carburant, l'un des nombreux sous-produits du pétrole brut. L'exploitation des ressources fossiles est progressivement devenue discutable sur le plan économique. Ensuite sont apparus deux aspects supplémentaires de la problématique : l'écologie et l'Environnement [2].

Dans ce contexte bien défini, l'intérêt croissant pour les technologies nouvelles appliquées à la biomasse est devenu indéniable. Actuellement, la recherche focalisée sur ce sujet explore différentes possibilités afin de transformer la biomasse en carburant, appelé sous cette condition « biocarburant ». Jusqu'à aujourd'hui, trois grandes notions ont été décrites : les biocarburants de première, seconde et troisième génération.

Les biocarburants de première génération désignent l'éthanol et le biodiesel (esters méthyliques d'acides gras) produits à partir de biomasse comestible dans la plupart des cas. Ces deux types de carburants sont les seuls à être produits à l'échelle industrielle en ce qui concerne les biocarburants de première génération. L'éthanol est majoritairement produit par la fermentation du glucose obtenu par l'hydrolyse de l'amidon ou du saccharose. Les matières premières sont principalement le blé, le sucre de canne et les betteraves. Les biocarburants de seconde génération sont issus de biomasse non comestible et d'origine très diversifiée. Le prix de ce type de matières est très inférieur à celui des matières premières utilisées pour produire le biocarburant de première génération. Deux types de traitements sont possibles : la voie thermique et la voie biochimique. La première voie consiste à effectuer un procédé thermique sur la matière, de manière à produire du charbon, de l'huile et du gaz de synthèse. Ensuite, une conversion catalytique permet de synthétiser du bioéthanol. La voie biochimique repose sur le traitement de la cellulose et des hémicelluloses de la matière. Les polymères sont

d'abord rendus disponibles par divers traitements : l'explosion à la vapeur, les prétraitements chimiques, etc. La cellulose et les hémicelluloses sont ensuite hydrolysées par voie enzymatique, puis le glucose et les autres monomères qui en résultent sont fermentés par des levures afin de produire du bioéthanol dit de seconde génération. Enfin, les biocarburants de troisième génération sont à ce jour les moins développés à l'échelle industrielle. Ils sont produits dans la plupart des cas à partir des lipides d'algues. Ceux-ci sont traités par trans-estérification pour produire du biodiesel. Pour plus d'informations, la revue de Lee et Lavoie [3] peut être consultée.

Le présent travail s'inscrit dans le cadre général du développement des biocarburants de seconde génération. Il est essentiellement basé sur la recherche de nouvelles enzymes de type cellulases et hémicellulases. Les procédés faisant appel à de telles enzymes sont incontournables dans le domaine des biocarburants de seconde génération. La fermentation des sucres simples en bioéthanol, en revanche, n'est pas considérée dans ce travail.

Il est utile de citer ici l'étendue des applications des cellulases et des hémicellulases au-delà du domaine des biocarburants. Les cellulases sont produites par des bactéries, actinobactéries et mycètes. Elles sont utilisées notamment dans l'industrie textile, la fabrication des détergents, le secteur alimentaire, l'alimentation animale et l'industrie papetière [4]. Les hémicellulases sont beaucoup plus nombreuses que les cellulases en raison de la complexité des hémicelluloses. Les principales enzymes sont les xylanases, α-glucuronidases, α-arabinofuranosidases, arabinases, endo-mannanases, β-mannosidases, acétyl-xylane estérases et féruloyl-xylane estérases. Les xylanases sont les hémicellulases les plus exploitées à l'échelle industrielle. Elles sont produites par les mêmes types de microorganismes que les cellulases. Elles sont principalement utilisées dans le domaine de l'industrie textile, le blanchiment biologique de la cellulose, le recyclage du papier, la bioconversion du xylane, la clarification des jus, la boulangerie, l'alimentation animale, etc. [5] Les applications des autres hémicellulases sont assez comparables : alimentations humaine et animale, détergents, industrie papetière… [6]

Les **Tableaux 1** et **2** présentent les propriétés de quelques xylanases et cellulases produites à l'échelle industrielle. Les applications possibles de ces enzymes sont citées ainsi que les conditions d'utilisation en termes de température et de pH. Ces tableaux seront analysés plus en détail au **Chapitre VIII**.

Tableau 1. Liste non exhaustive de xylanases industrielles et caractéristiques générales. L'activité enzymatique est exprimée en Unités Internationales par gramme.

Firme	Application(s)	Gamme de pH	Gamme de température	Activité (UI/g)
Mianyang Habio Bioeng. Co., Ltd.	*Alimentation animale*	*Optimal : 6,5*	*Optimal : 37°C*	*10,000*
Mianyang Habio Bioengineering Co., Ltd.	*Alimentation animale*	*Optimal : 6,5*	*Optimal : 37°C*	*200,000*
Shaanxi Top Pharm Chemical Co., Ltd.	*Alimentation animale*	*3,5-6,5* *Optimal : 5,3*	*45-60°C*	*40,000*
Sunson Industry Group Co., Ltd.	*Boulangerie*	*5-7*	*50-80°C*	*2,000,000*
Wuxi General Corp. of Supply & Marketing	*Boulangerie*	*3,5-6,5*	*45-60°C*	*20,000*
Sukahan (Weifang) Bio-Technology Co., Ltd.	*Général sauf alimentaire*	*3-6*	*35-60°C*	*non spécifié*
Mianyang Habio Bioengineering Co., Ltd.	*Industrie papetière*	*5,5-10,5*	*40-70°C*	*5,000*
Sukahan (Weifang) Bio-Technology Co., Ltd.	*Industrie papetière*	*6,5-9,5*	*45-55°C*	*77,000*
Wuhan Rison Trading Co., Ltd.	*Industrie papetière*	*5-7*	*50-80°C*	*2,000,000*
Sukahan (Weifang) Bio-Technology Co., Ltd.	*Industrie papetière* *Industrie textile* *Secteur alimentaire* *Traitement de l'eau*	*3-6*	*35-60°C*	*85,000*
Changzhou Comwin Fine Chemicals Co., Ltd.	*Secteur alimentaire*	*3-5,5*	*35-55°C*	*5,000*
Suntaq International Limited	*Secteur alimentaire*	*3,5-6,5* *Optimal : 4,5-5,5*	*35-70°C* *Optimal : 35-50°C*	*50,000*
Mianyang Habio Bioeng. Co., Ltd.	*Industrie papetière* *Industrie textile* *Traitement de l'eau*	*étendue*	*80% à 80°C*	*10,000*
Mianyang Habio Bioeng. Co., Ltd.	*Industrie papetière* *Industrie textile* *Traitement de l'eau*	*5,5-10,5* *Optimal : 7*	*40-70°C* *Optimal : 65°C*	*150,000*

Tableau 2. Liste non exhaustive de cellulases industrielles et caractéristiques générales. L'activité enzymatique est exprimée en Unités Internationales par gramme.

Firme	Application(s)	Gamme de pH	Gamme de température	Activité (UI/g)
JinanJunda Industrial Technology Co., Ltd.	*Alimentation animale*	*4-7* *Optimal: 4,5-5,5*	*30-70°C* *Optimal: 50-55°C*	*10,000*
Shenzhen Ideal Pharmtech Co., Ltd.	*Alimentation animale*	*5,5-7,5* *Optimal: 6*	*45-65°C* *Optimal: 55°C*	*10,000*
Shanghai Soyoung Biotech Company	*Alimentation animale* *Bioéthanol* *Industrie textile* *Secteur alimentaire*	*3,5-5,5* *Optimal: 4,6-4,9*	*40-60°C* *Optimal: 50°C*	*non spécifié*
Shandong Longda Bio-Products Co., Ltd.	*Bioéthanol* *Industrie textile* *Secteur alimentaire*	*4-6* *Optimal: 4,8-5,2*	*50-70°C* *Optimal: 55-60°C*	*10,000*
Suntaq International Limited	*Boulangerie*	*4-7* *Optimal: 4,5-5,5*	*30-70°C* *Optimal: 50-55°C*	*4,000*
Beijing Winovazyme Biol. Sci. & Technol. Co., Ltd.	*Industrie papetière* *Industrie textile*	*2,5-7,5*	*30-80°C*	*500*
Sukahan (Weifang) Bio-Technology Co., Ltd.	*Industrie papetière* *Industrie textile*	*4,5-5,5* *Optimal: 4,8*	*40-60°C*	*20,000/ml*
Enzymes Naveen	*Industrie textile*	*4,5-5,5*	*45-60°C*	*3,000*
Shi jiazhuang Huancheng Biochemical Factory	*Industrie textile*	*4-5*	*45-60°C*	*10,000/ml*
Sukahan (Weifang) Bio-Technology Co., Ltd.	*Industrie textile*	*4,5-5,5* *Optimal: 4,8*	*40-60°C* *Optimal: 55°C*	*non spécifié*
Sunson Industry Group Co., Ltd.	*Industrie textile*	*5,5-7,5* *Optimal: 6*	*45-65°C* *Optimal: 55°C*	*non spécifié*
Wuxi Colotex Bio-Tech. Co., Ltd.	*Industrie textile*	*5,5-7,5* *Optimal: 6,8*	*45-55°C* *Optimal: 50°C*	*84,000*
Shaanxi Fuheng (FH) Biotechnology Co.,Ltd	*Secteur alimentaire*	*4-6,5* *5,5-7*	*20-55°C* *30-55°C*	*20,000* *20,000*
Beijing Winovazyme Bio. Sci. & Technol. Co., Ltd.	*Traitement de l'eau*	*4-8* *Optimal: 4,8-5*	*40-60°C* *Optimal: 50-55°C*	*20,000*

Ces dernières années, de nombreuses recherches ont été menées afin de mettre au point des procédés enzymatiques de plus en plus performants. Une question qui émerge alors naturellement est la suivante : où peut-on trouver les enzymes les plus efficaces?

Comme il a été dit précédemment, les enzymes participant à l'hydrolyse de la cellulose et des hémicelluloses sont sécrétées par des bactéries, actinobactéries et mycètes. Il existe dans la nature des situations particulières où l'hydrolyse de ces polymères est réalisée non pas par un microorganisme mais par un réseau de souches liées entre elles. Ces associations symbiotiques sont des consortia microbiens. De tels réseaux ont pu être identifiés dans le rumen des ruminants et chez certains insectes [7, 8].

Les termites qui se nourrissent de bois, dits « xylophages », sont capables de métaboliser 85% du glucose contenu dans la cellulose et 83% du xylose contenu dans les hémicelluloses [9]. Cette efficacité surpasse celle des consortia qui se développent au sein du rumen. Par conséquent, le tube digestif du termite peut être considéré comme source potentielle de cellulases et d'hémicellulases. Il existe deux types de termites : les termites inférieurs et les termites supérieurs. Les termites inférieurs contiennent, en plus des bactéries, archéobactéries et mycètes communs aux termites supérieurs, des protistes supposés jouer un rôle clé dans la digestion de la cellulose au sein du tube digestif [10]. Ils sont capables de phagocyter les particules de bois pour les hydrolyser et les fermenter [11]. Par conséquent, les termites inférieurs possèdent un potentiel hydrolytique plus élevé que les termites supérieurs. L'essentiel de la microflore est contenu dans une zone élargie du tube digestif nommée « hindgut », en fin de tube digestif, après le « foregut » et le « midgut » [11].

Dans le cadre de ce travail, le choix a été fait de se concentrer sur un termite inférieur en particulier : le termite *Reticulitermes flavipes* (Kollar), anciennement nommé *Reticulitermes santonensis* (Feytaud). Cet insecte a été choisi comme source potentielle de nouvelles enzymes. A chaque essai, seul le hindgut du tube digestif, contenant la majeure partie de la microflore, a été prélevé. En effet, en plus d'être biologiquement très riche, cette portion est aussi le lieu de concentration des souches développant des activités cellulolytiques et hémicellulolytiques [11]. Le termite est appelé par son ancien nom dans les premiers chapitres de ce travail (*Reticulitermes santonensis*) et par son nouveau nom dans les derniers (*Reticulitermes flavipes*) ; il s'agit toutefois du même organisme.

I.2. Méthodologie du travail

La méthodologie générale adoptée dans ce travail peut être résumée selon le schéma suivant (**Figure 1**). Deux axes de recherche peuvent être décrits, tous deux basés sur la dissection des termites et l'extraction de leurs tubes digestifs. Dans les deux cas, un enrichissement en milieu liquide a été nécessaire.

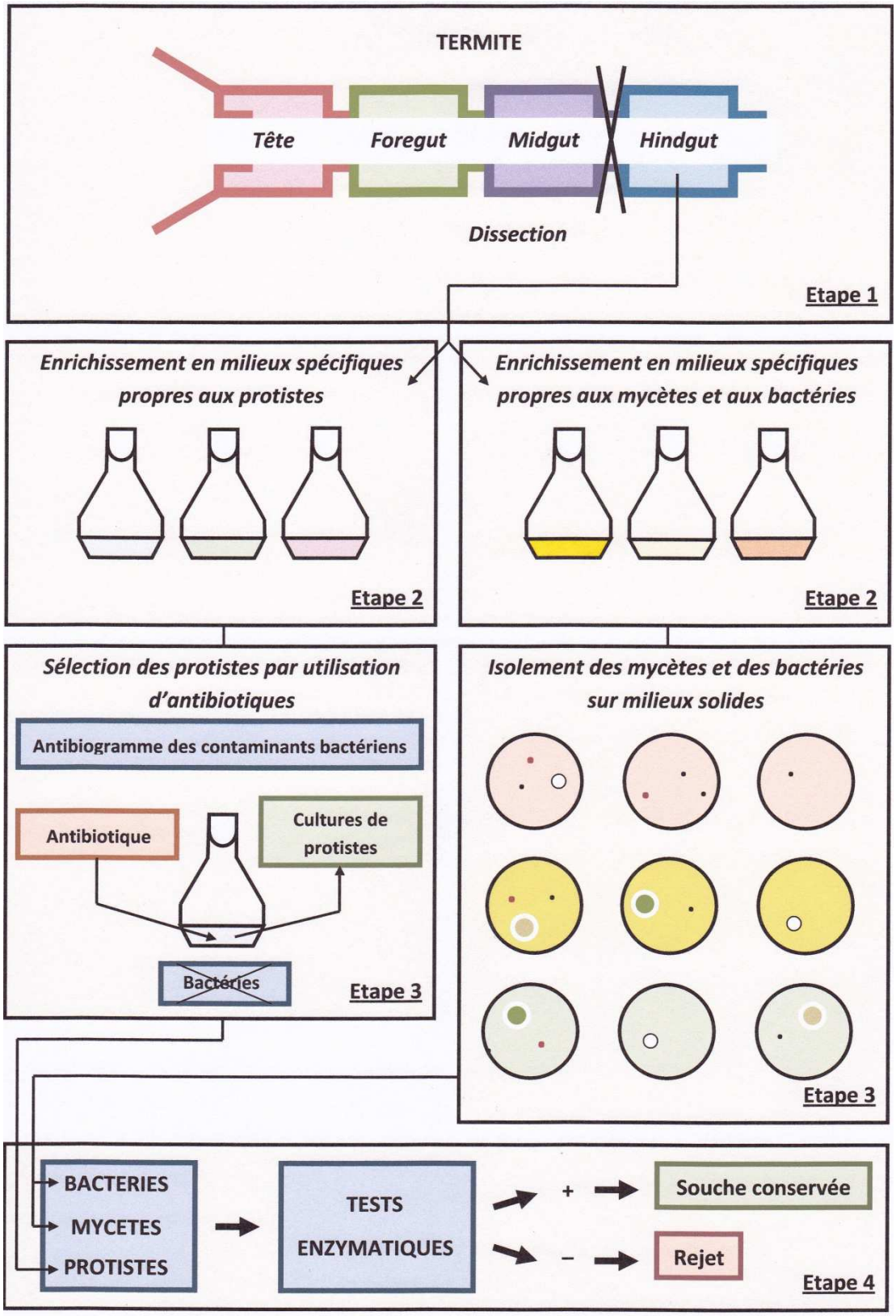

Figure 1. Stratégie générale utilisée pour isoler les souches de protistes, mycètes et bactéries à partir de l'intestin du termite *Reticulitermes flavipes*.

Un premier aspect de la recherche s'est concentré sur l'isolement de microorganismes en milieu liquide. Cette méthode a permis de cibler les protistes. Il est important de noter que leur isolement n'est pas possible selon la méthodologie appliquée aux bactéries et aux mycètes, pouvant être isolés facilement sur milieux gélosés. Un second aspect s'est donc focalisé sur des isolements en milieu solide, après l'étape d'enrichissement en milieu liquide. Cette méthode a permis de cibler les bactéries et les mycètes.

La première étape a conduit à la conclusion que des essais en atmosphère micro-aérobie, voire aérobie, étaient bien plus prometteurs. Un second aspect de la recherche, par conséquent beaucoup plus développé, s'est focalisé sur des isolements en atmosphère aérobie. Dans cette seconde partie, des diètes artificielles ont été appliquées au termite, en plus de la diète originale de bois de peuplier, dans le but d'enrichir la microflore intestinale en souches cellulolytiques et hémicellulolytiques.

Ce travail se décline en un chapitre introductif (le présent chapitre), un chapitre de revue bibliographique, 5 chapitres de résultats, un chapitre de discussion globale et un dernier chapitre de conclusion.

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CHAPITRE II.

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Review – The termite gut as a source of new enzymes: an approach by microbiological and "omics" techniques

Ce chapitre correspond à l'article intitulé *"Review – The termite gut as a source of new enzymes : an approach by microbiological and "omics" techniques"* (Julien Bauwens*, Cédric Tarayre*, Frédéric Francis, Éric Haubruge, Jacqueline Destain, Philippe Thonart, Edwin De Pauw, Micheline Vandenbol, Daniel Portetelle) actuellement soumis à la revue **World Journal of Microbiology and Biotechnology**.

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Les aspects abordés dans cette revue bibliographique sont les suivants :

- En premier lieu, l'intérêt que présentent les insectes par rapport à la problématique de l'hydrolyse de la cellulose et des hémicelluloses est investigué; le but étant d'obtenir à partir de matériaux lignocellulosiques des sucres fermentescibles exploitables notamment dans le domaine des biocarburants de seconde génération.
- Ensuite, l'intérêt se focalise sur le termite *Reticulitermes flavipes* (équivalent de *Reticulitermes santonensis*), termite inférieur, en caractérisant les conditions régnant au sein de son système digestif ainsi que les microorganismes qui y vivent. Le termite inférieur a été choisi comme sujet d'étude en raison de sa microflore hautement diversifiée.
- La dernière partie, plus pratique, passe en revue les techniques applicables pour isoler les microorganismes cellulolytiques et hémicellulolytiques associés aux termites dans le but de produire ces nouvelles enzymes.

Reticulitermes flavipes, termite inférieur, est à la base de tous les chapitres expérimentaux de ce travail. Par conséquent, une partie importante de la synthèse bibliographique lui est consacrée.

Résumé

Les biocarburants de seconde génération sont aujourd'hui devenus une alternative sérieuse aux carburants fossiles. Ils sont basés sur l'exploitation de la biomasse lignocellulosique. Un tel substrat n'est pas facile à degrader, et des pré-traitements peuvent être nécessaires à l'hydrolyse des composants de la lignocellulose. De nombreuses études ont été réalisées afin de mettre au point un système lignocellulolytique efficace. Malheureusement, cette recherche ne s'est pas à proprement parler soldée par un succès. Par conséquent, la recherche de nouvelles enzymes s'est avérée nécessaire.

L'intestin des termites inférieurs et supérieurs abrite de nombreux microorganismes liés par une symbiose complexe. Chez les termites inférieurs, les bactéries, protistes et mycètes synthétisent un véritable arsenal enzymatique qui dégrade presque totalement les hémicelluloses et la cellulose. La recherche d'enzymes dans l'intestin des termites peut être réalisée par différentes méthodes. L'isolement suivi de la culture de microorganismes est un premier moyen de criblage. Cependant, cette approche mène à une perte d'informations inévitable car de nombreux microorganismes ne peuvent pas être cultivés. Les méthodes "culture-indépendantes" permettent de contourner cette difficulté. La génomique et la métagénomique fournissent plus d'informations et sont basées sur l'analyse de l'ADN des microorganismes. Elles peuvent être utilisées pour cibler des gènes spécifiques. La transcriptomique est plus précise et cible l'information contenue dans l'ARN, passant outre l'étape de transcription. Enfin, la protéomique est sûrement la technique la plus puissante car elle s'intéresse jusqu'à la structure même des protéines.

Cette revue bibliographique décrit les différentes études qui ont été effectuées afin de trouver de nouvelles enzymes cellulolytiques et hémicellulolytiques chez la microflore du termite, tout en considérant l'immense complexité de la symbiose qui y est installée. Les différentes méthodes sont décrites : la culture de microorganismes et l'isolement d'enzymes par la génomique et la protéomique.

Mots-clés : Enzyme, Termite, Bio-carburant, Culture, Génomique, Protéomique

Abstract

Second-generation biofuels have now become a serious alternative to the use of fossil fuels. They are based on the exploitation of lignocellulosic biomass. Such a substrate is not easy to degrade, and pre-treatments may be necessary before enzymatic hydrolysis of lignocellulose components. Many studies have focused on finding an efficient lignocellulolytic system without much success. Consequently, looking for new efficient enzymes is necessary.

The gut of lower and higher termites harbors many microorganisms bound by a complex symbiosis. In lower termite guts, bacteria, protists and mycetes synthesize a real enzymatic arsenal, which degrades hemicelluloses and cellulose almost totally. Recovering enzymes from termite guts can be achieved through different methods. Isolation followed by cultivation of microorganisms is a first way to achieve that screening. However, this approach leads to an unavoidable loss of data because many microorganisms cannot be cultivated. “Culture-independent” methods circumvent that difficulty. Genomics and metagenomics provide more information and are based on DNA analysis from microorganisms. They can be used to detect specific genes. Transcriptomics is more accurate and targets RNA information, passing through a transcription step. Finally, proteomics is certainly the most powerful technique because it considers the protein structure directly.

This review describes the different studies that were carried out to find new cellulolytic and hemicellulolytic enzymes in the termite microflora, considering the huge complexity of symbiosis. The following pathways are described : cultivation of microorganisms and isolation of enzymes by genomics and proteomics.

Keywords : Enzyme, termite, biofuel, culture, genomics, proteomics

II.1. Introduction

Evidence of the need for sustainable alternatives to usual petroleum-based energy sources has been accepted for a few decades. The first biofuels were based on the use of starch and vegetable oils. However, their use competes with food and depends on the amount of available fertile soil. Moreover, energy is necessary to grow crops and convert them to biofuel. Consequently, this solution is not sustainable in the long term, and the use of lignocellulosic materials leading to second-generation bioethanol has become necessary [1, 2].

Second-generation biofuel then came out as a key solution in view of the relatively low valorization of lignocellulosic materials in human activities and on the other hand, the growing need for new and sustainable energy sources, particularly in the field of transportation.

Lignocellulose and 2nd generation bioethanol → process → improvement → enzymes

The typical composition of biomass is 45% of cellulose, 25% of hemicelluloses, 25% of lignin and 5% of other components [3]. Cellulose can reach 30 to 50% of the plant cell walls in stems and roots as well as in foliage [4]. Lignocellulose is now an important natural resource that can be used in various fields : biofuels, heat, pyrolysis, gasification, biopolymers, biolubricants, biosurfactants and biosolvents [2, 3]. Lignocellulosic biomass could be valued from agro-residues, forestry residues, municipal solid wastes and various industrial wastes [1]. In addition to valorization of diverse wastes, direct lignocellulosic biomass production for biofuels has been suggested. However, this point of view is more debatable as it applies more to first-generation biofuels. Life cycle assessment of such productions has been reviewed [5]. Potential optimization ranges from biomass selection to pretreatment or conversion yield itself. One of the key points pertaining to yield is enzyme efficiency [6]. This suggests that new enzymatic systems need to be found or engineered.

Hydrolysis of lignocellulose followed by sugar conversion is essential to synthesize biofuel. This requires removing lignin and hemicelluloses from the lignocellulosic matrix. Then, the fermentation of sugars is necessary to obtain bioethanol [2]. The pretreated biomass can be processed through different ways : separate hydrolysis and fermentation, simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation (**Figure 1**) or consolidated biomass processing.

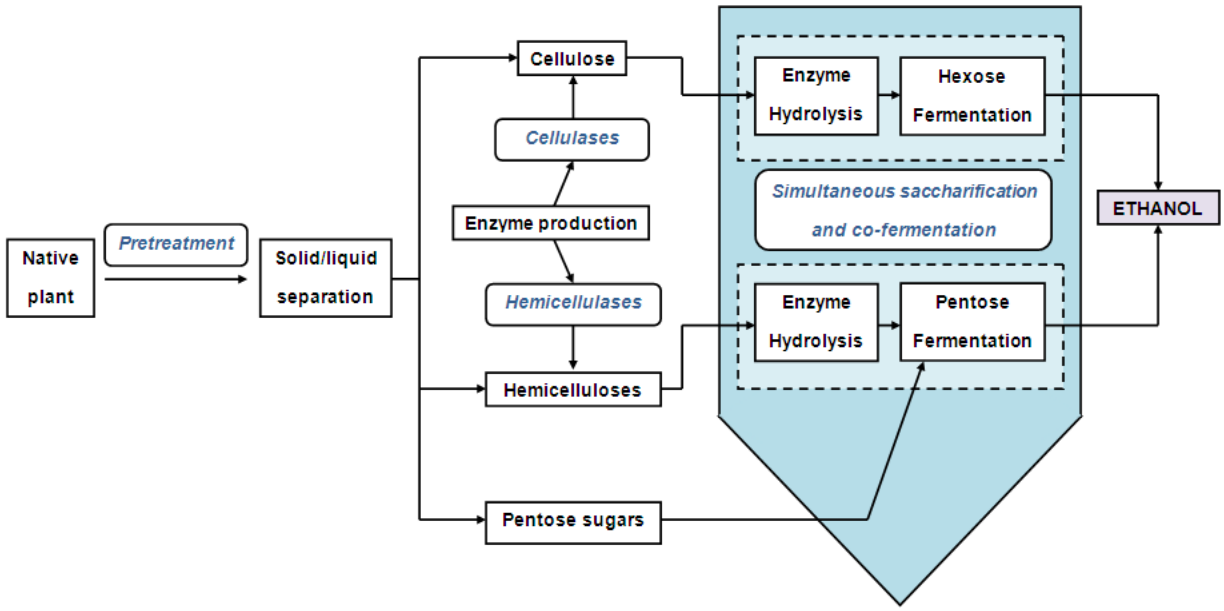

Figure 1. Bioethanol production process from lignocellulosic materials by simultaneous saccharification and co-fermentation (figure derived from [1]). In that system, the fermentation of pentoses and hexoses is achieved in the same vessel, by contrast with the simultaneous saccharification and fermentation process which requires two separate vessels.

The production of fermentable sugars is processed in two steps :

1. Pretreatment : steam explosion, ammonia fiber explosion, microwave-chemical pretreatment, chemical pretreatments (acid, alkali, organic acids, pH-controlled hot water and ionic liquids) or biological pretreatment.
2. Enzymatic hydrolysis to fermentable sugars and fermentation into bioethanol [1].

Other interesting substances can be derived from lignocellulose components. **Table 1** presents the different components that can be obtained from cellulose, hemicelluloses and lignin [1].

Table 1. Products derived from cellulose, hemicelluloses and lignin [1].

Lignocellulose component	Derived molecules
Cellulose	Polymers, levulinic acid, ethanol, lactic acid, 3-hydroxypropanoic acid, itaconic acid, glutamic acid, glucuronic acid, succinic acid
Hemicelluloses	Xylitol, ethanol, butanol, hydrogen, 2,3-butanediol, ferulic acid, lactic acid, furfural, chitosan, xylo-oligosaccharides
Lignin	Syngas, syngas products, hydrocarbons, phenols, oxidized products, macromolecules

Some animals are able to metabolize lignocellulose efficiently. Termites are able to digest up to 85 and 83% of glucosyl and xylosyl residues from lignocellulose, respectively [7]. However, ruminants are able to digest up to only 40% of parietal polysaccharides [8].

II.2. Structure of lignocellulose

Typical lignocellulose is mainly composed of cellulose, lignin and hemicelluloses [1]. Cellulose consists of polymers of glucose units, held together in bundles by hemicelluloses. In higher plants, the degree of polymerization of cellulose is about 14,000. Cellulose is not bound to hemicelluloses, but their association results from hydrogen bonds [9].

Hemicelluloses are more diverse and consist of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid [1, 10]. Hemicelluloses are soluble and can be hydrolyzed by acids. Their degree of polymerization is usually between 100 and 200. Xylan is the most abundant hemicellulose and is composed of a central chain of β-1,4-xylopyranose residues, linked to α-D-glucuronic acid, 4-O-methyl-α-D-glucuronic acid or α-L-arabinofuranose. Xylosyl residues can be substituted with acetylated groups. Hemicelluloses are linked to lignin by ester and ether bonds [9].

Finally, lignin is a complex macromolecule composed of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol [1]. Hemicelluloses are esterified with lignin components [10]. The principal bond in lignin is a phenylglycerol-β-arylether, followed by phenylcoumaran (ring 3-2), diarylpropane (ring 9-11), and biphenyl (ring 3-5) linkages. The degree of polymerization is about 100. Lignin comes from complex reactions of polymerization, and its structure depends on the plant considered. The removal of lignin from the lignocellulosic complex causes a partial denaturation, which is due to the breaking of covalent bonds [9].

II.3. Herbivorous and xylophagous insects as sources of new enzymes

Enzymes can be used to hydrolyze lignocellulose, and these biochemical catalysts can be found in many places. Some insects can be considered as interesting enzyme sources. Willis et al. [11] reviewed the methods applicable to the research of enzymes in insects. Large-scale prospection for degradation of carboxymethyl cellulose (CMC) and microcrystalline cellulose (MCC) activity in insects highlighted insect orders showing significant cellulase activity in the gut or head fluids [12]. Particularly, *Blattodea* (cockroach and termite), *Coleoptera* and *Orthoptera* showed the highest activity on CMC, while MCC was more degraded in *Coleoptera*, *Hymenoptera*, *Lepidoptera* and *Orthoptera*. These insects

have developed the ability to digest cellulose on their own or in association with microbial populations, like some scarab larvae producing endogenous proteinases in the midgut and benefitting from cellulolytic enzymes produced by symbiotic microorganisms [13]. The hindguts of such scarab larvae contain high concentrations of volatile fatty acids, fermenting bacteria and typical anaerobic activities, such as methanogenesis [13].

Wood-eating termites digest lignocellulose with a high efficiency [14] and represent a relevant insect model. Some studies advocate for parallel and somewhat independent cellulose-digestive systems represented by the host and the symbiotic community [14]. However, recent work demonstrated a synergistic effect between enzymes from host and native gut tissues [15].

Termites are symbiotically associated with various microorganisms including bacteria, protists and mycetes. Bacteria can be found in higher and lower termites, while protists live in the lower termite gut only. Mycetes, also present in some termites, are supposed to play different roles in the termite gut : they are a source of proteins, are able to degrade lignin, decrease the carbon/nitrogen ratio and finally secrete cellulases and xylanases [16]. Mycetes can degrade lignin into carbon dioxide, which is not attackable by bacteria and protists [7]. The digestion of lignocellulose by lower and higher termites depends on various strains, producing enzymes able to hydrolyze lignocellulosic components.

What makes termites good candidates for the isolation of cellulolytic, xylanolytic and lignolytic strains is the fact that they can remove most neutral polysaccharides and more than half of the acidic sugars contained in lignocelluloses [17].

Wood digestion is mainly performed inside the termite hindgut, which harbors 10^{11} microbial cells/ml [17]. The termite ingests small wood particles by the action of its mandibles (20–100 µm). Flagellated protists can phagocytose these fragments and ferment them [17].

Wood-eating termites ferment sugars into acetate, carbon dioxide and hydrogen. The highest concentrations of hydrogen are found inside the hingut in lower termites. Lactate, formate, succinate and propionate remain a minority inside the termite gut. Carbon dioxide and hydrogen can be further transformed into acetate or methane [7]. Cellulose metabolism has been observed in the protist *Trichomitopsis termopsidis*. This microorganism can ferment cellulose into acetate, carbon dioxide and hydrogen. Hydrogen production was found to be

very common in parabasalid flagellates. Bacterial symbionts use the sugars released by the action of the protist enzymes. *Candidatus Endomicrobium trichonymphae* and *Candidatus Azobacteroides pseudotrichonymphae*, endosymbionts of *Trichonympha agilis* and *Pseudotrichonympha grassii*, ferment the sugars released by the action of protist enzymes into acetate. However, the metabolism of oxymonads is not known because no oxymonad has been cultivated in pure culture up to now [17].

Lactate is thought to be an important intermediate between primary and secondary fermentations. It is released by protists, lactococci and enterococci. These two are able to produce lactate from xylose and cellobiose. Then, lactate is consumed by peripheric bacteria, which ferment it into butyrate, propionate and mainly acetate. Fermentative bacteria are able to change their metabolic pathways in favor of acetate when oxygen is available. This was proven in the case of the gut of *Reticulitermes santonensis* [17].

Large amounts of hydrogen accumulate in the gut lumen of lower and higher termites. Hydrogen is formed by protists and bacteria in lower and higher termites, respectively. It can be metabolized into acetate or methane [17]. The digestion process occurring in the gut of the termite *Reticulitermes santonensis* is illustrated in **Figure 2**.

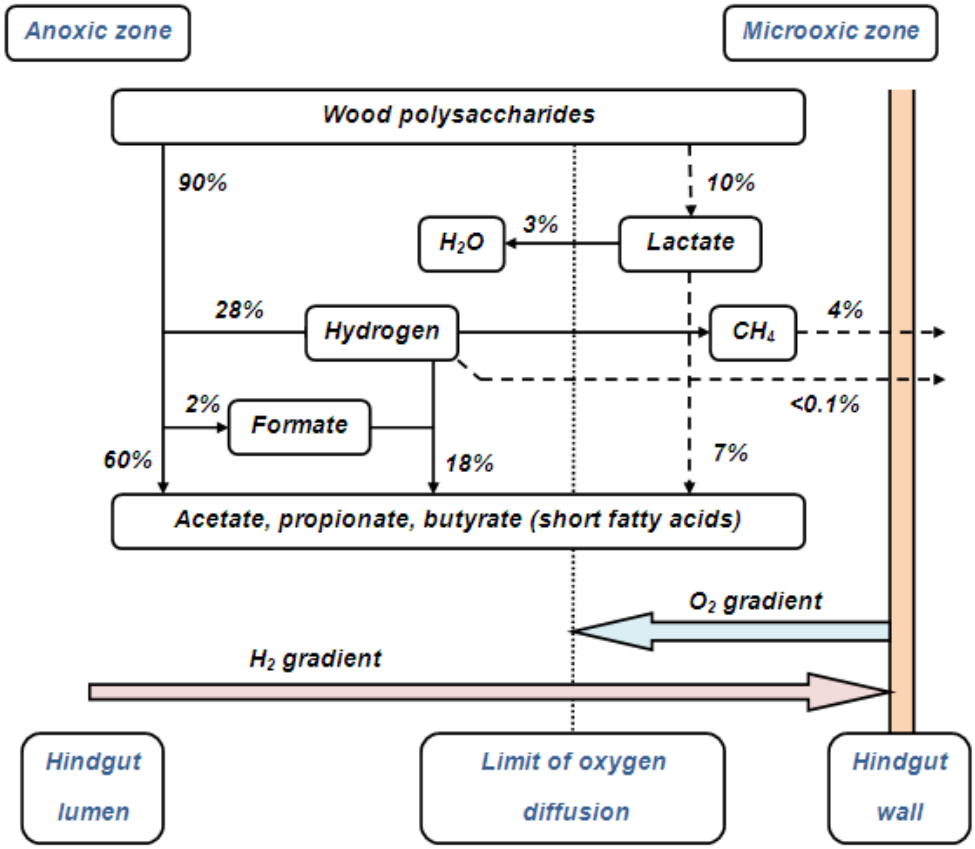

Figure 2. Digestion of wood polysaccharides occurring in the digestive tract of the termite *Reticulitermes santonensis* (figure derived from [17]). Wood polysaccharides are hydrolyzed by the action of microbial enzymes, releasing soluble sugars. Pentoses and hexoses are then fermented and generate short fatty acids, formate, lactate and hydrogen. This hydrogen is produced in the gut lumen by symbiotic bacteria (higher termites) or protists (lower termites). It diffuses to the gut wall, creating a gradient. Oxygen diffuses in the opposite direction (from the gut wall to the lumen).

II.4. Enzymatic activities developed in the termite gut

The degradation of cellulose depends on three enzymes. Endo-1,4-β-glucanase hydrolyzes internal bonds of cellulose and is active on its amorphous areas and carboxymethylcellulose. Cellobiohydrolase is more active on microcrystalline cellulose, while exoglucohydrolase is active on cellodextrins. Finally, β-glucosidase hydrolyzes cellobiose and cellodextrins to release glucose. Other secondary enzymes participate in the final step of cellulose hydrolysis, such as cellobiose oxidase, cellobiose-quinone oxydoreductase, cellobiose phosphorylase and cellodextrin phosphorylase. The degradation of hemicelluloses is performed by endo-1,4-β-xylanases which hydrolyze the xylose backbone, β-xylosidase which hydrolyzes xylobiose, and debranching enzymes, such as α-glucuronidase, α-L-arabinofuranosidase and acetylesterase. The degradation of lignin involves peroxydases secreted by mycetes. The reaction is initiated by the generation of an aryl cation and phenoxy

radicals, formed by the action of lignin peroxydases or Mn-peroxydases, which cause a fragmentation of lignin. However, those reactions require oxygen [9].

Wood-eating termites offer many possibilities in the research of new enzymes. Indeed, those termites contain numerous enzyme-producing strains able to digest lignocellulose [18]. Cellulases produced by the termite itself are “incomplete”. Endogenous cellulases of termites are mostly found in salivary glands and midgut [18]. These enzymes belong to the glycosyl hydrolase family (GHF) 9 and can hydrolyze amorphous cellulose and carboxymethylcellulose, but are not efficient on crystalline cellulose [17]. The hydrolysis of cellulose could not be achieved without the exogenous enzymes coming from microbial strains [18]. The cellulases secreted by the termite consist of endoglucanases and/or β-glucosidase. However, the termite is not able to secrete cellobiohydrolase, which is necessary for a complete hydrolysis. This enzyme is found in bacteria, mycetes and protists [18]. Protists synthesize enzymes belonging to the GHF 5, 7, and 45, while bacteria release enzymes belonging to the GHF 10 and 11 [17]. Hemicellulases, such as xylanase, mannanase and arabinosidase, are also necessary for the degradation of lignocelluloses. Indeed, the removal of hemicelluloses increases cellulose accessibility [18]. Xylanase, arabinosidase, mannosidase and arabinofuranosidase activities were detected in the termite *Reticulitermes speratus* [17]. The genetic diversity of endogenous termite cellulase systems is poor compared with the one of cellulolytic microbes, such as *Trichoderma reesei* or *Clostridium cellulovorans* [18].

Termites themselves are able to produce cellulases, but also secrete lysozyme in their salivary glands, able to destroy the bacterial cell wall peptidoglycan. This was observed in the termite *Reticulitermes hesperus*. High concentrations of proteases were also found in the midgut [7].

II.5. The debate relating to lignin digestion in termites

The digestion of lignin by termites is an important topic because this component is highly resisting in the lignocellulosic complex. Ligninases can degrade lignin, which is a problematic macromolecule of lignocellulose. Some authors studied the degradation of lignin inside the termite gut. Katsumata et al. [19] studied the modification of lignin structure through the termite gut by nuclear magnetic resonance. It was possible to conclude that, in the case of the termite *Cryptotermes brevis*, no significant change of structure is observable. Aliphatic groups were hydroxylated, and new bonds were detected in guaiacyl nuclei. The

degradation of lignin can be achieved if oxygen, which is necessary to break aromatic cycles, is sufficiently available [20]. All the studies confirm that only lateral chains of lignin are modified [7]. The degradation of lignin was detected in the termite *Reticulitermes santonensis*, but the mechanism was aerobic and consequently insignificant. However, ligninase activities, although weak, were detected [7]. That degradation comes from the secretion of laccases released by some microorganisms, and monomers released from the hydrolysis can be further metabolized by specific strains [7]. Some species of *Burkholderia* and *Citrobacter* are able to degrade lignin through a detoxifying pathway [7]. Actinobacteria and mycetes are also thought to play a role in the degradation of lignin [7]. Some actinomycetes have been isolated from termite guts and were able to solubilize lignin and secrete extracellular peroxidases [9]. Ke et al. [21] studied the degradation products of lignin after the digestion of different biomass substrates by the lower termite *Coptotermes formosanus* by pyrolysis followed by GC/MS analysis. The study concluded that lignin coming from hardwood and barley straw was modified through the gut, leading to an increase in solubility. These transformations depended on the source of lignin. Significant lignin transformations were detected in the second part of the intestine (midgut). Reactions of carbonylation, methoxylation, esterification and dehydroxylation were reported. Transformations on the side chains were also revealed, such as oxidations, esterifications and carbonylations. That study also revealed a possible ring breaking happening in the midgut [22]. Consequently, the termite gut can be a potential source of new ligninases.

II.6. Difficulties relating to the research of enzymes in the termite gut

The research of enzymes in termites can be done by using different techniques. Some techniques lead to the isolation of enzymes, while other techniques can be very useful to understand the interactions existing between the microorganisms composing the complex microflora of termites. This field is particularly important because the termite symbionts degrade lignocellulose over a complex symbiotic pathway.

The simplest method consists in extracting enzyme-producing organisms from the gut of termites before cultivating them. The isolation followed by the cultivation of enzyme-producing strains requires the elimination of all the interactions between those strains and their complex environment. Indeed, the isolation of microorganisms potentially interesting in the field of enzyme production involves breaking associations between the microbes, leading to an unavoidable loss of microbial strains [23]. The isolation of microorganisms must take account of the physicochemical properties of the termite gut.

The termite gut is full of microbiological and physiological heterogeneities, making the isolation more difficult. The termite gut is highly compartmentalized, creating specific ecological niches. In higher termites, some parts of the gut display a highly alkaline pH. In all termite genera, the concentrations of hydrogen and oxygen show important gradients [7]. The microbial microflora is spread over the termite gut, and the setting of the different microorganisms depends on their optimal conditions for growth [23]. Finally, metabolite gradients are also created due to these heterogeneities. All these gradients (**Figure 3**) allow the development of anaerobic and aerobic strains [17].

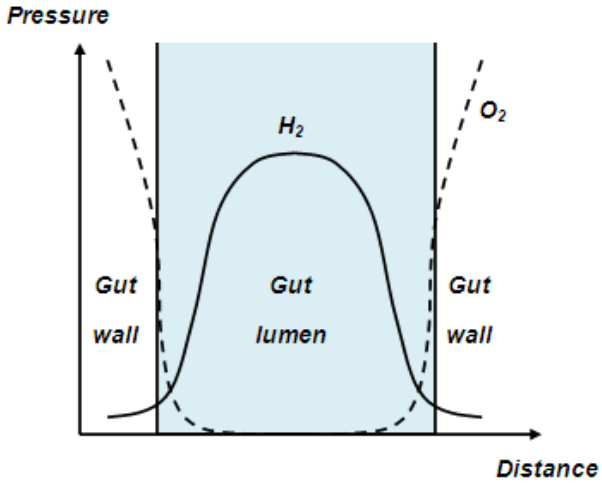

Figure 3. H_2 and O_2 concentration profiles as a function of the distance to the gut walls in termites (figure derived from [17]). Hydrogen is produced by the symbiotic microorganisms in the gut lumen and diffuses to the gut wall, which can be considered as a microoxic medium. Oxygen diffuses from the gut wall to the gut lumen, which is an anoxic medium because oxygen is consumed before reaching the central part of the gut.

The majority of microorganisms living in the termite gut are attached to the epithelial wall. Valves and muscular constrictions regulate the movements of the contents of the gut, creating a continuous culture chemostat. Flagellated protists tend to concentrate in the gut lumen, where they ingest bacteria and wood particles and use their cellulases and xylanases to digest lignocellulose. Protists themselves constitute a specific habitat and are able to adjust their redox potential thanks to their endo- and ectosymbionts [7].

The termite gut is divided into three parts : foregut, midgut and hindgut. The latter part is the most important, particularly in the case of lower termites. The hindgut displays a dilation, which slows down the gut transit and increases the exposure to the symbionts. Given that the microorganisms can colonize different sections of the termite gut in accordance with their optimal environment, specific activities are found in specific zones. For example, in

Reticulitermes flavipes, xylanase activities are found in the hindgut only [17]. The hindgut of termites usually displays a redox potential from -270 to -50 mV, and the pH value is close to neutrality (6.2–7.6), except for soil-feeding termites. In that case, the pH increases drastically between the midgut and hindgut to reach a value of 12 [9, 24]. The redox potential is mainly influenced by bacteria [24]. The junction between the midgut and the hindgut, called "mixed segment", is particular. In the case of *Microcerotermes edendatus*, it harbors a pure culture of spore-forming bacteria [24]. Such an observation further highlights the compartmentalization of termite guts.

Those characteristics illustrate the complexity of the termite gut and must be considered in the field of enzyme research.

II.7. Complexity of the termite gut microflora

The weight of the termite microflora can reach up to 40% of the total weight of termites. The termite microflora is supposed to play key roles in termite metabolism. At first, the hydrolysis of lignocellulosic materials brings usable carbon sources to the termite and the microflora itself. Nitrogen can also be fixed by specific microorganisms, providing assimilable nitrogen inside the termite gut. Secondly, the consumption of oxygen creates ecological niches with a central anaerobic zone and an aerated peripheric zone in the gut. Redox balances are created with hydrogen released by the microorganisms, thereafter consumed through acetogenesis or methanogenesis. Aromatic molecules are also partially degraded inside the gut by demethylation, desacetylation and decarboxylation. Finally, soil-eating termites need their microflora to improve humification through their guts [7]. The digestion of cellulose in the termite gut can reach a yield of 99%. This digestion is partially achieved by the termite itself, but mainly by its symbionts [18]. The composition of the gut microflora is influenced by the termite diet. As an example, strong differences can be observed between the wood-eating termite *Reticulitermes speratus* and the soil-eating termite *Cubitermes orthognathus* [24].

Protists

Protists, if present, can reach up to 60% of the total weight [7]. In *Mastotermes darwiniensis*, protists occupy 95% of the hindgut paunch volume, and 90% of the bacteria living in that zone are associated with them [23]. Protists are able to absorb wood particles through endocytosis. Their removal from inferior termite guts causes the death of termites [18]. Protists live in the hindgut of lower termites, and belong to the class of *Parabasalia* or

the order of *Oxymonadida*. About 400 parabasalids and 70 oxymonads have been listed up to now. Oxymonads are composed of five families, of which four are found exclusively in termites and *Cryptocercus*. Parabasalids include hypermastigids and trichomonads [23]. Protists living in the termite hindgut do not have mitochondria, but parabasalids harbor hydrogenosomes [23]. Protists can produce cellulases and hemicellulases [25].

Bacteria

Bacteria can be associated with protists, attached to the epithelial wall or free in the gut medium [17]. The majority of bacteria living in the intestine of termites are not being cultivated yet. In the *Reticulitermes* species, the bacterial diversity is distributed over 15 phyla. Spirochetes (particularly those belonging to the genus *Treponema*) are the most abundant phylum and are dominant in various inferior and superior wood-eating termites [23]. Most spirochetes are acetogenic and consume hydrogen to release acetate, such as *Treponema primitia* [17]. Moreover, they are associated with protists in various symbioses. They can propel the protist cell by a synchronous waving motion, being attached to its surface. Several spirochete species can colonize specific areas on a cell of a protist [23]. The protist *Streblomastix strix* is associated with six or seven species of bacteria, colonizing the cell surface through a structured organization [24]. Another example is *Trichonympha agilis*, which harbors *Desulfovibrio trichonymphae* in the front part of the cell, while *Endomicrobia* remain in the back zone. *Bacteroidetes*, *Firmicutes* and the TG1 phylum are also abundant. *Bacteroidales* share a symbiosis with the protist *Pseudotrichonympha grassii* as endosymbionts. Bacteria belonging to an uncultivable group were classified in *Endomicrobia* and are endosymbionts of parabasalids and oxymonads [23]. Some bacteria reduce sulfate but are in the minority because of the low concentration in sulfate inside the termite gut. *Desulfovibrio desulfuricans* and *Desulfovibrio giganteus* consume hydrogen and some organic molecules, while *Desulfovibrio termitidis* oxidizes sugars [9]. Some bacteria can also reduce Fe^{3+} in soil-eating and wood-eating termites. Clones isolated from genomic banks of *Reticulitermes* species also contained sequences related to *Mycoplasmatales*, *Streptococcus* and *Proteobacteria*. *Thermoplasmales* and *Thermococcales* were also detected in the termites *Reticulitermes speratus* and *Cubitermes orthognathus* [24].

Archaea

Methanogenic archaea can also be found in lower and higher termites. Lower termites only contain the genus *Methanobrevibacter*, while superior termites also harbor the family

Methanosarcinacea and the order *Methanomicrobiales*. Soil-eating termites contain more archaea bacteria than wood-eating termites [23]. Methanogenic archaea and acetogenic bacteria consume hydrogen released by other bacteria and protists in higher and lower termites, respectively. Archaea fixed to the epithelial wall lack hydrogen in the gut of wood-eating termites, while acetogenic bacteria turn it into acetate in the gut lumen, rich in hydrogen. The location of methanogens on the epithelial wall is enigmatic because of the oxygen and the lack of hydrogen, which is necessary for methanogenesis. However, this attachment protects archaea from washout and predation [17]. Methanogenesis in termites is far from negligible and constitutes 5% of global emissions of methane [9]. Methanogenic strains offer possibilities in the field of methane production.

Mycetes

Associations between mycetes and termites are observable in inferior and superior termite genera, and both partners depend on each other. The fungus-growing termites belonging to the subfamily *Macrotermitinae* developed a symbiosis with the fungus *Termitomyces*. Each genus of fungus-growing termite harbors specific strains of fungus [16]. Mycetes show hemicellulase, lignolytic and cellulase activities [9]. Some wood-eating termites also feed on wood colonized by mycetes, to improve the digestion of lignocellulose [24].

Termites harbor a specific lignocellulose-degrading microflora. The different microorganisms that were described in this section can be considered as potential sources of useful enzymes, such as cellulases and hemicellulases.

II.8. Techniques used to isolate and study the enzymes of termite guts

II.8.1. *Isolation of microorganisms from the termite gut*

The termite gut is a complex culture medium containing numerous microorganisms interacting through symbiotic relationships. Many attempts of isolation of potential enzyme-producing strains were made. Bioethanol can be obtained through the fermentation of sugars coming from the hydrolysis of cellulose and hemicelluloses. Consequently, the microbial strains which degrade lignin will not be considered here. König et al. [26] reviewed all the strains that were isolated from termite guts. In *Reticulitermes flavipes*, most isolated bacteria were *Streptococcus*, *Enterococcus*, *Bacteroides* species and *Enterobacteriaceae*. Coccoid lactic acid bacteria were also isolated from *Mastotermes darwiniensis* and *Cryptotermes*

primus. *Staphylococci* were isolated from *Nasutitermes graveolus* and *Nasutitermes walkeri*. The isolates from termite guts are mostly aerobic or aerotolerant anaerobes. However, it was determined that about 90% of microbial cells contained in the gut of *Reticulitermes flavipes* escaped cultivation attempts [24].

Cases of isolation of microorganisms from termite guts are summarized in **Table 2** (this list in not exhaustive). The many isolated strains were able to produce cellobiohydrolase, β-glucosidase, endoglucanase, β-xylosidase, endoxylanase, α-amylase and filter paper-cellulase activities.

Table 2 – Part 1. Microorganisms isolated from digestive tracts of termites and their enzymatic activities. EG : Endoglucanase activity – FP : Filter Paper-cellulase activity – βG : β-glucosidase activity – CBH : Cellobiohydrolase activity – βX : β-xylosidase activity – EX : Endoxylanase activity – αA : α-amylase activity – C : Cellulase activity without specification – X : Xylanase activity without specification.

Strain	Activity	Source	Reference
BACTERIA			
Acinetobacter sp.	EG, FP	*Microcerotermes diversus*	[27]
Acinetobacter sp.	EG, βG	*Coptotermes curvignathus*	[28]
Afipia sp.	EG, FP	*Zootermopsis angusticollis*	[29]
Bacillus cereus	EG, FP	*Zootermopsis angusticollis*	[29]
Bacillus cereus	EG	*Reticulitermes hesperus*	[30]
Bacillus cereus	EG, βG	*Coptotermes curvignathus*	[28]
Bacillus circulans-related isolate	EG, FP	*Zootermopsis angusticollis*	[29]
Bacillus megaterium	EG, FP	*Zootermopsis angusticollis*	[29]
Bacillus sp.	βG, αA	*Reticulitermes santonensis*	[31]
Bacillus sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]
Bacillus subtilis	EX, αA	*Reticulitermes santonensis*	[33]
Brevibacillus brevis	EG, FP	*Zootermopsis angusticollis*	[29]
Brucella melitensis	EG, FP	*Zootermopsis angusticollis*	[29]
Cellulomonas sp.	EG, FP	*Zootermopsis angusticollis*	[29]
Cellulosimicrobium variabile	EG, X	*Mastotermes darwiniensis*	[34]
Chryseobacterium kwangyangense	EG, βG	*Coptotermes curvignathus*	[28]
Chryseobacterium sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]
Citrobacter sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]
Clostridium beijerinckii	βG	*Coptotermes formosanus*	[35]
Clostridium termitidis sp.	EG, βG	*Nasutitermes lujae*	[36]

Table 2 – Part 2. Microorganisms isolated from digestive tracts of termites and their enzymatic activities. EG : Endoglucanase activity – FP : Filter Paper-cellulase activity – βG : β-glucosidase activity – CBH : Cellobiohydrolase activity – βX : β-xylosidase activity – EX : Endoxylanase activity – αA : α-amylase activity – C : Cellulase activity without specification – X : Xylanase activity without specification.

Strain	Activity	Source	Reference
BACTERIA			
Comamonas sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]
Dyella sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]
Enterobacter aerogenes	EG, βG	*Coptotermes curvignathus*	[28]
Enterobacter cloacae	EG, βG	*Coptotermes curvignathus*	[28]
Enterobacter sp.	βG, βX	*Reticulitermes santonensis*	[37]
Enterobacter sp.	βG	*Reticulitermes santonensis*	[38]
Enterococcus faecalis	βG	*Reticulitermes flavipes*	[39]
Klebsiella sp.	βG	*Reticulitermes santonensis*	[38]
Klebsiella sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]
Kocuria varians	EG, FP	*Zootermopsis angusticollis*	[29]
Lactococcus lactis	βG	*Thoracotermes macrothorax*	[39]
Lactococcus sp.	EX, αA	*Reticulitermes santonensis*	[31]
Microbacterium sp.	EG, FP	*Zootermopsis angusticollis*	[29]
Micromonospora sp.	EG, FP	*Armitermes, Microcerotermes, Macrotermes and Odontotermes spp.*	[40]
Ochrobactrum sp.	EG, FP	*Zootermopsis angusticollis*	[29]
Paenibacillus sp.	EG, FP	*Zootermopsis angusticollis*	[29]
Paenibacillus sp.	EX	*Sinocapritermes mushae*	[41]
Pilibacter sp.	EX, αA	*Reticulitermes santonensis*	[31]
Pseudomonas sp.	EG, FP	*Microcerotermes diversus*	[27]
Rhizobium etli	EG, FP	*Zootermopsis angusticollis*	[29]
Serratia marcescens	EG	*Reticulitermes hesperus*	[30]
Serratia sp.	CBH, βG, EG	*Reticulitermes speratus*	[32]

Table 2 – Part 3. Microorganisms isolated from digestive tracts of termites and their enzymatic activities. EG : Endoglucanase activity – FP : Filter Paper-cellulase activity – βG : β-glucosidase activity – CBH : Cellobiohydrolase activity – βX : β-xylosidase activity – EX : Endoxylanase activity – αA : α-amylase activity – C : Cellulase activity without specification – X : Xylanase activity without specification.

Strain	Activity	Source	Reference
BACTERIA			
Sphingomonas echinoides	EG, FP	*Zootermopsis angusticollis*	[29]
Sphingomonas sp.	EG, FP	*Zootermopsis angusticollis*	[29]
Staphylococcus saprophyticus	EG, EX	*Odontotermes obesus*	[42]
Staphylococcus sp.	EG, FP	*Microcerotermes diversus*	[27]
Streptomyces sp.	EX, αA	*Reticulitermes santonensis*	[31]
Streptomyces sp.	EG, FP	*Armitermes, Microcerotermes, Macrotermes* and *Odontotermes* spp.	[40]
Zymomonas mobilis	EG, FP	*Zootermopsis angusticollis*	[29]
PROTISTS			
Trichomitopsis termopsidis	C	*Zootermopsis* termites	[43]
Trichomitopsis termopsidis	C	*Zootermopsis* termites	[44]
Trichonympha sphaerica	C	*Zootermopsis* termites	[45]
MYCETES			
Sporothrix sp.	C	*Zootermopsis nevadensis*	[29]

The most common cellulase activity which is observed in microorganisms extracted from termites is endoglucanase. However, the most problematic part of the cellulose in the lignocellulosic complex is crystalline, not degraded by the action of endoglucanase. This enzymatic activity only degrades amorphous cellulose, which is more easily accessible than crystalline cellulose. Xylanases are also necessary to degrade the lignocellulosic complex, acting on hemicelluloses. Such enzymes were found to be produced by many termite gut bacteria. Protists also produce useful enzymes but are more difficult to obtain in pure cultures. Only a few examples can be cited, although such microorganisms are thought to play a key

role in cellulose digestion. There are also several yeast species that were isolated from termites. The study of Schäfer et al. [46] mentions some cases of yeast isolation from termite guts.

The isolation of pure enzyme-producing microorganisms focuses on one strain only. However, another method consists in considering microbial consortia in order to improve the efficiency of the enzymatic complex. Such heterogeneous microbial systems are already being used in foodstuffs, beverages, biogas and bioethanol industries. They could be exploited to produce more complete enzymatic systems. Of course, the use of microbial consortia requires the knowledge of the symbiotic relationships between the different microorganisms [47]. For instance, the study of Nanthakumar et al. [48] used artificial consortia composed of 12 different bacteria extracted from *Macrotermes* sp. In that study, bacteria were used to decolorize dyes. Those bacteria were tested singly, and different combinations were then tried. Those artificial associations gave better results than the microorganisms considered alone. Although this study relates to the textile industry, it illustrates a technique that could be used in the field of enzyme production. Zuroff et al. [49] studied the feasibility of creating symbiotic consortia for lignocellulosic biofuel production. The study concluded that it would be possible to create symbiotic cultures by assembling the metabolic capabilities of different microbial strains. Quorum sensing and biofilm formation should also be considered in the field of industrial applications because natural consortia are usually found in surface-attached communities.

II.8.2. *Research of enzymes through microbial community alteration*

Some studies analyzed the effect of termite feeding habits and concluded that the composition of the consortia depends on the diets. Other studies compared the microbial communities in a single insect host species fed on different diets. Those diets induced significant modifications in the microbial composition [50, 51]. In this approach, the termites can be considered as bioreactors in which the diet composition leads to a specific microbial consortium. Consequently, even though most analyses were achieved by using culture-independent techniques, this approach could be considered as an intermediate method between the cultivation of microbial consortia and culture-independent approaches. The alteration of original consortia may lead to the characterization of new enzymes.

II.8.3. *Omics approaches*

The isolation of enzymes and the understanding of the microbial interactions can be achieved through some culture-independent methods. These approaches can be considered as more holistic because “non-cultivable” microorganisms may be partially included. In this part of the review, we will describe some “omics” approaches with an emphasis on proteomics. The main outcomes provided by such analyses applied to termites are also being discussed.

The “omics” techniques refer to global approaches. A common genetic analysis focuses either on a single gene or on several ones cloned separately from DNA by the polymerase chain reaction (PCR) or from RNA by reverse PCR. By contrast, genomics encompasses the whole genome, which represents the total DNA of an organism. This approach is particularly interesting to characterize uncultivable microorganisms, such as TG-1 bacteria [52]. Those organisms were described as endosymbionts, providing useful amino acids and cofactors to the termite. Microbial diversity can be determined by using targeted amplification. This technique was used to characterize taxonomic diversity of prokaryotic microbiota depending on gut fractions using terminal restriction fragment length polymorphism (T-RFLP) analysis applied to 16S rRNA [53]. This study revealed the relative abundance of *Firmicutes* associated with the midgut, whereas representatives of the Termite Group 1-phylum were mostly located in the protist-associated fractions. Major phylogenetic groups from hindgut fluid or hindgut wall fractions were representatives of the TG-1 phylum, *Firmicutes* and *Bacteroidetes*. More recently, 454 pyrosequencing of 16S V5-V6 amplicons has been done after differential feeding treatments in order to highlight lignin-poor and lignin-rich specific microbial communities [54] and showed that taxonomic diversity was previously underestimated. Symbiotic flagellates have also been studied at the genetic level [55]. However, most studies use a morphological or phylogenetic characterization.

Genomic results made the investigation of the genetic potential of probed organisms possible, and the subsequent amount of data collected enabled scientists to target a gene to quantify its copy number, characterize polymorphism [55] or “visualize” it by fluorescence in situ hybridization [56]. Metagenomics is a genomic approach applied to samples containing several species, such as microbial communities and symbionts. Metagenomics applied to higher termites (*Nasutitermes* sp.) illustrated the complexity of a symbiotic microbial community free from flagellates [57]. No further information about the dynamics of gene expression is available. Consequently, the transcriptome is a better basis to study stress responses, implications in symbiotic systems [58] and adaptations of the hindgut microbial

community to termite diet [59, 60]. Phylogenetic analysis of clones related to cellulolytic enzymes from protists of several termite species revealed the specificities of co-evolved termite species and their protist communities [61]. Transcriptomic data on two co-cultured termite-isolated *Spirochaetes* also revealed comprehensive and mutualistic interactions [62]. Thanks to transcriptomics, the analysis of non-coding RNA is possible as well. However, this approach is thought to be limited because of the short half-life of RNA or the weak correlation between RNA and functional protein quantities [63]. Proteomics offers the possibility to override some of these problems by working with the final product of gene expression. This discipline encompasses qualitative and quantitative analysis of the proteome expressed in an organism, an organ, a cell or an organelle. It takes account of the interactions, post-translational modifications and turnover, at a particular time that, considering our current knowledge, cannot be simply translated from genetic material. So far, few protein-based studies were achieved on termites compared to nucleic acid-based ones. As an example, the metaproteome of a *Nasutitermes* species has been investigated, particularly for proteins belonging to CAZy families [64]. More recently, an interdisciplinary study based on 454 pyrosequencing of metatranscriptomic cDNA bank and metaproteomics revealed different profiles of expression in *Reticulitermes flavipes* depending on the polymerization state of lignin [60]. It is also important to mention that several enzymes which do not show cellulase, hemicellulase or ligninase activity were shown to significantly increase lignocellulose saccharification. Two termite genera, *Nasutitermes* and *Reticulitermes*, can be considered as models of higher and lower termites, respectively. *Reticulitermes flavipes* and *Reticulitermes santonensis*, which are now considered as synonymous species [65], have been the topic of many studies [50, 53, 58, 60, 66].

By considering proteins instead of RNA or DNA, we can detect or quantify the enzymatic activities. Common enzymatic activity assays can be used with a variety of substrates that are specific to those enzymatic activities [50, 67]. Separation steps may be necessary and can be achieved by liquid chromatography [68]. Enzymatic activities can be highlighted through zymograms, based on gel electrophoreses [69]. Zymography can be performed in native or denaturing conditions [70]. This technique can be used for detection but also identification using mass spectrometry [71] or N-terminal sequencing [72]. Such high-throughput technologies, especially mass spectrometry, show more and more applications, such as MALDI-imaging [73]. The different techniques often require protein separation steps which can be performed in gel or in solution. Gel separation is generally done

through isoelectric focusing (IEF) or sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Two-dimensional gel electrophoresis (2DE) is a combination of these two independent features. However, protein 2D gel separation shows variability [74]. By contrast, Coomassie Brillant Blue staining methods provide a quantitative signal and are MS-compatible though generally less sensitive than silver staining with a relatively low dynamic range. The development of differential gel electrophoresis (DIGE) circumvented most of these drawbacks. Samples are pre-labeled with fluorescent cyanines that can be used on the same gel, combining highly sensitive detection with a wide dynamic range and limiting the variability between gels and an extensive use of samples. Data analysis is achieved by using specific software, and spot picking for mass spectrometry analysis can be automated. The separation and purification of proteins can also be achieved through liquid chromatography which can be based on size, electrostatic or hydrophobic interactions. Sequence-tag affinity is very specific and commonly used in recombinant protein purification [75].

After separation, protein spots or fractions can be used in a wide range of enzymatic activity quantification or other bio-assays. The identification of proteins is generally a major step. Mass spectrometry is mostly used to identify proteins either after gel or gel-free separation. The analysis of entire proteins is called “top-down” analysis. However, in most applications, peptides are analyzed after proteolysis in a “bottom-up” analysis. “Shotgun proteomics” is a particular approach consisting of a bottom-up analysis of a protein mixture and has been reviewed by Zhang et al. [74]. It consists in hydrolyzing protein mixtures, separating them by liquid chromatography and analyzing them by tandem mass spectrometry. This technique is generally used in the gel-free analysis of protein mixtures [76]. Finally, mass spectrometry datasets are compared to an in silico digested protein database, scoring peptides and protein matches.

Many different workflows can be considered by combining separation with ionization and detection methods. Gel separation is mostly combined with matrix assisted laser desorption/ionization (MALDI) and time of flight (TOF) analyses [77]. Relative quantification is then provided by comparison of protein spot intensities, particularly in DIGE experiments. By contrast, liquid samples of proteins are often analyzed by electrospray ionization (ESI) and ion trap or orbitrap analyses. Relative quantification is possible using stable isotopes or the less-costly dimethyl labeling [78]. Technical improvements in LC-MS operation and data processing have recently set marks for label-free quantitative proteomics

[74, 79]. Those different techniques can be used to characterize enzymes or other proteins which can be extracted from the gut of termites.

II.9. From the digestive tract of termites to industrial enzyme production

Industrial enzyme production is the final aim of many studies. The efficiency of xylanases and cellulases depends on their peptide sequences, which depend on the DNA sequences. These sequences can be processed by original strains, or improved to increase the secretion in industrial reactors.

The simplest way to produce an enzyme consists in isolating a microorganism, cultivating it and recovering enzymes (cellulases or xylanases). The optimal conditions for enzyme production must also be investigated. Common industrial processes related to the production of extracellular enzymes are achieved with aerobic submerged cultures in stirred-tank reactors. Three elements must be considered : the strain, the equipment and the fermentation protocol. They must be selected in order to reach the highest production rates, and make it possible to achieve an optimal transcription, expression and posttranslational processing of enzymes to be optimal. A microorganism can be improved through mutagenesis, using chemical agents or UV radiation. However, this method is not easy to apply because of the high number of colonies to screen. In vivo recombination has been used to increase the efficiency of mutation techniques. Genetic engineering has considerably improved the performance of original strains. It is now possible to produce the enzyme in the strain that initially contained the gene, or to insert the gene into a specific strain, such as *Escherichia coli*. A modification of regulation is also possible, removing inhibitory mechanisms and enhancing positive regulation [80]. Many microorganisms have been reported as cellulase and xylanase producers. The enzymes showing a strong tolerance to temperature are particularly desirable. Indeed, these enzymes can be used in a wide range of temperatures, are resistant to high temperatures, and the cultivation of thermophilic microorganisms reduces contamination risks. These conditions also reduce viscosity and increase substrate solubility. Some multifunctional enzymes can degrade both cellulose and xylan simultaneously. This property has been observed in *Cellulomonas flavigena* and *Terendinibacter turnerae* T7902. Other microorganisms can also produce enzymatic complexes called cellulosomes, characterized by very high hydrolysis efficiency. Artificial cellulosomes have already been designed, and recombinant sequences have been inserted in *Saccharmoyces cervisiae* and *Bacillus subtilis* [81].

Once the cultivation of an isolated strain has been scaled up, enzymes still need to get recovered or purified without losing their activity. The complexity of this step depends on the production process that has been chosen, the degree of purity needed and some features of the enzymes.

II.10. Conclusions

The research of new enzymes in termite guts seems original and interesting. Many enzymatic activities have been reported : xylanases, cellulases and amylases. These activities, especially cellulases, are being looked for while second-generation biofuels are emerging. Hemicelluloses, which contain C_5-sugars, are also important, but their fermentation is not as simple as for C_6-sugars. Glucose is the most common C_6-sugar and the only component of cellulose, making cellulases particularly important. Although the understanding of lignocellulose degradation is becoming more and more precise, efficient enzymatic systems still need to be found.

Lignocellulose digestion in termite guts can reach very high levels and is partially achieved by symbiotic microorganisms : bacteria, protists and mycetes. However, it is difficult to understand such an efficiency. The termite gut is a very complex environment, and some paradoxes still need to be explained. The isolation of microorganisms could be a solution. Unfortunately, the opportunities are limited. Protists, supposed to play a key role in cellulose digestion, are often bound to bacteria in precarious symbioses. Consequently, a study based on isolation and further cultivation requires the conservation of fragile biochemical and physical balances through those steps. Another point is the lack of studies relating to mycetes, which are usually excellent enzyme producers. The knowledge in that field needs to be improved.

Nucleic acid-based methods, metagenomics and metatranscriptomics open a wide and highly detailed frame. They also generate precious data, useful to databanks. This high resolution combined with the more realistic snapshot provided by metaproteomics reflects trends in “omics” approaches and leads to more comprehensive studies of biological mechanisms. Integrated omics also progressively include metabolomics, corresponding to the study of metabolites present in a defined environment at a given time or produced by an enzyme activity on a substrate. Complex biological systems such as termite gut microbial communities require integrated approaches for characterization.

The cultivation of microbial strains, based on the isolation of purified organisms or not, is still a source of enzyme discovery. New findings in the field of the physicochemical environment and the biological components of the termite gut should reach beyond the limits of the "uncultivable". This highlights an optimistic and somewhat speculative point of view. Metaproteomic analysis of cultivated consortia could help in the characterization of possible interactions and lead to an understanding of the possible roles of microorganisms, considered as marginal, in buffering alimentary changes.

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Objectifs de la partie expérimentale

Les objectifs des manipulations entreprises dans ce travail sont les suivants :

- Identifier les conditions d'isolement des microorganismes les plus prometteuses à partir du tube digestif du termite *Reticulitermes flavipes*, en tenant compte du fait que les tentatives d'isolement vont inévitablement rompre certaines symbioses.
- Identifier les microorganismes les plus exploitables dans le cadre de la production d'enzymes parmi les bactéries, les mycètes et les protistes.
- Décrire quels sont les types d'activités enzymatiques développés par ces différents microorganismes, l'intérêt étant porté sur les cellulases et les hémicellulases.
- Déterminer quelles sont les sources de carbone les plus adaptées pour développer les activités enzymatiques recherchées dans les cultures pures des souches microbiennes (cellulases et hémicellulases).
- Décrire les propriétés des enzymes : effets du pH, de la température, conditions de travail optimales, poids moléculaires, produits d'hydrolyse.
- Observer l'effet de diètes artificielles appliquées aux élevages de termites. Cet aspect se décline en deux sous-objectifs. Le premier est l'étude pluridisciplinaire des communautés microbiennes résultantes de ces diètes à travers diverses techniques : microscopie, protéomique, métagénomique et microplaques BIOLOG®. Le second sous-objectif est l'isolement de microorganismes producteurs d'enzymes à partir des consortia microbiens issus des diètes artificielles appliquées.

Chaque chapitre de résultats est introduit par une courte présentation décrivant le contexte des manipulations ainsi que leurs objectifs spécifiques.

CHAPITRE III.

-

Isolation of amylolytic, xylanolytic, and cellulolytic microorganisms extracted from the gut of the termite *Reticulitermes flavipes* by means of a micro-aerobic atmosphere

Ce chapitre correspond à l'article intitulé "*Short communication - Isolation of amylolytic, xylanolytic, and cellulolytic microorganisms extracted from the gut of the termite Reticulitermes santonensis by means of a micro-aerobic atmosphere*" (Cédric Tarayre, Alison Brognaux, Julien Bauwens, Catherine Brasseur, Christel Mattéotti, Catherine Millet, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart) publié dans la revue **World Journal of Microbiology and Biotechnology** Volume 30, Issue 5, pp 1655-1660 (mai 2014).

Le but de cette première étape de la recherche est d'isoler des microorganismes capables de produire des enzymes intervenant dans la dégradation de la cellulose et des hémicelluloses à partir du termite.

La technique d'isolement utilisée doit tenir compte de plusieurs paramètres spécifiques au matériel biologique exploité :

- Premièrement, isoler des souches microbiennes les unes des autres va mener à une rupture des relations symbiotiques multiples établies au sein du tube digestif du termite.
- Deuxièmement, l'isolement suivi de la culture des microorganismes peut engendrer une ségrégation parmi les souches présentes. En effet, toutes ne sont certainement pas cultivables, qu'il s'agisse des bactéries comme des protistes.
- Enfin, il faut essayer de reproduire les conditions régnant dans l'intestin du termite.

Cette dernière observation est à la base de notre expérimentation. Les principales conditions régnant dans le tube digestif du termite et importantes dans le cadre des manipulations en laboratoire sont le pH, la température, l'oxygène et la composition du milieu. Dans le cas du termite *Reticulitermes santonensis*, le pH est proche de la neutralité, quelle que soit la portion de l'intestin considérée. La température a été fixée à 30^{o}C pour tous les essais. Cette température est souvent utilisée dans les essais de mise en culture, et est aussi très proche de la température appliquée à l'élevage des termites. Les milieux de culture destinés à obtenir un enrichissement en souches d'intérêt ont été choisis sur base de publications antérieures. Ces milieux comportaient toujours un substrat cellulosique ou hémicellulosique. Enfin, une des contraintes les plus complexes à considérer est sans nul doute celle de la teneur en oxygène car il existe un gradient de concentration dans le tube digestif des termites. C'est pourquoi des atmosphères contrôlées micro-aérobies ou anaérobies ont été utilisées. L'hydrogène est aussi présent dans le système digestif et sa concentration est également conditionnée par un gradient. Une atmosphère enrichie en hydrogène a aussi été employée. Les souches isolées par ces différentes techniques ont été identifiées par voie génétique. Elles ont ensuite été soumises à différents tests de mise en évidence d'activités enzymatiques sur milieux gélosés.

Résumé

Le but de ce travail était d'isoler des micro-organismes producteurs d'enzymes à partir de l'intestin du termite *Reticulitermes santonensis*. Les souches microbiennes ont été extraites des intestins et des atmosphères anaérobies (CO_2 ou CO_2/H_2) et micro-aérobie ont été utilisées pour tenter de stimuler leur croissance. Trois stratégies ont été testées. Tout d'abord, la suspension a été étalée sur un milieu gélosé contenant de la carboxyméthylcellulose, de la cellulose microcristalline ou du cellobiose. Cette méthode a permis d'isoler 2 bactéries : *Streptomyces* sp. ABGxAviA1 et *Pseudomonas* sp. ABGxCellA. La seconde stratégie consistait à enrichir la suspension microbienne dans un milieu liquide spécifique contenant soit de la carboxyméthylcellulose, soit de la cellulose microcristalline, soit du cellobiose. Des échantillons de ces cultures ont ensuite été étalés sur le milieu solide précédent contenant les 3 substrats précités. Cette méthode a mené à l'isolement d'*Aspergillus* sp. ABGxAviA2. Enfin, une troisième stratégie consistait à chauffer l'échantillon primaire puis à l'étaler sur milieu solide riche. Cette méthode a permis l'isolement de *Bacills subtilis* ABGx. Toutes ces opérations ont été menées en atmosphères contrôlées. Les 4 souches productrices d'enzymes isolées ont été obtenues en conditions aérobies uniquement. Par la suite, des tests enzymatiques ont été effectués sur ces 4 souches. *Streptomyces* sp. ABGxAviA1 a produit de l'amylase, tandis que *Pseudomonas* sp. ABGxCellA produisait de la β-glucosidase en plus de l'amylase. *Aspergillus* sp. ABGxAviA2 a montré des activités de type β-glucosidase, amylase, cellulase, et xylanase. Pour terminer, *Bacillus subtilis* ABGx s'est avéré produire de la xylanase et de l'amylase.

Mots-clés : Termite, *Bacillus*, *Pseudomonas*, *Streptomyces*, *Aspergillus*, atmosphère

Abstract

The aim of this work was to isolate enzyme-producing microorganisms from the tract of the termite *Reticulitermes santonensis*. The microorganisms were extracted from the guts and anaerobic (CO_2 or CO_2/H_2) and micro-aerobic atmospheres were used to stimulate growth. Three different strategies were tried out. First, the sample was spread on Petri dishes containing solid media with carboxymethylcellulose, microcrystalline cellulose or cellobiose. This technique allowed us to isolate two bacteria : *Streptomyces* sp. strain ABGxAviA1 and *Pseudomonas* sp. strain ABGxCellA. The second strategy consisted in inoculating a specific liquid medium containing carboxymethylcellulose, microcrystalline cellulose, or cellobiose. The samples were then spread on Petri dishes with the same specific medium containing carboxymethylcellulose, microcrystalline cellulose, or cellobiose. This led to the isolation of the mold *Aspergillus* sp. strain ABGxAviA2. Finally, the third strategy consisted in heating the first culture and spreading samples on agar plates containing rich medium. This led to the isolation of the bacterium *Bacills subtilis* strain ABGx. All those steps were achieved in controlled atmospheres. The four enzyme-producing strains which were isolated were obtained by using a micro-aerobic atmosphere. Later, enzymatic assays were performed on the four strains. *Streptomyces* sp. strain ABGxAviA1 was found to produce only amylase, while *Pseudomonas* sp. strain ABGxCellA was found to produce β-glucosidase as well. *Aspergillus* sp. strain ABGxAviA2 showed β-glucosidase, amylase, cellulase, and xylanase activities. Finally, *Bacillus subtilis* strain ABGx produced xylanase and amylase.

Keywords : Termite, *Bacillus*, *Pseudomonas*, *Streptomyces*, *Aspergillus*, atmosphere

III.1. Introduction

Termites are able to break the lignocellulosic complex. Lignocellulose is composed of lignin, cellulose, and hemicelluloses. Cellulose is a polymer of glucose units bound together by hemicelluloses. These macromolecules are more complex and made of various monomers : D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, and D-glucuronic acid. Hemicelluloses are linked to lignin by ester or ether bonds. Lignin is a complex macromolecule resulting from the polymerization of p-coumaryl, coniferyl, and sinapyl alcohols [1].

The termite *Reticulitermes santonensis*, a wood-eating termite, contains a highly diversified microflora composed of bacteria, mycetes, and protists. These microorganisms act as a microbial consortium which is able to break the lignocellulose components. In 2005, Yang et al. [2] published an excellent review about the strains living in the gut of *R. santonensis*. The termite gut is divided into three parts : foregut, midgut, and hindgut. That study concluded that there are differences in microbial communities in accordance with the location considered as well as the distance from the gut wall. The major phyla of prokaryotes which were identified were *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Proteobacteria*. Tartar et al. [3] identified the fungi genera *Aspergillus*, *Neurospora*, *Verticillium*, *Trichoderma*, and *Thermomyces*.

The conditions relating to pH and O_2 in the gut of *R. flavipes*, which can be considered as *R. santonensis* [4], were studied previously [5]. The pH values in the gut vary from 6 to 7. However, the repartition of oxygen is more complex. It can penetrate the peripheral hindgut up to 50 to 200 µm. The central zone of the hindgut was found to be completely anoxic because of the oxygen consumption by the hindgut microflora [6].

This study relates to the isolation of enzyme-producing strains isolated from the gut of the termite *R. santonensis*. Specific atmospheres were used to simulate micro-aerobic and anaerobic environments in order to copy the conditions which can be found inside the termite gut. The most interesting microorganisms able to synthesize enzymes were identified on the basis of 16S and 28S rDNA sequences.

III.2. Materials and methods

III.2.1. *Termites*

Reticulitermes santonensis Feytaud (Rhinotermitidae) was harvested on the Island of Oleron (France). The termites were kept in darkness at a temperature of 27°C with a relative humidity of 70%. The termites were first fed on pinewood which was gradually replaced by poplar wood.

III.2.2. *Isolation of microorganisms*

Some termites were washed in ethanol to prevent surface contaminations, then in water and held with two dissection forceps, as described before [7]. Intestines were extracted under sterile conditions and put into an Eppendorf tube containing 200 µl of YPD medium (10 g/l peptone, 5 g/l yeast extract, and 10 g/l glucose). The sample was kept at 40°C for 24 hours. The intestines were then sterilely crushed and the volume was adjusted to 1.5 ml with sterile YPD medium. The volume was divided into three parts each of which was put into a tube containing 5 ml of medium. The first tube was saturated with an atmosphere composed of 16% of CO_2 and 84% of air to simulate a micro-aerobic atmosphere (atmosphere A). The second atmosphere consisted of 100% of CO_2 (atmosphere B) and the third atmosphere containing 85% of H_2/15% CO_2 (atmosphere C) were used to create anaerobic conditions. The tubes were put on an agitator at 30°C for 6 days. The first group of microorganisms was isolated by spreading 100 µl of various dilutions of the contents of each tube on agar plates containing modified GBG medium [8]. Different substrates were added as carbon sources to this medium : carboxymethylcellulose 2.5 g/l (CMC), microcrystalline cellulose 2.5 g/l (MC), or cellobiose 2.5 g/l (CELL). The agar plates were kept in darkness at 30°C and the microorganisms were grown in the atmospheres A, B or C for 3 days before isolation. The second group of microorganisms was obtained by pouring the contents of the tubes into flasks containing liquid GBG medium with the three carbon sources described above. The aim of this step was to enrich the microbial population able to hydrolyze cellulose or cellobiose. After 14 days, 100 µl of different dilutions of each liquid medium were spread on agar plates containing solid GBG medium with the three carbon sources (same concentrations). The agar plates were kept in the same conditions as before, prior to isolating the second group of strains. The third group of microorganisms was obtained by heating the microbial suspension stemming from the crushed intestines (10 min, 80°C). Then, 100 µl of culture at different dilution rates were spread on agar plates containing rich solid medium (glucose 20 g/l, yeast

extract 10 g/l, casein peptone 10 g/l, agar 17 g/l). This method led to the isolation of spore-developing bacteria. The whole method is illustrated in **Figure 1**.

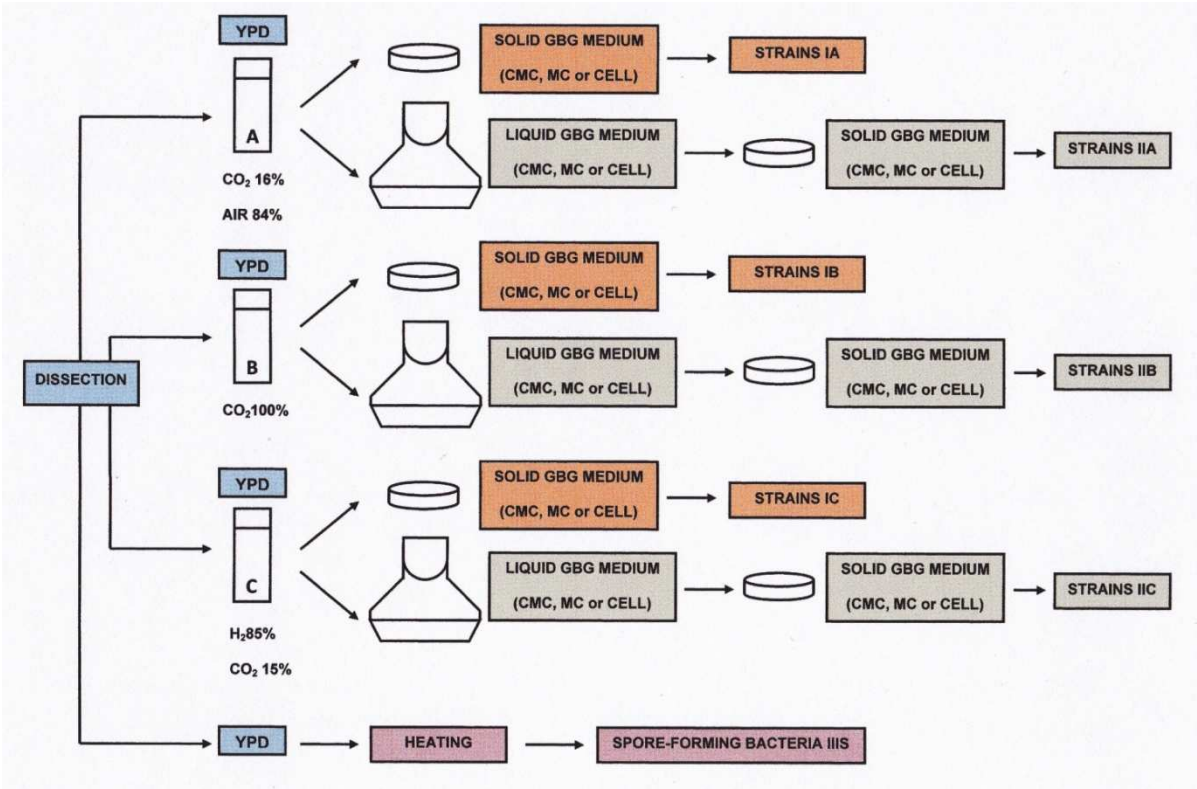

Figure 1. Strategies of isolation of enzyme-producing microorganisms from the gut of *R. santonensis*. The isolation of microorganisms was performed in different atmospheres and in solid or liquid media added with carboxymethylcellulose (CMC), microcrystalline cellulose (MC), or cellobiose (CELL). Spore-forming bacteria were isolated after heating the sample.

III.2.3. *Investigation of enzymatic activities*

The following activities were observed in each strain : endo-1,4-β-D-xylanase, endo-1,4-β-D-glucanase, β-glucosidase, and α-amylase. The medium used for β-glucosidase investigation consisted of casein peptone (8 g/l), esculin sesquihydrate (1 g/l), ammonium ferric citrate (1 g/l) and agar (17 g/l), pH 7.4. The basic medium used to detect all the other activities was derived from [9] and composed of $NaNO_3$ (2 g/l), $MgSO_4.2H_2O$ (0.5 g/l), KCl (0.5 g/l), casein peptone (0.2 g/l), agar (17 g/l). Trace elements were added to the medium (2.86 mg/l H_3BO_3, 1.81 mg/l $MnCl_2.4H_2O$, 0.222 mg/l $ZnSO_4.7H_2O$, 0.39 mg/l $NaMoO_4.2H_2O$, 0.079 mg/l $CuSO_4.5H_2O$, and 0.0494 mg/l $Co(NO_3)_2.6H_2O$). The following components were used as carbon sources : 0.5 g/l AZCL-xylan medium (endo-1,4-β-D-xylanase activity), 0.5 g/l AZCL-HE-cellulose medium (endo-1,4-β-D-glucanase), 0.5 g/l

AZCL-amylose medium (α-amylase activity). AZCL media were supplied by Megazyme (Megazyme International Ireland - Bray Business Park - Bray, Co. Wicklow, Ireland). All the strains were first cultivated in rich liquid media. Bacteria were grown in the following medium : glucose 20 g/l, yeast extract 10 g/l, casein peptone 10 g/l. Molds were cultivated in potato dextrose broth (24 g/l) containing chloramphenicol (0.5 g/l). After 24 hours of cultivation, 80 µl of each culture were spot plated on the different media (three replicates) under the atmospheres chosen in accordance with the conditions of isolation. The plates were incubated for 3 days at 30°C.

III.2.4. *Identification of enzyme-producing microorganisms*

DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega Benelux BV Branch Office - Schipholweg 1 - 2316 XB LEIDEN - The Netherlands). The primers were provided by Eurogentec (Eurogentec S.A. – Belgium). They were the universal primers 357F and 1100 R, 8F, and 1492R [10, 11], and LR0R and LR6 [12]. The primers 357F/1100R and 8F/1492R were used to amplify 16S rDNA of bacteria, while the primers LR0R/LR6 were used to amplify 28S rDNA of mycetes. The program used for the amplification of 16S rDNA (primers 357F/1100R) and 18S rDNA (primers LR0R/LR6) consisted of 5 min of denaturation (95°C), 25 cycles of amplification composed of 30 s at 95°C, 30 s at 54°C, 2 min at 72°C, and a final extension of 10 min at 72°C. The program used for 16S rDNA (primers 8F/1492R) amplification included 10 min of denaturation (95°C), 30 cycles of amplification composed of 1 min at 95°C, 1 min at 54°C, 2 min at 72°C, and a final extension of 10 min at 72°C. PCR products were used for the sequencing reaction. Sequencing was achieved by Progenus® (Rue des Praules, 2 – 5030 Sauvenière – Belgium) with a Genetic Analyzer 3130 designed by Applied Biosystems®. The sequences were aligned with the Vector NTI® program and the homologous sequences present in the GenBank database were identified using the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) BlastN program [13].

III.3. Results and discussion

III.3.1. *Isolation and identification of microorganisms*

This strategy enabled us to isolate 12 strains. However, the identification was only performed with the strains displaying enzymatic activities. All the assays were performed in culture media with low concentrations of nitrogen to reconstitute the conditions found in the termite gut. No strain was isolated in anaerobic atmospheres (CO_2 100% and H_2 85%/CO_2

15%). This observation may be due to acidification caused by CO_2. Another gas could have been used to check that, such as N_2. On the other hand, it was established that oxygen penetrates the outer part of the hindgut lumen by 50-200 µm. Consequently, anoxic conditions are limited to the dilated compartments of the central hindgut portion, which is mainly colonized by anaerobic protists in lower termites [6]. Another point is that it is particularly difficult to prevent oxygen transfers during the crushing step of the intestines. Therefore, it is not surprising to isolate only aerobic strains from the termite gut.

Three strains were isolated in micro-aerobic atmospheres and one spore-forming bacterium was found after heating the sample. It is important to note that no anaerobic strain was isolated. All the strains were identified on the basis of rDNA sequences. The first strain belonged to the group of *Strains IAMC* (isolated in micro-aerobic atmosphere, on solid GBG medium containing microcrystalline cellulose, see **Figure 1**). It was identified as *Streptomyces* sp., and was named *Streptomyces* sp. strain ABGxAviA1 (97% identity with *S. sampsoni*, *S. somaliensis*, *S. exfoliatus*, *S. albus*, *S. coelicolor*, *S. lividens*, *S. griseus*, *S. viridochromogenes* ; GenBank ID : KF297884). The second strain was isolated in the group of *Strains IACELL* (isolated in micro-aerobic atmosphere, on solid GBG medium containing cellobiose, see **Figure 1**) and was identified as *Pseudomonas* sp., and further named *Pseudomonas* sp. strain ABGxCellA (99% identity with *P. citronellolis*, *P. aeruginosa*, *P. delhiensis*, *P. knackmussii*, *P. denitrificans*, *P. nitroreducens*, *P. azelaica*, *P. multiresinivorans* ; GenBank ID : KF297883). The third strain was a mold isolated in the group of *Strains IIAMC* (isolated in microaerobic atmosphere, on liquid GBG medium containing microcrystalline cellulose, see **Figure 1**) and was found to be an *Aspergillus* sp. The strain was given the name *Aspergillus* sp. strain ABGxAviA2 (95% identity with *A. fumigatus* ; GenBank ID : KF297885). The identity of the strain was confirmed by the mycotheque of Louvain-la-Neuve (Croix du sud 2, bte L7.05.06, 1348 Louvain-la-Neuve, Belgium). The spore-developing bacterium (*Group III*, see **Figure 1**) was identified as a *Bacillus* sp. (99% identity with *B. subtilis*, *B. methylotrophicus*, *B. tequilensis*, *B. licheniformis*). Further DNA analysis was described in another article (Tarayre et al. 2013). Gyrase (GenBank ID : JX545344) and xylanase (GenBank ID : JX545345) sequences confirmed that the strain was a *Bacillus subtilis*. The strain was named *Bacillus subtilis* strain ABGx (16S GenBank ID : KF297882).

III.3.2. *Enzymatic activities*

All the strains which were isolated were aerobic. Consequently, the enzymatic assays were performed in aerobic conditions. The following enzymatic activities were tested on the isolated strains : endo-1,4-β-D-xylanase, endo-1,4-β-D-glucanase, β-glucosidase, and α-amylase. The results are presented in **Table 1**. Many examples of α-amylase- and β-glucosidase-producing strains of *Pseudomonas* can be found in literature. For instance, Kimura et al. [14] studied the maltotetraose-producing strain of *Pseudomonas stutzeri*. Rickard et al. [15] studied the production of a cloned β-glucosidase from *Pseudomonas* sp. strain PS2-2. *Pseudomonas* species have already been found in the termite gut, such as *P. aeruginosa* and *P. cepacia* [16]. Pourramezan et al. [17] isolated an endo-1,4-β-D-glucanase-producing strain of *Pseudomonas* sp. from the xylophagous termite *Microcerotermes diversus*. *Pseudomonas* sp. strain ABGxCellA was isolated on a medium containing cellobiose. Although it was logical to detect a β-glucosidase activity hydrolyzing esculin, an amylase activity was also observed.

Table 1. Enzymatic activities detected in the four enzyme-producing strains.

Strain	**Endo-1,4-β-D-xylanase**	**Endo-1,4-β-D-Glucanase**	**β-glucosidase**	**α-amylase**
***Streptomyces* sp. strain ABGxAviA1**	-	-	-	***YES***
***Pseudomonas* sp. strain ABGxCellA**	-	-	***YES***	***YES***
***Aspergillus fumigatus* strain ABGxAviA2**	***YES***	***YES***	***YES***	***YES***
***Bacillus subtilis* strain ABGx**	***YES***	-	-	***YES***

Streptomyces species producing xylanase were also found in *R. santonensis* [18], and α-amylase-producing strains were studied previously [19]. Watanabe et al. [20] isolated

actinomycetes from the termites *Coptotermes formosanus*, *Reticulitermes speratus*, *Neotermes koshunensis*, *Odontotermes formosanus* and *Hodotermopsis japonica*. The strains which were isolated were closely related to *Streptomyces* and able to degrade lignin and carboxymethylcellulose. Pasti et al. [21] extracted actinomycetes belonging to the genera *Streptomyces* and *Micromonospora* from the hindgut of the termites *Macrotermes*, *Armitermes*, *Odontotermes* and *Microcerotermes* spp. In the present case, *Streptomyces* sp. strain ABGxAviA1 was only able to produce amylase, although it had been isolated on a medium containing microcrystalline cellulose.

Another strain of *Bacillus subtilis* was extracted from *R. santonensis* [16]. Bashir et al. [22] also isolated cellulolytic strains of *Bacillus subtilis*, *B. cereus*, *B. pumilus* and *B. licheniformis* showing endo-1,4-β-D-glucanase activity from termite guts. Dheeran et al. [23] isolated a xylanolytic strain of *Paenibacillus macerans* from wood-eating higher termites. In another study, it was possible to identify by DGGE some strains of *Bacillus* able to degrade xylan and carboxymethylcellulose from the gut of the termite *Odontotermes formosanus* [24]. It was also possible to identify a symbiosis between *Bacillus* and *Clostridium*. Cellulolytic *Bacilli* were found to create the anaerobic environment necessary for the growth of *Clostridium* [25]. However, this is the first time that a strain of *Bacillus subtilis* has been extracted from the gut of a termite and showed xylanase and amylase activities.

Other strains of *Aspergillus fumigatus* were found to produce amylase, cellulase, and xylanase [26, 27]. As far as we know, this is however the first time that this strain has been isolated from the gut of this termite. Other filamentous fungi belonging to the *Aspergillus* genus, such as *A. niger* and *A. oryzae*, have been found to be excellent enzyme producers [28, 29]. Moreover, Tartar et al. [3] also identified a xylanase-producing strain of *A. nidulans* in the gut of *R. flavipes* by transcriptomics. This is nevertheless the first time that a strain close to *A. fumigatus* (95 % of identity) has been found inside termite guts. Several species of *Aspergillus* cause the disease of aspergillosis. *A. fumigatus* is considered as the most common etiological agent in that disease [28].

This article provides a new method of isolation of microorganisms focusing on a specific group of strains living in the termite gut. Although it was used for termites, such a method can apply to other sources. The main problem encountered was the difficulty in simulating the original conditions of the termite gut. This is particularly complex because it is a culture medium the composition and the microflora of which depend on location. It can be

considered as a natural continuous-flow fermentor divided into organized sections. Therefore, termites offer huge prospects as regards the isolation of enzyme-producing microorganisms.

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CHAPITRE IV.

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Xylanase production by a strain of *Bacillus subtilis* extracted from the gut of the termite *Reticulitermes flavipes*

Ce chapitre correspond à l'article intitulé "*Isolation and cultivation of a xylanolytic Bacillus subtilis extracted from the gut of the termite Reticulitermes santonensis*" (Cédric Tarayre, Alison Brognaux, Catherine Brasseur, Julien Bauwens, Catherine Millet, Christel Mattéotti, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart) publié dans la revue **Applied Biochemistry and Biotechnology** Volume 171, Issue 1, pp 225-245 (septembre 2013).

L'utilisation d'atmosphères contrôlées pour isoler les microorganismes du système digestif des termites a abouti aux différents résultats et considérations suivants :

- Seule l'atmosphère micro-aérobie a permis d'isoler des microorganismes. Ensuite, les souches isolées de cette atmosphère ont pu être cultivées en conditions aérobies classiques. Cette observation est paradoxale étant donné qu'une portion non négligeable du système digestif se trouve en anaérobiose.
- Notre méthode a permis d'isoler des bactéries et une moisissure. Cette dernière présente des activités endoglucanase et endoxylanase intéressantes. Cependant, l'identification génétique a montré qu'il s'agit d'une souche d'*Aspergillus fumigatus*, un organisme de classe II, donc difficilement exploitable.
- Des activités cellulases n'ont pas pu être mises en évidence chez les bactéries isolées du termite.
- Une souche de *Bacillus subtilis* (nommée ABGx) sporulante et xylanolytique a pu être isolée et purifiée.

Le chapitre suivant porte sur la caractérisation de cette souche de *Bacillus subtilis* et la production de la xylanase.

Résumé

Le but de ce travail était d'isoler et cultiver des micro-organismes xylanolytiques extraits du tractus intestinal du termite *Reticulitermes santonensis*. Les sucres réducteurs libérés suite à l'hydrolyse du xylane peuvent par la suite être fermentés en bioéthanol. Une souche xylanolytique de *Bacillus subtilis* a été isolée du hindgut du termite et a montré des activités amylase et xylanase. La bactérie a été cultivée en milieu liquide contenant des déchets agricoles : du son de blé, de la drêche de blé et du tourteau de colza. Le son de blé a mené à l'activité xylanase la plus élevée, bien que le développement de la souche ait été plus lent que dans les autres milieux de culture. Il a été possible d'atteindre des activités xylanase de 44.3, 33.5, and 29.1 U.I./ml dans les milieux contenant respectivement du son de blé, de la drêche de blé et du tourteau de colza. La spectrométrie de masse a permis d'identifier une large gamme d'oligomères de xylose, suggérant une activité de type endo-xylanase. L'enzyme a montré une stabilité jusqu'à 45^{o}C et un pH optimal proche de 8.

Mots-clés : Xylanase, *Bacillus subtilis*, termite, intestin de termite, *Reticulitermes santonensis*

Abstract

The aim of this work was the isolation of xylanolytic microorganisms from the digestive tract of the termite *Reticulitermes santonensis*. The reducing sugars released after the hydrolysis of xylans can be further fermented to provide bioethanol. A xylanolytic strain of *Bacillus subtilis* was isolated from the hindgut of the termite and displayed amylase and xylanase activities. The bacterium was grown on media containing agricultural residues : wheat bran, wheat distiller's grains, and rapeseed oil cake. Wheat bran led to the highest induction of xylanase activity, although the development of the strain was less fast than in the other media. It was possible to reach maximal xylanase activities of 44.3, 33.5, and 29.1 I.U./ml in the media containing wheat bran, wheat distiller's grains, and rapeseed oil cake, respectively. Mass spectrometry identified a wide range of xylose oligomers, highlighting an endoxylanase activity. The enzyme was stable up to 45^{o}C and displayed an optimal pH close to 8.

Keywords : Xylanase, *Bacillus subtilis*, termite, termite gut, *Reticulitermes santonensis*

IV.1. Introduction

Reticulitermes santonensis is a lower termite symbiotically related to numerous microorganisms such as bacteria, fungi, and protists. These symbiotic microorganisms living in the termite gut play a key role in lignocellulose digestion [1]. Hydrolytic bacteria, fungi, and protists help the termite to digest the wood fibers [2]. The biodiversity of the termite gut was studied previously, and different enzymatic activities coming from the symbionts and the termite itself were identified. It was demonstrated that the termite gut contains amylases, cellulases, and xylanases [3].

Wood is mainly composed of cellulose, hemicelluloses, and lignin. Plants are usually composed of 40-50% cellulose, 27-31% hemicelluloses and 20-30% lignin in the dry biomass [4, 5]. Xylans are hemicelluloses that consist of polysaccharides containing a central chain of β-1,4-linked D xylosyl residues. Arabinofuranosyl, 4-O-methylglucuronosyl, acetyl groups, and phenolic acid residues substitute the central chain at various frequencies, depending on the source of xylan [6, 7]. Xylan structure can be simple (1,4-β-linked polyxylose) or more complex (highly branched polysaccharides). Hardwoods and softwoods are made of 15-30% and 7-12% xylan, respectively [8].

The degradation of wood fibers involves three types of enzymes. The first group is composed of oxidative enzymes able to attack lignin. The second group comprises enzymes able to degrade hemicelluloses and cellulose (such as xylanase), which finally lead to monosaccharides. The enzymes of the third group, such as superoxide dismutase and glyoxalate oxidase, cooperate with the enzymes of the first group without targeting the wood directly. They oxidize glucose coming from cellulose hydrolysis and reduce quinones [9]. Xylanases are mainly used in the paper, feed, and baking industries [10]. They can be used to reduce the use of chlorine and chlorine dioxide produced during the paper-making process [11]. The use of xylanases in the paper industry involves a very low cellulase activity [12]. They are also used in animal feed, improvement of plant cells, clarification of juices, and can be used in the production of biosurfactants [13]. Xylanases can also be used on lignocellulosic materials to hydrolyze xylans and to produce xylose, which can be fermented to produce bioethanol by microorganisms such as *Pichia stipitis* [14].

Xylanases have been reported from various microorganisms : bacteria, fungi, yeasts, and actinomycetes [12, 13, 15-19]. Some species of *Bacillus* are able to produce xylanase and have been reported earlier, such as *Bacillus subtilis*, *circulans*, *cereus*, *pumilus*,and

stearothermophilus [11, 20-24]. Microbial enzymes are highly specific for their substrates and no chemical modification of the substrate is observable. Moreover, reaction conditions are easy to obtain. Enzymes that are able to hydrolyze at neutral and alkaline pH are particularly interesting [20].

The termite gut provides opportunities to isolate potential wood-degrading strains and can be used as a source of hydrolytic microorganisms.

IV.2. Materials and Methods

IV.2.1. *Organisms*

Reticulitermes santonensis Feytaud (*Rhinotermitidae*) was obtained from the Island of Oleron (France). The termites were cultivated in darkness at 27°C with a relative humidity of 70%. The wood used as a diet was mostly pinewood and was gradually replaced by poplar wood.

IV.2.2. *Isolation of Bacillus subtilis from Reticulitermes santonensis*

50 termites were washed in ethanol, then water and held with two dissection forceps. One was used to hold the head and the second one was used to remove the cuticle at the back of the termite. The digestive tracts were then extirpated and put in a tube containing 200 µl YPD medium (10 g/l peptone, 5 g/l yeast extract, and 10 g/l glucose). A disinfected needle was used to pierce the digestive tracts. Different dilutions were prepared in a solution containing 1 g/l peptone, 5 g/l NaCl, and 0.2% (w/v) Tween80. The different dilutions were then heated at 80°C for 10 minutes. 100 µl of each microbial suspension were finally spread on Petri dishes containing the following medium : 10 g/l peptone, 20 g/l glucose, 10 g/l yeast extract, and 16 g/l agarose.

IV.2.3. *Genetic identification*

DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega Benelux BV Branch Office - Schipholweg 1 - 2316 XB LEIDEN - The Netherlands). The primers were provided by Eurogentec (Eurogentec S.A. – Belgium). The primers used were gyrase primers to amplify the gyrase gene (GyrA : 5'-CAGTCAGGAAATGCGTACGTCCTT-3'; GyrB : 5'-CAAGGTAATGCTCCAGGCATTGCT-3' [25]), and xylanase primers to amplify the xylanase gene :

- XylA : 5'-ACGAATTCCATGTTTAAGTTTAAAAAGAATTTCTTAGTT-3';
- XylB : 5'-GAGGATCCTTACCACACTGTTACGTTAGAACTTCCACT-3'.

The primers were derived from [26] and used by [27]. The program used for xylanase amplification was 5 min of denaturation (94°C), 35 cycles of amplification composed of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C [27]. The program used for gyrase amplification was 5 min of denaturation (95°C), 25 cycles of amplification composed of 30 sec at 95°C, 30 sec at 47°C, 2 min at 72°C, and a final extension of 10 min at 72°C. PCR products were used for the sequencing reaction. The sequences were aligned with the Vector NTI® program and the homologous sequences present in GenBank database were identified by using BlastN as software. The amino acid sequence of xylanase was deduced from the nucleotide sequence using Mega 5.0® software. InterProScan® (www.ebi.ac.uk/Tools/Inter-ProScan) was applied for the analysis of the xylanase sequence.

IV.2.4. *Culture conditions*

Preliminary tests were performed to investigate the best pH conditions for an optimal bacterial development and a maximal production of xylanase. These tests were done in a rich culture medium containing 10 g/l peptone, 20 g/l glucose, and 10 g/l yeast extract. A pH value of 7 provided the best results and was consequently chosen for further analysis. Four media were used to investigate the growth of *Bacillus subtilis* and xylanase production. These media were modified from [21]. Each culture medium was prepared in a solution containing 1 g/l tryptone, 2 g/l KH_2PO_4, 0.2 g/l NH_4Cl, 0.75 g/l $MgSO_4$, 2.5 g/l sodium citrate, 2.86 mg/l H_3BO_3, 1.81 mg/l $MnCl_2.4H_2O$, 0.222 mg/l $ZnSO_4.7H_2O$, 0.39 mg/l $NaMoO_4.2H_2O$, 0.079 mg/l $CuSO_4.5H_2O$, and 0.0494 mg/l $Co(NO_3)_2.6H_2O$. Four substrates were separately used to increase xylanase activity : Wheat Bran (WB), Wheat Distiller's Grains (WDG), Rapeseed Oil Cake obtained after Mechanical pressing (ROCM), and Rapeseed Oil Cake obtained after Solvent extraction (ROCS) at a concentration of 30 g/l.

IV.2.5. *Investigation of enzymatic activities*

The following activities were tested : endo-1,4-β-D-xylanase, cellulase, and amylase. The following media were used : 1) Endo-1,4-β-D-xylanase activity : 1 g/l AZCL-xylan medium (Megazyme), Na acetate 25 mM (pH 4.7), 16 g/l agar; 2) Cellulase activity : 1 g/l AZCL-HE-cellulose medium (Megazyme), Na acetate 25 mM (pH 4.5), 16 g/l agar; 3) Amylase activity : 1 g/l AZCL-amylose medium (Megazyme), Na acetate 100 mM (pH 6), 16 g/l agar. The samples (80 µl of crude culture and 80 µl of a sample filtered on a cellulose acetate membrane; 0.45-µm porosity) were dropped on Petri dishes containing the different solid media. Anti-protease (Complete, Mini, EDTA-Free Protease Inhibitor Cocktail Tablets, Roche) was added to the culture samples to avoid degradation of enzymes caused by potential

proteases. Buffers and pH values were chosen according to the manufacturer's suggestions (Megazyme International Ireland - Bray Business Park - Bray, Co. Wicklow, Ireland). Petri dishes were kept at 30°C for one day before reading the results.

IV.2.6. *Enzymatic assay of xylanase*

Xylanase activity was determined using dinitrosalicylic acid by a method developed previously [28]. Each sample was centrifuged (10,000 g; 5 minutes) and the determination of xylanase activities was performed on the supernatant. A solution containing birchwood xylan (10 g/l) prepared in a phosphate buffer (0.2M, pH 7) was used as a substrate. A volume of 1750 µl of xylan solution was added to 250 µl of enzyme samples (three repetitions). The reaction was performed at 40°C for five minutes before adding 3 ml 3,5-dinitrosalicylic acid (DNS) reagent [28]. The contents were placed in a water bath (100°C, 5 minutes) before cooling at room temperature. 10 ml of distilled water was added to the samples before centrifugation (10000 g, 5 minutes). The absorbance was measured at a wavelength of 550 nm and xylanase activities were calculated on the basis of a calibration curve built using xylose. One International Unit of xylanase activity is defined as the amount of enzyme able to provide 1 µmol of xylose in one minute. Controls were also performed on the solution of xylan and enzymes samples considered separately.

IV.2.7. *Xylanase extraction*

Xylanase was extracted from the culture medium containing wheat bran, which led to the highest amount of xylanase. Culture media were filtered through a nylon cloth (porosity of 0.1mm) and the filtrate was conserved at 4°C. The bran was rinsed two times with 20 ml of phosphate buffer (0.2M, pH 7) and filtered through the nylon cloth. The liquid fraction was added to the first filtrate before centrifugation (10000 g, 5 minutes). Enzyme was kept at -20°C and used for pH and temperature investigations and SDS-PAGE analysis.

IV.2.8. *Zymographic assay*

Cultivation samples were centrifuged, and the pellets were resuspended in 100 mM Tris-HCl buffer pH 8, followed by sonication, and then 12 µg of protein (3.9 µl) was mixed with 7.8 µl Laemmli Sample Buffer (Bio-Rad), with 5% β-mercaptoethanol. Electrophoresis was performed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.2% xylan from birchwood (Sigma-Aldrich). Zymography was done as previously [29] with slight modifications. PageRuler Prestained Protein Ladder (Fermentas) was added to check the molecular weight of the bands observed. To visualize enzymatic activity, the gel was rinsed

twice for 30 min in 100 mM Tris-HCl buffer pH 8, containing 1% Triton X-100 to remove SDS and then incubated for 2 h in 100 mM Tris-HCl buffer pH 8 to allow enzymatic activity on xylan. The substrate was then stained with Congo red (Sigma-Aldrich) for 30 minutes and destained with 1 M NaCl. Destained bands were visible and the gel was imaged under UV light.

IV.2.9. *Detection of xylose oligomers*

Xylose oligomers were measured with a mass spectrometer. A matrix of 2,5-dihydroxybenzoic acid (DHB) was used at 20 $mg.ml^{-1}$ in acetonitrile/water 0.1% trifluoroacetic acid. Samples were prepared with a mixture of 1 µl hydrolysate solution and 1 µl matrix. Measurements were performed with a time-of-flight mass spectrometer, Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen Germany), equipped with a frequency-tripled Nd : YAG laser (355 nm). Calibration was performed with peptide calibration standard II solution (Bruker). The spectra were recorded in positive reflectron ion mode, with an accelerating voltage of 25 kV and a laser shot rate of 100 Hz. The voltages of the electrodes 1 and 2 were set at 21.8 kV and 9.5 kV, respectively to carry out the pulsed ion extraction. The time delay before the ion extraction was set at 30 ns. A total of 10 000 shots was accumulated for each mass spectrum. The acquisition m/z range started at 400 to exclude high intensity signals from matrix ions.

IV.3. Results and Discussion

IV.3.1. *Genetic identification*

DNA was extracted from 2 ml of a one-day culture. The DNA sequencing led to two fragments of 778 nucleotides (gyrase gene) and 662 nucleotides (xylanase gene). The sequences are available on GenBank (gyrase DNA GenBank ID : JX545344, xylanase DNA GenBank ID : JX545345). The NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/)-BlastN program [30] was used to align and identify the sequences. The genes coding for gyrase and xylanase led to the identification of *Bacillus subtlis* with 100 % and 99 % identity, respectively. The strain was named *Bacillus subtilis* strain ABGx.

The autoradiography of the xylanase gene is presented in **Figure 1**.

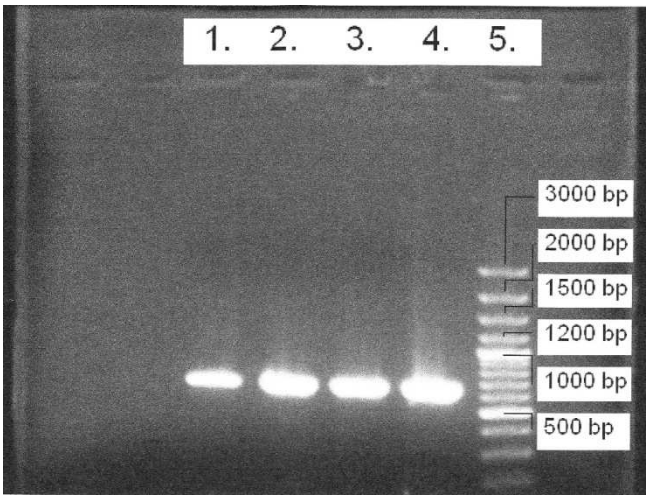

Figure 1. PCR products viewed on an agarose gel (1%) after electrophoresis. Row 5 : Weight Markers, length of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, and 3000 nucleotides. Row 1, 2, 3, and 4 : PCR products obtained with xylanase primers (XylA and XylB) by amplification of the xylanase gene.

It was possible to deduce the amino acid sequence from the nucleotide sequence using Mega 5.0®. The amino acid sequence is available on the site of NCBI (Genpept GI : 410519079). InterProScan® classified the xylanase as being in the Glycoside Hydrolase Family 11 (Pfam classification : PF00457, www.pfam.sanger.ac.uk). The enzymes of Glycoside Hydrolase Family 11 are monospecific and consist only of xylanases. These enzymes are also most active on long-chain xylo-oligosaccharides and display large substrate-binding clefts. Family 11 enzymes are generally characterized by a high isoelectric point and a low molecular weight [31]. The amino acid sequence possesses several tryptophan residues, which are thought to play a key role in the active sites of the enzyme. This was demonstrated by [32] who used N-bromosuccinimide, a specific oxidizing agent, to modify tryptophan residues. Tryptophan residues in sugar binding proteins are usually involved in the interaction with the sugar moiety. The amino acid sequence of the xylanase shares 99% identity with a xylanase produced by *Bacillus subtilis* strain 168 (1A1) [33].

IV.3.2. *Investigation of enzymatic activities*

AZCL solid media were used to investigate the enzymatic activities developed by *Bacillus subtilis* strain ABGx. No cellulase activity was detected. However, xylanase and amylase activities were highlighted and the intensity of coloration was the same for both filtered and crude samples. Consequently, these enzymes are extracellular. The primer XylA contains a DNA sequence 5'-ATGTTTAAGTTTAAAAAGAATTTCTTAGTT-3' relating to a signal peptide. This sequence codes for the amino acid sequence Met-Phe-Lys-Phe-Lys-

Lys-Asn-Phe-Leu-Val, a part of a sequence reported by [26]. In that previous study, a peptide of 28 amino acids (Met-Phe-Lys-Phe-Lys-Lys-Asn-Phe-Leu-Val-Gly-Leu-Ser-Ala-Ala-Leu-Met-Ser-Ile-Ser-Leu-Phe-Ser-Ala-Thr-Ala-Ser-Ala) was found to be a signal peptide. In the present study, the amino acid sequence deduced from the DNA sequence displays the same structure. Consequently, the xylanase sequence explains the extracellular character of the enzyme.

IV.3.3. *Xylanase production by B. subtilis ABGx using lignocellulosic materials*

The growth of *Bacillus subtilis* ABGx was evaluated with optical densities (OD). Initial OD values were subtracted from each value to consider the initial turbidity of each culture medium (**Figure 2**). Agro-residues were tested in this study to avoid the use of purified xylanase inducers such as commercial xylan, which is very expensive. Wheat bran (WB) is the outer layer of wheat grains, composed of aleurone and pericarp [34]. Wheat distiller's grains (WDG) are a dried by-product obtained after wheat ethanol distillery composed of grains, soluble fractions, and bran [35]. Rapeseed oil cake is a residue coming from the oil extraction from rape. A first extraction is performed through a mechanical pressing, which leads to oil and the first solid residue (Rapeseed Oil Cake obtained after Mechanical pressing; ROCM). Sometimes, a second extraction is performed to recover the remaining oil trapped in the cake. This extraction leads to a second type of oil cake (Rapeseed Oil Cake obtained after Solvent extraction; ROCS) [36].

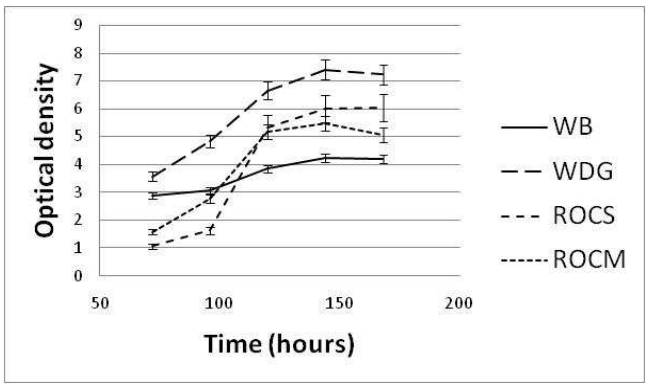

Figure 2. Evolution of optical densities in the four culture media of *Bacillus subtilis* strain ABGx. WB = Wheat Bran, WDG = Wheat Distiller's Grains, ROCS = Rapeseed Oil Cake obtained after Solvent extraction, ROCM = Rapeseed Oil Cake obtained after Mechanical pressing.

The results were analyzed with MINITAB16® statistical software. Normality tests and test for equal variances were positive ($p>0.05$). *Bacillus subtilis* strain ABGx developed significantly better in wheat distiller's grains and rapeseed oil cake media. The optical

densities increased progressively until about 100 hours of culture. The growth in a medium containing agro-residues is possible if the strain can hydrolyze xylan to release xylose, but it can also metabolize proteins. It was possible to calculate biomass yields for each culture medium on the basis of the biomass produced in each flask (**Table 1**).

Table 1. Biomass yields (Yxs) of *Bacillus subtilis* strain ABGx obtained as a function of the agro-residues used as carbon sources and xylanase inducers. Protein and hemicellulose contents are also indicated for each substrate [37-40].

Carbon source	Proteins (%)	Cellulose (%)	Hemicelluloses (%)	Yxs
WB	18	24	41	15.0 ± 0.5
WDG	33	7	17	34.2 ± 2.3
ROCS	32	12	6	34.0 ± 1
ROCM	32	12	6	30.6 ± 2.7

Biomass yields were analyzed with MINITAB16® statistical software. Normality tests and test for equal variances were positive ($p>0.05$). Wheat distiller's grains led to the highest biomass yield. The biomass yields resulting from the media containing rapeseed oil cakes were lower but not significantly different. Although wheat bran contains the highest quantity of hemicelluloses, it led to the lowest biomass yield and optical density, which was significantly lower than the other substrates. Biomass yields are closely related to protein contents (see **Table 1**). Nitrogen is not only necessary for growth but also enzyme production. Wheat bran does not provide enough nitrogen to reach a higher biomass yield, but the enzyme production was the best. Sodium citrate and tryptone initially put in the medium can also be used as carbon sources by the strain, and tryptone also provides nitrogen in a small quantity. The effect of increasing xylanase activity by tryptone, sodium citrate, KH_2PO_4, and $MgSO_4$ on *Bacillus circulans* AB16 was demonstrated before [21].

Xylanase activities were evaluated in each medium (**Figure 3**) and no cellulase activity was detected in our experiments. The results were analyzed with MINITAB16® statistical software. Normality tests and test for equal variances were positive ($p>0.05$). The composition of the culture media influenced xylanase production directly.

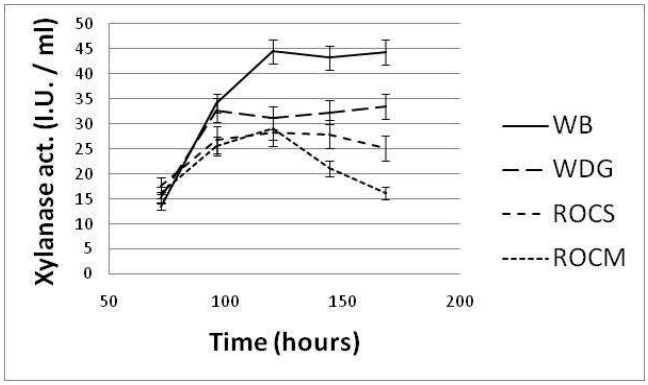

Figure 3. Evolution of xylanase activities in the four culture media of *Bacillus subtilis* strain ABGx. WB = Wheat Bran, WDG = Wheat Distiller's Grains, ROCS = Rapeseed Oil Cake obtained after Solvent extraction, ROCM = Rapeseed Oil Cake obtained after Mechanical pressing.

It was possible to reach a maximal xylanase activity of 44.3I.U./ml with the wheat bran medium, which was significantly higher than in the other media. Other studies relating to xylanolytic strains are summarized in **Table 2**. Many bacterial strains can produce xylanases with specific properties. The negative effect caused by a too high concentration in agro-residue in the culture medium was studied before [20]. In that study, xylanase activity increased from a concentration of agro-residue of 0.25 to 2% (w/v) but decreased when wheat bran concentration was higher because of the formation of a thick suspension. The substrate was not able to mix freely in the culture medium. However, that problem was not observed here given that the culture media were homogeneous under a good agitation. The four xylanase activities were significantly different after 148 hours of cultivation. The values of xylanase activity can be compared with other strains. Many publications cite bacteria able to produce xylanase (see **Table 2**).

Table 2 - Part 1. Xylanolytic activities produced by bacterial strains from other studies. Xylanase activities are expressed in International Units/ml or /mg of protein. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited.

Strain	Xylanase activity (IU/ml or /mg pr.)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Acidobacterium capsulatum [41]	-	7,3	41	5	65	3-8	20-50
Acinetobacter junii F6-02 [42]	317 IU/ml	-	70-85	7	60	-	up to 50
Bacillus arseniciselenatis DSM 15340 [43]	299,25 IU/mg	-	29,8	8	50	6-11	up to 40
Bacillus cereus [24]	31150 IU/ml	-	32	6	40	-	-
Bacillus circulans AB16 [21]	55 IU/ml	-	Xyl A : 30 Xyl B : 22	A: 6 B: 6	A :75-80 B :65-70	46% at pH 8	at 65, pH 9 (2h) A: 70% B : 34%
Bacillus circulans BL 53 [44]	37 IU/mg	8,8	38	5-8	40-80	-	up to 60
Bacillus circulans Teri 42 [45]	36000 IU/mg	-	71	7	50	-	up to 50
Bacillus circulans WL-12 [46]	-	9,1	15	5,5-7	-	-	-
Bacillus halodurans C-125 [47]	3361 IU/ml	-	43	9	70	5,5-10	up to 65
Bacillus halodurans S7 [48]	5,1 IU/ml	4,5	43	9-10	70-75	5,5-10,5	up to 65
Bacillus licheniformis MS5-14 [49]	-	-	23	5-7	40-50	-	up to 50
Bacillus licheniformis P11 (C) [50]	29,7 IU/ml	-	17,5-23	8	60	8	up to 60
Bacillus mojavensis AG137 [51]	302,5 IU/ml	-	-	9	55	up to 10	up to 45
Bacillus polymixa CECT 153 [52]	4,5 IU/mg	4,7	61	6,5	50	-	-
Bacillus pumilus strains 13_a, 5_2, 5_{14} et 4_a [53]	328, 131, 90 and 167 IU/ml	-	-	9 (13_a) 8 (5_2, 5_{14}, 4_a)	60 (13_a) 55 (5_2, 5_{14}, 4_a)	-	5_2 40 % (40, 2h) 5_{14}, 4_a, 13_a: 60 %

Table 2 - Part 2. Xylanolytic activities produced by bacterial strains from other studies. Xylanase activities are expressed in International Units/ml or /mg of protein. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited.

Strain	Xylanase activity (IU/ml or /mg pr.)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Bacillus pumilus ASH [20]	5407 IU/ml	-	-	-	-	neutral-alkaline	up to 70
Bacillus pumilus MK001 [54]	4000 IU/ml	-	-	-	-	-	-
Bacillus pumilus SV-85S [55]	2995,2 IU/ml	-	-	6	50	5-11	up to 65
Bacillus pumilus SV-205 [56]	7382,7 IU/ml	-	-	6,5	37	5-11	up to 60
Bacillus sp. [57]	16 IU/ml	-	-	6,5-7	90	6	30-50
Bacillus sp. [58]	16 IU/ml	-	44	6,5-8,5-10,5	50	6-10,5	up to 55
Bacillus sp. AR-009 [59]	38,89 IU/mg	-	23-48	9-10	60-75	8-9	60-65
Bacillus sp. BP-23 [60]	1,12 IU/ml	9,3	32	5,5	50	9,5-11	up to 55
Bacillus sp. BP-7 [61]	-	7-9	22-120	6	55	8-9	up to 65
Bacillus sp. K-1 [62]	4,8 IU/mg	-	23	5,5	60	5-12	50-60
Bacillus sp. K-8 [63]	3 IU/ml	-	24	6-7	-	-	-
Bacillus sp. NCIM-59 [64]	-	4,8	15,8-35	6	50-60	7	up to 50
Bacillus sp. NG-27 [65]	-	-	-	7-8,4	70	6-11	40-90
Bacillus sp. SPS-0 [66]	3 IU/ml	-	-	6	75	6-9	up to 85
Bacillus sp. W-1 [67]	-	8,5	21,5	6	65	3-9	up to 40
Bacillus sp. XTR 10 [68]	70,7 IU/ml	-	-	-	-	-	-
Bacillus species StA, StB, StC [69]	52 IU/ml	-	-	StA: 9 StB: 9 StC: 8	StA: 55 StB: 50 StC: 55	StA: 9 StB: 9 StC: 9	StA: 50 StB: 50 StC: 50
Bacillus stearathermo-philus T-6 [23]	1,45 IU/ml	7,9	43	6,5	55	6,5-10	up to 70
Bacillus subtilis [70]	128 IU/ml	-	36	9	55	-	up to 55
Bacillus subtilis [71]	12 IU/ml	-	-	6	60	-	up to 60
Bacillus subtilis ASH [72]	400 IU/ml	-	-	-	-	-	-
Bacillus subtilis, by E. coli [73]	448 IU/ml	9,6	23,3	5,8	60	6	up to 50

Table 2 - Part 3. Xylanolytic activities produced by bacterial strains from other studies. Xylanase activities are expressed in International Units/ml or /mg of protein. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited.

Strain	Xylanase activity (IU/ml or /mg pr.)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Bacillus thermanta-rcticus DSM 9572 [74]	-	4,8	45	5,6	80	-	up to 60
Bacillus trypoxylicola [75]	-	-	-	-	-	-	-
Cellulomonas fimi [76]	-	4,5-8,5	14-150	5-6,5	40-45	-	-
Cellulomonas flavigena [77]	-	-	35	6,5	55	-	not stable
Cellulomonas sp. N.C.I.M. 2353 [78]	-	8	22-33-53	6,5	55	-	-
Cellulosimicrobium sp. HY13 [79]	4067 IU/mg	4,7	36	6	55	-	-
Cellulosimicrobium sp. MTCC 10645 [43]	96,33 IU/ml	-	78	7	50	up to 11	8 % at 60°C, 4h
Chromohalobacter sp. TPSV 101 [80]	0,5 IU/mg	-	15	9	65	7-9	50-70
Clostridium absonum CFR-702 [81]	421,5 IU/ml	-	-	-	-	-	-
Clostridium strain PXYL1 [82]	50 IU/ml	-	-	5,3	20	-	up to 20
Cohnella laeviribosi HY-21 [83]	88,6 IU/mg	5,8	39,33	7,5	50	-	up to 37
Enterobacter sp. MTCC 5112 [84]	90 IU/ml	-	43	9	100	up to 10	up to 50
Geobacillus thermoleo-vorans [85]	10,2 IU/mg	-	48	8,5	80	-	up to 70
Gracilibacillus sp. TSCPVG [86]	18,44 IU/ml	-	55	7,5	60	5-10,5	50-70
Jonesia denitrificans BN-13 [87]	10,81 IU/ml	-	-	7	50	-	up to 50

Table 2 - Part 4. Xylanolytic activities produced by bacterial strains from other studies. Xylanase activities are expressed in International Units/ml or /mg of protein. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited.

Strain	Xylanase activity (IU/ml or /mg pr.)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Kluyvera sp. OM 3 [88]	5,12 IU/ml	-	-	8	70	5-9	up to 60
Micrococcus sp. AR-135 [89]	0,49 IU/mg	-	56	7,5-9	55	6,5-10	up to 40
Paenibacillus campina-sensis BL11 [90]	2392 IU/mg	-	41	7	60	up to 9	up to 60
Paenibacillus sp. 2S-6 [91]	4,93 IU/ml	-	33	6	50	not stable	up to 50
Paenibacillus sp. HPL-001 [92]	-	-	38,1	5,5-9,5	50	-	-
Promicromonospora sp. MARS [93]	42,2 IU/ml	-	-	8	65	up to 8	up to 65
Pseudobutyrivibrio xylanivorans [94]	-	5,1-5,9	30	5,6	38	-	-
Staphylococcus sp. SG-13 [95]	0,28 IU/ml	-	60	7,5-9,2	50	7,5-9,5	up to 50
Streptomyces cyaneus SN32 [96]	716 IU/ml	8,5	20,5	6	60-65	6	up to 50
Streptomyces rameus L2001 [97]	3236,6 IU/mg	-	21,1	5,3	70	4,3-6,7	up to 55
Streptomyces sp. S 38 [98]	31 IU/ml	4,5-5 8,5-9	24,5-37 5-38	6	60	-	up to 50
Streptomyces sp. AMT-3 [99]	70 IU/ml	-	170-240-600	6	55-65	-	up to 55
Streptomyces sp. QG-11-3 [100]	96 IU/ml	-	-	8,6	60	5,4-9,4	up to 50
Thermoanaerobacterium saccharolyticum NTOU1 [101]	91 IU/mg	-	50	6,4	63	6,5-7	up to 65

Table 2 - Part 5. Xylanolytic activities produced by bacterial strains from other studies. Xylanase activities are expressed in International Units/ml or /mg of protein. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited.

Strain	Xylanase activity (IU/ml or /mg pr.)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Thermoanaerobacterium sp. JW/SL-YS 485 [102]	0,36 IU/ml	4,4	80	6,2	80	-	-
Thermotoga maritima MSB8 [103]	12,2 IU/mg	5,6	40-120	5,4-6,2	92-105	-	-

Xylanase producing strains of *Acidobacterium*, *Acinetobacter*, *Bacillus*, *Cellulomonas*, *Cellulosimicrobium*, *Chromohalobacter*, *Clostridium*, *Cohnella*, *Enterobacter*, *Geobacillus*, *Gracilibacillus*, *Jonesia*, *Kluyvera*, *Micrococcus*, *Paenibacillus*, *Promicromonospora*, *Pseudobutyrivibrio*, *Staphylococcus*, *Streptomyces*, *Thermoanaerobacterium* and *Thermotoga* were cited before. Very high enzymatic activities were detected in *Bacillus cereus* [24], *Bacillus halodurans* C-125 [47] and *Bacillus pumilus* SV-205 [56] with values of 31150 IU/ml, 3361 IU/ml and 7383 IU/ml, respectively. *Bacillus* species are common producers of xylanases. Some articles cite *Bacillus subtilis* strains as xylanase producers with activities of 128, 12, 400 and 448 IU/ml [70-73]. The endoxylanase activity observed in the present study is quite weak compared with those values.

Xylanase activity values increase with the concentration in hemicelluloses provided by the agro-residues. Wheat bran was the best xylanase inducer and provides a large surface area under high moisture conditions. The composition of wheat bran consists of 42.5% glucose, 15.4% xylose, 3.1% arabinose, and 2.7% galactose. All these monosaccharides can be used as nutrients by microorganisms [104]. However, glucose is not totally available because the hydrolysis of cellulose requires cellulase activity. The strain also produces amylase and can hydrolyze starch (19% of dried matter in wheat bran [39]).The effect of different sugars on xylanase production by *Bacillus sp*. was investigated in a previous study [105]. This effect depended on the type and the concentration of monosaccharides. Lactose, sucrose, and glucose repressed the enzyme production. Xylan and xylose are the most reported xylanase inducers [22]. The wheat bran/water ratio can also affect xylanase production [105].

The efficiency relating to the enhancement of xylanase activity by wheat bran was reported in previous studies with several species of *Bacillus* [24, 45, 56]. However, the

induction of xylanase activity is not well understood. Xylan is too voluminous to enter microbial cells and it is thought that xylanase activity depends on smaller xylan fragments, which would be produced by the action of a constitutively produced enzyme [13]. The development of xylanase activity observed here is slow compared with other studies. A maximal activity was reached after 36 h with *Bacillus pumilus* B20 [104]. However, in that study, xylanase activity started decreasing after 36 hours of culture and no activity remained after 120 hours. A stable value of xylanase activity was obtained after 50 h of culture by *Bacillus* sp. [105]. In another study, a maximal xylanase activity was reached after 72 h [22]. In this case, xylanase activity was conserved until 168 hours with wheat bran and wheat distiller's grains.

Xylanase activities can also be expressed as a function of dried matter of agro-residues. Wheat bran provided a maximal xylanase activity of 1632±156 I.U./g dried matter, wheat distiller's grains provided 1268±260 I.U./g dried matter, rapeseed oil cake obtained after solvent extraction provided 1028±68 I.U./g dried matter, and rapeseed oil cake obtained after physical pressing led to an activity of 1072±51 I.U./g dried matter.

The evolution of pH in the culture media depended on the carbon source (**Figure 4**). A decrease of pH was observed with wheat bran whereas pH showed a tendency to increase with other substrates. The composition of the substrate seems to modify the pH evolution. Wheat bran contains the lowest amount of proteins (18%). More proteins are available in wheat distiller's grains and rapeseed oil cakes. Aminoacids resulting from the hydrolysis of proteins coming from agro-residues or tryptone can also be metabolized in the citric acid cycle [106]. The enhancement of xylanase production increased by amino acids was shown in *Bacillus sp.* No C-125 [107] and *Bacillus sp.* NCL-87-6-10 [108]. This effect was not observed here although wheat bran contains the highest protein concentration. Maximal xylanase activities were observed after 120 hours of culture, corresponding to a pH value close to neutrality.

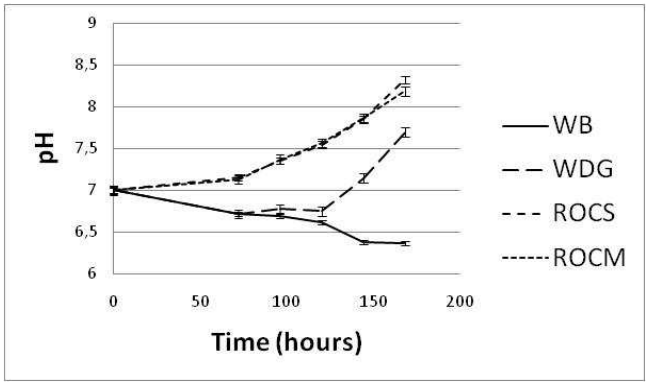

Figure 4. Evolution of pH in the four culture media of *Bacillus subtilis* strain ABGx. WB = Wheat Bran, WDG = Wheat Distiller's Grains, ROCS = Rapeseed Oil Cake obtained after Solvent extraction, ROCM = Rapeseed Oil Cake obtained after Mechanical pressing.

IV.3.4. *Optimal pH and pH stability*

The optimal pH conditions were investigated at different pH values. Solutions of xylan (10 g/l) were prepared in a citrate-phosphate buffer (B1, 0.2M, pH 3 to 5.5), phosphate buffer (B2, 0.2M, pH 6 to 8) and carbonate-bicarbonate buffer (B3, 0.2M, pH 9.2 to 10.8). Xylanase activities were measured by the method described before for each pH. The reaction was carried out at 40^{o}C for 5 minutes. The effect of pH on xylanase activity was investigated on samples of enzyme adjusted to pH values ranging from 3 to 10.8 with buffers B1, B2 and B3 at 0.02M. These samples were agitated for 4 hours at 25^{o}C. Then, residual xylanase activities were measured by the method described before for each sample. The optimal conditions of pH and the effect of pH are shown in **Figure 5**. Optimal pH is slightly alkaline and close to neutrality. A pH of 8 led to the maximal activity and is the optimal pH. Alkaline pH values were more suitable for enzyme activity. However, the enzyme conserved its activity in a wide range of pH values. Indeed, more than 70 % of xylanase activity was measured from a pH 3 to 10.8. This resistance to pH conditions makes the xylanase produced by *Bacillus subtilis* strain ABGx interesting for industrial processes. The curve of optimal pH does not converge on the curve of the effect of pH perfectly. Such an observation was reported previously (see **Table 2**). Bacterial strains often produce xylanases showing an optimal pH value comprised between 5 and 10.5 (see **Table 2**). Some *Bacillus* species produce alkalophilic xylanases, such as *Bacillus halodurans* S7 (optimal pH of 9-10) [48], *Bacillus mojavensis* AG137 (optimal pH of 9) [51] and *Bacillus* sp. AR-009 (optimal pH of 9-10) [59]. Other bacterial genera were also found to produce alkalophilic xylanases, like *Chromohalobacter* sp. TPSV 101 (optimal pH of 9) [80], *Enterobacter* sp. MTCC 5112 (optimal pH of 9) [84] or *Streptomyces* sp. QG-11-3 (optimal pH of 8.6) [100]. Other strains of *Bacillus subtilis* produce a xylanase with an

optimal pH value of 9, 6 and 5.8 [70, 71, 73]. Optimal pH values observed here are comparable to those of *Bacillus subtilis* described by [70, 71, 73]. Concerning pH stability, less than 40% of xylanase activity were retained at pH 3 compared to more than 80% of activity at pH 11 in the present study. The enzyme shows a good resistance after 4 hours of incubation at different pH values. The enzyme secreted by the transformed strain of *Escherichia coli* that incorporated the xylanase gene of a strain of *Bacillus subtilis* in the study of [73] withstood acidic pH better than alkaline pH. In that study, the enzyme conserved more than 60 % at pH 3.5 against less than 40 % at pH 7.5. The enzyme produced by *Acidobacterium capsulatum* [41] displayed a good resistance to pH conditions from 2.5 to 8, but the enzyme was incubated for 30 minutes only at each pH value. *Bacillus arseniciselenatis* DSM 15340 [43] produced a xylanase that lost about 20 % and 30 % of its activity at pH 10 and 11 after 4 hours, respectively. The enzyme of *Bacillus halodurans* C-125 [47] displayed a strong resistance to pH values from 5.5 to 10 after 12 hours. However, no stability was observed when pH was less than 5. The enzyme secreted by *Bacillus halodurans* S7 [48] displayed the same characteristics. *Bacillus licheniformis* P11(C) [50] produced an enzyme that lost 40 % of its activity at pH 11 after 60 minutes. However, the enzyme conserved 80 % of its activity at pH 5. The xylanase of *Bacillus mojavensis* AG137 [51] conserved about 80, 70 and 60 % of its activity after an incubation of 3 hours at pH values of 8, 9 and 10, respectively. *Bacillus pumilus* SV-85S [55] produced an enzyme that was 100 % stable at a pH range of 5-11 after 60 minutes of incubation. After 3 hours, the residual activities were 85 and 67 % at pH values of 10 and 11. *Bacillus pumilus* strain SV-205 [56] produced a xylanase showing 100 % of stability at a pH range of 6-11 after 24 hours.

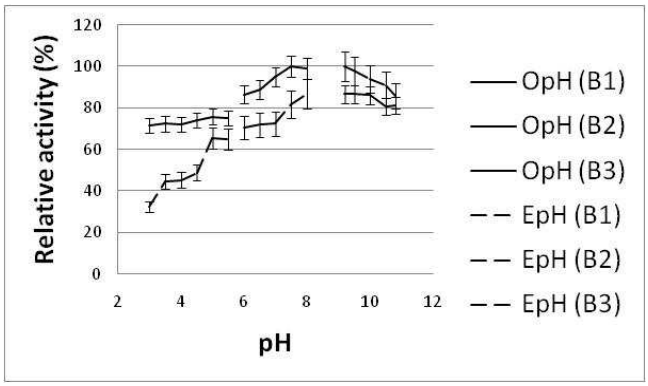

Figure 5. Optimal pH (time : 5 minutes, temperature : 25°C) and pH stability (time : 4 hours, temperature : 25°C) of the xylanase produced by *Bacillus subtilis* strain ABGx; OpH = Optimal pH, EpH = Effect of pH. B1 refers to Buffer 1 (citrate-phosphate), B2 refers to Buffer 2 (phosphate) and B3 refers to Buffer 3 (carbonate-bicarbonate). The values are expressed as percentages of the maximal activities.

IV.3.5. *Optimal temperature and thermal stability*

The optimal conditions of temperature were investigated at temperature values ranging from 35 to 85°C. Xylanase activities were measured in accordance with the method described before at the different temperature values. The reaction was carried out at pH 7 for 5 minutes. The effect of temperature was investigated using enzyme samples kept at different temperature values under agitation for 30 minutes at pH 7. Then, residual xylanase activities were measured in accordance with the method described before. The optimal conditions of temperature and the effect of temperature are shown in **Figure 6**. The enzyme was not stable above 45°C. Thermal stability remained up to 45°C and started decreasing above that temperature. 80% of enzymatic activity was conserved under 55°C, but less than 60% of activity was retained above 60°C.The enzyme was totally inactivated by temperatures above 80°C. The optimal temperature is between 50 and 60°C. However, thermal stability is not complete in these conditions. Optimal conditions of temperature are usually comprised between 40 and 65°C (see **Table 2**).

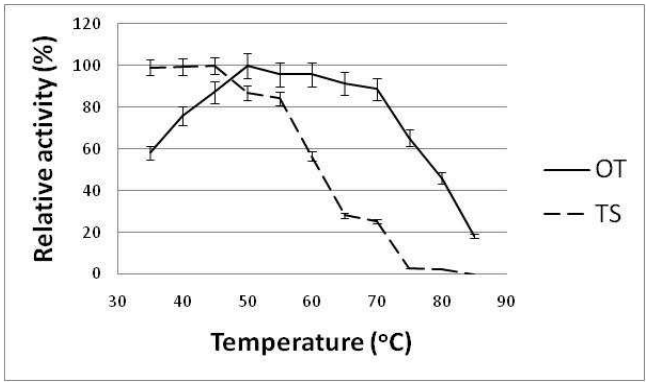

Figure 6. Optimal temperature (time : 5 minutes, pH : 7) and thermal stability (time : 30 minutes, pH : 7) of the xylanase produced by *Bacillus subtilis* strain ABGx; OT = Optimal Temperature, TS = Thermal Stability. The values are expressed as percentages of the maximal activities.

However, some *Bacillus* species display high optimal temperatures such as *Bacillus circulans* AB 16 (80°C) [21], *Bacillus halodurans* C-125 (70°C) [47], *Bacillus* sp. (90°C) [57] and *Bacillus* sp. SPS-0 (75°C) [66]. Other bacterial genera also produce thermotolerant xylanases, like *Enterobacter* sp. MTCC 5112 (optimal temperature of 100°C) [84], *Geobacillus theroloeovorans* (80°C) [85], *Thermoanaerobacterium* sp. (80°C) [102] and *Thermotoga maritima* MSB8 (92-105°C) [103]. However, the strains of Bacillus subtilis display lower optimal temperatures (55-60°C) [70, 71, 73]. *Bacillus subtilis* strain ABGx display the same characteristics. Concerning thermal stability, the xylanase produced by

Bacillus subtilis strain ABGX is comparable to other *Bacillus subtilis* strains. Residual xylanase activities of *Bacillus subtilis* strain ABGx after a heating of 30 minutes were more than 80 % up to 55°C, decreased to 55 % at 60°C, and more than 20 % at 70°C. No activity was preserved when temperature was more than 70°C. Residual xylanase activities of another strain of *Bacillus subtilis* were 80 % after a heating of 30 minutes at 60°C, but decreased drastically to about 5 % at 70°C [70]. In the present study, the loss of activity is less rough. The thermal stability of the xylanase of *Bacillus subtilis* strain ABGx is comparable to the strain of *Bacillus subtilis* studied by [71]. In both cases, the loss of activity increased strongly when the temperature reached 65°C. In another study, the strain of *Bacillus subtilis* secreted an enzyme more thermostable, keeping 80 % of its activity after a heating of 30 minutes at 75°C [73]. Other strains of *Bacillus* usually produce thermostable xylanases up to 55, 60 or 65°C (see **Table 2**).

IV.3.6. *Amino acid sequence of the xylanase*

The amino acid sequence of the enzyme deduced from the nucleotide sequence was compared with other xylanase sequences. The enzyme produced by *Bacillus subtilis* ABGx shares 99% identity with a xylanase produced by *Bacillus subtilis* strain 168 (1A1) (GenBank ID : NP389765.1). A special domain in the amino acid sequence of that enzyme formed by glutamic acid and tyrosine residues was described before [33] (**Figure 7**).

ABGx	**1**	**KKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVN**	**53**
168(1A1)	**5**	**KKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVN**	**57**
ABGx	**55**	**WSNTGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYV**	**108**
168(1A1)	**59**	**WSNTGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYV**	**112**
ABGx	**109**	**VDSWGTYRPTGTYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQT**	**158**
168(1A1)	**113**	**VDSWGTYRPTGTYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQS**	**162**
ABGx	**163**	**KRPTGSNATITFSNHVNAWKSHGMNLGSNWAYQV L ATEGYQSSGSSTV**	**206**
168(1A1)	**167**	**KRPTGSNATITFSNHVNAWKSHGMNLGSNWAYQVMATEGYQSSGSSNV**	**210**

Figure 7. Comparison between the two xylanase sequences of *Bacillus subtilis* strain ABGx and *Bacillus subtilis* strain 168(1A1). The glutamic acid and tyrosine residues (E and Y, respectively) are framed and underlined, and the amino acid differences are only underlined.

In that study, it was demonstrated that a temperature of 55°C was necessary to activate the catalytic domain. The enzyme produced by *Bacillus subtilis* strain ABGx displays the same characteristics concerning the optimal conditions of temperature. Consequently, the

enzyme has probably a similar mode of action. The enzyme described in [33] possesses a special sequence of glutamic acid and tyrosine that can also be found in the xylanase produced by *Bacillus subtilis* strain ABGx (see **Figure 7**). The enzyme is not stable above 50^{o}C but could be implemented in industrial processes using low temperatures. On the other hand, when the xylanase produced by *Bacillus subtilis* ABGx was frozen or lyophilized, it conserved all its activity.

IV.3.7. *Zymographic assay*

Enzyme activity was successfully restored after denaturation by SDS-PAGE. Two activity bands were visible, the most intense (**Figure 8**) corresponding to a molecular weight expected from the sequenced xylanase.

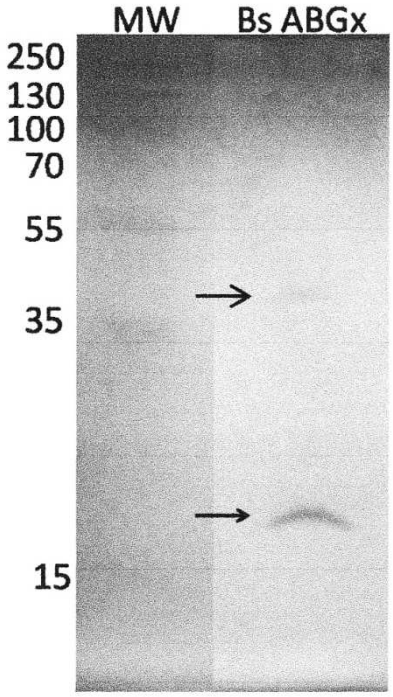

Figure 8. Zymogram of the xylanase produced by *Bacillus subtilis* strain ABGx. Left row : molecular weight markers. Right row : sample. Molecular weights are indicated in kDa. A strong activity was detected between 15 and 35 kDa. Another xylanase activity was detected at a higher molecular weight (>35 kDa), but the intensity of the band was weak.

Another xylanase produced by *Bacillus subtilis* strain ABGx was detected, but its activity was weak. Such an observation was also described in other articles (see **Table 2** [21, 50, 59, 61, 64]). *Bacillus circulans* AB16 secreted two different xylanases of different molecular weights (22 and 30 kDa). These enzymes also displayed different thermal and pH stabilities [21]. *Bacillus licheniformis* P11(C) was found to produce two xylanases of 17.5 and 23 kDa [50]. *Bacillus* sp. AR-009 secreted two xylanases of 23 and 48 kDa [59]. *Bacillus* sp.

BP-7 was found to produce different xylanases from 22 to 120 kDa. However, the highest molecular weights were described as xylanase aggregates, and two xylanases of 22 and 45 kDa were highlighted [61]. Finally, a strain of *Bacillus* sp. NCIM-59 was found to secrete two xylanases of 15, 8 and 35 kDa [64]. In many cases, *Bacillus* strains that produce two xylanases secrete a basic and an acid pI xylanase [61]. In the present study, two xylanases of approximately 20 and 40 kDa were identified. Other bacteria were also found to produce several kinds of xylanases, such as *Cellulomonas*, *Streptomyces* and *Thermotoga* strains (see **Table 2** [76, 78, 98, 99, 103]).

IV.3.8. *Hydrolysis products of birchwood xylan*

The hydrolysis of xylan using the enzyme produced by *Bacillus subtilis* strain ABGx was performed for 24 hours, and different dilutions of the enzyme were tested (1.12 I.U./ml, 2.24 I.U./ml, 3.36 I.U./ml, 4.48 I.U./ml, and 5.6 I.U./ml). Birch xylan contains mainly xylose (92.6 %) and uronic acids (4.1 %). The structure of birch xylan consists of a backbone of β-D-xylopyranose with residues of 4-O-methyl-D-glucuronic acid every 15 xylose units [48]. These residues were detected with mass spectrometry. The mass spectrum is displayed in **Figure 9**. The number of xylose units is indicated above each peak. The intensity of the peaks represents semi-quantitively the abundance of the corresponding ions. Each oligomer contains a residue of 4-O-methyl-D-glucuronic acid. Consequently, no glucuronidase activity was detected. However, the spectrum displays an endoxylanase activity. The mass-to-charge ratios are indicated under the numbers of xylose residues. The distribution of xylose residues is normal. An increase of the enzyme concentration causes a shift of that Gaussian curve. Indeed, the enzyme releases oligomers with lower molecular weight. When the enzyme concentration increases, more linkages can be hydrolyzed.

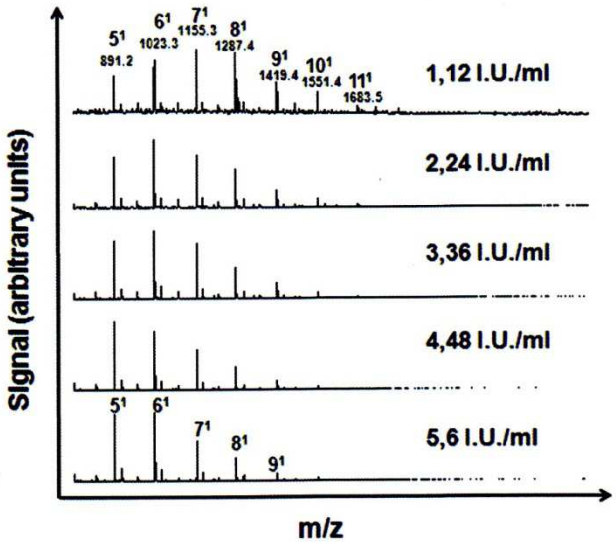

Figure 9. Mass spectrum obtained after the hydrolysis of birch xylan by the enzyme of *Bacillus subtilis* strain ABGx at different dilutions (1.12 I.U./ml, 2.24 I.U./ml, 3.36 I.U./ml, 4.47 I.U./ml, and 5.6 I.U./ml). The exponents represent the number of methylglucuronic acids substituting the oligomers.

The same experience was realized with an endo-1,4-β-xylanase from *Trichoderma longibrachiatum* (Sigma, ≥1 I.U./mg) (**Figure 10**). The enzyme was tested at different concentrations : 0.01, 0.1, 1.0, and 10 g/l. A Gaussian distribution of the xylose residues was also observed with the shift for higher concentrations.

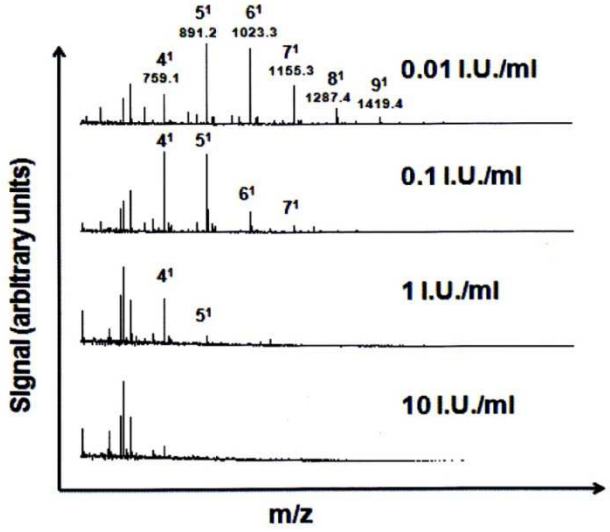

Figure 10. Mass spectrum obtained after the hydrolysis of birch xylan by the enzyme of *Trichoderma longibrachiatum* (Sigma) at different dilutions (0.01 I.U./ml, 0.1 I.U./ml, 1.0 I.U./ml, and 10 I.U./ml). The exponents represent the number of methylglucuronic acids substituting the oligomers.

IV.4. Conclusion

The following conclusions can be reached from the experiments :

- The termite gut provides opportunities for isolation of interesting microorganisms that produce enzymes. It was possible to isolate a xylanolytic *Bacillus subtilis* from the hindgut of the termite.
- Agro-residues can be used as cheap inducers of enzymatic activities. These materials allowed an enzyme production without using expensive highly purified products.
- Wheat bran provided the best induction of xylanase activity and allowed to reach a maximal activity of 44.3 I.U./ml, although the development of the strain was better in the other media. The xylanase production increased with the concentration of hemicelluloses found in the different agro-residues.
- The biomass yields depended on the composition of the agro-residues. The protein content influenced the biomass directly.
- Wheat bran is a significantly better xylanase inducer than wheat distiller's grains, which are a significantly better inducer than rapeseed oil cake.
- The enzyme produced was stable up to 45^{o}C and displayed a slightly alkaline optimal pH (7.5-8).
- Mass spectrometry was a useful method to identify the type of enzymatic activities. In this case, it allowed identifying an endoxylanase activity developed by *Bacillus subtilis* strain ABGx. The results were confirmed by testing a commercial endoxylanase from *Trichoderma longibrachiatum* used as a reference.

IV.5. Acknowledgements

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IV.6. References

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CHAPITRE V.

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Isolation of an amylolytic chrysophyte, *Poterioochromonas* sp. from the digestive tract of the termite *Reticulitermes flavipes*

Ce chapitre correspond à l'article intitulé "*Isolation of an amylolytic chrysophyte, Poterioochromonas sp. from the digestive tract of the termite R. santonensis*" (Cédric Tarayre, Julien Bauwens, Catherine Brasseur, Christel Mattéotti, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart) publié dans la revue **Biotechnologie, Agronomie, Société et Environnement** Volume 18, Issue 1, pp 1-13 (2014).

Les travaux présentés ci-avant concernent principalement les bactéries. Toutefois, les protistes devraient aussi constituer une source d'enzymes capitale car ils sont considérés comme étant majoritairement responsables de la dégradation de la cellulose au sein du système digestif des termites inférieurs.

C'est dans cette optique que se situe l'étape suivante de la recherche. En utilisant différents milieux de culture enrichis en matières lignocellulosiques incubés en conditions aérobie et anaérobie (milieu dégazé à l'azote), il a été possible de cultiver différents protistes. Cependant, chaque culture était contaminée par des bactéries. L'utilisation de différents antibiotiques n'a pas permis de purifier ces cultures en maintenant le protiste vivant. A cet égard, il est à noter que la littérature a bien démontré que de nombreux protistes du tube digestif des termites entretiennent des relations symbiotiques avec des bactéries.

En utilisant un milieu à base d'amidon, il a été possible d'isoler et d'étudier un protiste identifié comme un chrysophyte. La méthodologie d'isolement présentée dans le chapitre suivant est relativement originale.

Résumé

Le but de ce travail était d'isoler et cultiver des protistes vivant dans le tube digestif du termite *Reticulitermes santonensis* (Feytaud). Un chrysophyte identifié comme *Poterioochromonas* sp. en a été isolé dans un milieu spécial contenant des grains de riz comme source carbonée et azotée. Ensuite, le protiste a été cultivé dans un milieu contenant de l'amidon comme source de carbone, de la tryptone, et un tampon phosphate à différents pH (5, 6 et 7). De l'extrait de levue a été ajouté ou non. La ciprofloxacine a été employée pour éviter le développement bactérien. D'autres antibiotiques ont aussi été testés mais ont montré un effet inhibiteur sur la croissance de *Poterioochromonas* sp. L'extrait de levure a permis d'augmenter les concentrations cellulaires finales d'un facteur 1.9 (pH 5), 2.3 (pH 6) et 2.2 (pH 7), ainsi que les rendements en biomasse d'un facteur 2.8 (pH 5), 2.8 (pH 6) et 2.2 (pH 7). La concentration en amidon n'a pas diminué dans le milieu jusqu'à 3 et 4 jours de culture, respectivement avec et sans extrait de levure. Huit jours de culture ont été nécessaires à l'hydrolyse complète de l'amidon, avec et sans extrait de levure. Du maltose et du maltotriose ont été détectés dans les milieux et ont été progressivement hydrolysés. Les concentrations maximales en maltose ont atteint 0.68, 0.66 et 0.51 $g.l^{-1}$ dans le milieu contenant l'extrait de levure. Les concentrations en maltotriose étaient seulement de 0.17, 0.14 et 0.12 $g.l^{-1}$. D'autres oligomères de glucose ont aussi été détectés mais en quantités plus faibles. Il a été montré que le protiste a développé une faible activité amylase, particulièrement à pH légèrement acide (5-6). Un tel pH a aussi permis une meilleure croissance du protiste. Une activité amylase maximale de 112 $nkat.l^{-1}$ a été mesurée à pH 5 en présence d'extrait de levure. Aucune autre activité enzymatique (protéase, cellulase, xylanase) n'a été détectée à l'exception de l'amylase. Les produits de dégradation de l'amidon qui ont été obtenus par hydrolyse enzymatique permettent d'identifier des activités enzymatiques de type α-amylase, amyloglucosidase et éventuellement β-amylase.

Mots-clés : Amylases, Chrysophyceae, culture in vitro, milieu de culture, protiste, *Reticulitermes*

Abstract

The aim of this work was the isolation and cultivation of amylolytic protists living in the digestive tract of the termite *Reticulitermes santonensis* (Feytaud). A chrysophyte identified as *Poterioochromonas* sp. was isolated in a special medium containing rice grains as a source of carbon and nitrogen. Then, the protist was grown in a medium containing starch as a carbon source, tryptone, and a phosphate buffer at different pH values (5, 6 and 7). Yeast extract was added or not. Ciprofloxacin was used to avoid the bacterial development. Other antibiotics were also tested but showed an inhibitive effect on the growth of *Poterioochromonas* sp. Yeast extract allowed reaching 1.9 (pH 5), 2.3 (pH 6) and 2.2 (pH 7) times higher final cell concentrations, and 2.8 (pH 5), 2.8 (pH 6) and 2.2 (pH 7) times higher biomass yields. The starch concentration did not decrease in the medium until 3 and 4 days of culture, with and without yeast extract, respectively. Eight days of culture were necessary for hydrolyzing the starch completely, with and without yeast extract. Maltose and maltotriose were detected in the culture media and were hydrolyzed progressively. Maximal maltose concentrations were 0.68, 0.66 and 0.51 $g.l^{-1}$ in the medium containing yeast extract. Maltotriose concentrations were only 0.17, 0.14 and 0.12 $g.l^{-1}$. Other glucose oligomers were also detected but in lower quantities. It was determined that the protist developed a weak amylase activity, particularly at a weakly acidic pH (5-6). Such a pH also allowed a better growth of the protist. A maximal amylase activity of 112 $nkat.l^{-1}$ was measured with yeast extract at pH 5. No other enzymatic activity (protease, cellulase or xylanase) was detected except amylase. The degradation products of starch which were obtained by enzymatic hydrolysis allow the identification of α-amylase, amyloglucosidase and possibly β-amylase activities.

Keywords : Amylases, Chrysophyceae, in vitro culture, culture media, protista, *Reticulitermes*

V.1. Introduction

The symbiotic microorganisms living in the wood-feeding termite gut are necessary to lignocellulose digestion and nitrogen metabolism [1]. Termites are classified into two groups : lower termites, which harbor different kinds of protists in their guts, and higher termites, which do not contain any protists [2]. Lower termites can degrade lignocellulose by the action of their own enzymes combined with microbial enzymes [1].

The digestive tract of termites is divided into three parts : foregut, midgut and hindgut. The hindgut of lower termites is a bioreactor containing numerous packed symbionts : bacteria, molds, archaea and protists [2]. It is known that the termite hindgut harbors specialized microorganisms which help the insect to degrade wood fibers by secreting cellulases and hemicellulases [3]. Protists are thought to play a role in hydrolysis of cellulose and are associated with bacteria in complex symbioses [4]. The main flagellates living in the termite gut belong to three groups : *Oxymonadida*, *Trichomonodida* and *Hypermastigida*. The last two groups belong to the Class of *Parabasalia*. These protozoa are only found in the termite gut [5]. Those symbiotic microorganisms are thought to be necessary to the survival of termites [6].

Although poorly described in literature, amylases can also be produced by protists. Alpha-amylase activity was detected from the host gut tissue and hindgut symbionts [7]. Also, amylase activity was found in *Trichomitopsis termopsidis* (Cleveland) [8]. The termites *Macrotermes michaelseni* (Sjöstedt), *Macrotermes bellicosus* (Smeathman), *Macrotermes barneyi* (Light) and *Pseudacanthotermes militaris* (Hagen) also contain amylase activity in the mid- and hindgut [9]. Little is known about the protist metabolism because their culture is far from being easy [10].

There are few cases of protist isolations. The aim of this work is to isolate amylolytic protists from the gut of the termite *Reticulitermes santonensis*. Here, we suggest a method of isolation of amylolytic protists from the termite gut with rice grains, providing starch as a carbon source and proteins. An antibiotic purification is also necessary to prevent bacterial contaminations.

V.2. Materials and methods

V.2.1. *Organisms*

Reticulitermes santonensis (Feytaud) was obtained from the Island of Oleron (France). Trees showing characteristics of termite infestation (death or galleries) were cut into sections. Contaminated pieces were packed and transported in thick plastic bags. The termites were cultivated in darkness for 6 months at 27°C with a relative humidity level of 70%. They were fed with pine wood, which was gradually replaced by poplar wood.

V.2.2. *Dissection of termites*

The contents of digestive tracts from twenty-five termites were introduced into a tube containing 2 ml of medium. The termites were washed in ethanol, water and held with two dissection forceps. One of them was used to hold the head and the other to remove the cuticle at the back of the termite. The digestive tracts were then extirpated and inserted into the tube. A sterile needle was used to pierce the digestive tracts before putting the contents of the tube in a flask containing 150 ml of isolation medium.

V.2.3. *Isolation medium*

The choice of the isolation medium was based on a previous study [11]. It consisted of mineral water containing Ca^{2+} 65 $mg.l^{-1}$, Mg^{2+} 18 $mg.l^{-1}$, Na^{+} 44 $mg.l^{-1}$, K^{+} 2.5 $mg.l^{-1}$, SO_4^{2-} 40 $mg.l^{-1}$, HCO_3^{-} 305 $mg.l^{-1}$ and sterile rice grains (100 rice grains.l^{-1}). The flasks used for isolation contained 150 ml of medium and were incubated at 30°C and stirred 60 rpm. Different antibiotics (**Table 1**) were used to obtain pure cultures of protists. All of the antibiotic experiments were made in flasks containing culture medium. Antibiotic solutions were prepared at high concentrations, filter-sterilized and added to the flasks to reach a final volume of 150 ml. Antibiotic concentrations were chosen further based on the analysis of the results of antibiograms done on the two contaminant bacterial strains and involving a normalized method which led to the determination of Minimal Inhibitory Concentrations. Each antibiotic was tested at twice the concentration of the MIC value (for contaminating bacteria 1 or 2) and was added to the culture medium.

Table 1. Minimal Inhibitory Concentrations (M.I.C.) for the two main contaminant bacterial strains of the culture medium. Ciprofloxacin was effective on the two strains and had no negative effect on the protist. Other antibiotics were effective on bacteria and inhibited the growth of protists. Kanamycin and streptomycin were the most effective antibiotics but the protist proved unable to grow in the medium.

Antibiotic	CMI for Strain 1 (mg/l)	CMI for Strain 2 (mg/l)
Penicillin	512	< 0.25
Gentamicin	< 0.25	128
Ciprofloxacin	16	< 0.25
Streptomycin	0.5	8
Vancomycin	128	0.25
Kanamycin	< 0.25	< 0.25
Tetracycline	2	64
Erythromycin	64	< 0.25
Chloramphenicol	16	8
Rifampicin	2	16
Ampicillin	256	256

V.2.4. *Putative phylogenetic affiliation of the protist isolated*

DNA was extracted from a 7-day culture sample of protist cells with the Wizard Genomic DNA Purification Kit (Promega Benelux BV Branch Office - Schipholweg 1 - 2316 XB LEIDEN - The Netherlands). The method of DNA extraction from tissue culture cells was modified : Trypsin was not used and, after adding Nuclei Lysis Solution, the samples followed a specific temperature program (10 min at -80^0C, 10 min at 80^0C, 5 min at -80^0C, 5 min at 80^0C). The other steps were not modified. The primers used were ITS1 and ITS4 (amplification of 5.8S ribosomal DNA - large subunit), and two further primers were used to amplify 18S ribosomal DNA – small subunit :

- Primer 1 : 5'-TGATCCTTCTGCAGGTTCACCTAC-3' [12],
- Primer 2 : 5'-CTTTCGATGGTAGTGTATTGGACTAC-3' [13]).

Cycling conditions were : 2 min of denaturation (94°C), 35 cycles of amplification composed of 1 min at 94°C, 65 seconds at 37°C, 3 min at 72°C, and a final extension step of 6 min at 72°C [13]. PCR products were sequenced by the Sanger method of the firm Progenus (2, rue des Praules, Gembloux 5030-Belgium). The sequences were aligned with the Vector NTI® program and compared with the GenBank database. A phylogenetic tree was designed with the MEGA 5.0® software using the Neighbor-Joining method. Bootstrap values were expressed as percentages of 1000 replicates. The tree was drawn to scale and the distance bar indicates the number of base substitutions per site. The tree was developed with the sequences

of 18S rDNA of Chrysophyceae available on GenBank. The 18S rDNA sequences of protists extracted from the gut of termites were also added to the tree.

V.2.5. *Culture conditions*

Two media were used to investigate the growth of protozoa : Culture Medium 1 : 10 $g.l^{-1}$ soluble starch (Sigma-Aldrich), 5.5 $g.l^{-1}$ tryptone (Organotechnie, La Courneuve, France), 1.34 $g.l^{-1}$ $NaH_2PO_4.2H_2O$ and 30 $mg.l^{-1}$ ciprofloxacin (filter-sterilized before addition, Sigma-Aldrich), pH was adjusted to 4, 5, 6, 7, 8, 9 with H_3PO_4 1M or NaOH 1 M and Culture Medium 2 : 10 $g.l^{-1}$ soluble starch, 5.5 $g.l^{-1}$ tryptone, 1,34 $g.l^{-1}$ $NaH_2PO_4.2H_2O$, 4 $g.l^{-1}$ yeast extract (Organotechnie, La Courneuve, France) and 30 $mg.l^{-1}$ ciprofloxacin (filter-sterilized before addition), pH adjusted to 4, 5, 6, 7, 8, 9. The ciprofloxacin concentration was chosen in accordance with the results of the antibiograms. The flasks were filled with 150 ml of medium, inoculated at an initial concentration of 5.10^4 $cells.ml^{-1}$ and agitated (60 rpm) at a temperature of 30°C. All the quantitative results obtained from protist cultures (cell, glucose, maltose, maltotriose and starch concentrations) were analyzed with the MINITAB16® statistical software, and all the measurements were performed in triplicate. Normality tests and tests for equal variances were applied to all the series of data before achieving a variance analysis.

V.2.6. *Quantification of glucose, glucose oligomers and starch*

Glucose and glucose oligomers were quantified using a High-Performance Anion Exchange Chromatograph coupled with a Pulsed Amperometric Detector. The apparatus was a Dionex DX500 chromatographic system operating at 1 $ml.min^{-1}$. The stationary phase consisted of a CarboPac PA 100 column (250 x 4 mm) with a pre-column PA 100 (50 x 4 mm) (DIONEX Corp, Sunnyvale, USA). The volume injected was always 25 µl. Temperature was set to 35°C. Four mobile phases were used : NaOH 100 mM (Solvent A), NaOH 100 mM + sodium acetate buffer 600 mM (Solvent B), NaOH 500 mM (Solvent C) and H_2O MilliQ (Solvent D). An acetate gradient was used to elute the glucose oligomers (from 100 to 70% of Solvent A, from 0 to 30% of Solvent B) for 10 minutes. The column was washed with 50% of Solvent B and 50% of Solvent C for 9.9 minutes. Finally, conditioning was performed with 50% of Solvent A and 50% of Solvent D for 9.9 minutes. Maltose and maltotriose were chosen as calibration oligomers. Higher mass glucose oligomers were measured with a mass spectrometer. A matrix of 2,5-dihydroxybenzoic acid (DHB) was used at 20 $mg.ml^{-1}$ in acetonitrile/water 0.1% trifluoroacetic acid. Samples were prepared with a mixture of 1µL of hydrolysate solution and 1 µL of matrix. Measurements were taken with Ultraflex II

TOF/TOF (Bruker Daltonics) equipped with smartbeam. Calibration was achieved with peptide calibration standard II solution (Bruker). Starch concentrations were determined using a spectrophotometric measurement after iodine staining, using a method developed before [14]. The degradation of starch was measured in uninoculated flasks at pH values of 5, 6, 7 and 8 to check a potential degradation. This experiment was carried out in triplicate.

V.2.7. *Enzymatic assays*

The following activities were tested : endo-1,4-β-D-xylanase, endo-1,4-β-D-glucanase, amylase and protease. The samples (80 µl of crude culture and 80 µl of a sample filtered on a cellulose acetate membrane; 0.45 µm of porosity) were dropped on Petri dishes filled with different agar media. Anti-protease (Complete, Mini, EDTA-Free Protease Inhibitor Cocktail Tablets, Roche) was added to the culture samples except for protease assays. For each Petri dish, water (80 µl, negative answer) and diluted enzyme (80 µl of concentrated endo-1,4-β-D-xylanase, cellulase, amylase or bromelain $2.5.10^3$ $nkat.ml^{-1}$, positive answers) were both added. Enzymatic activities were calculated on the basis of the number of $nkat.g^{-1}$ mentioned for each enzyme. The following media were used : Endo-1,4-β-D-xylanase activity : 1 $g.l^{-1}$ AZCL-xylan medium, Na acetate 25 mM (pH 4.7), 16 $g.l^{-1}$ agar - Cellulase activity : 1 $g.l^{-1}$ AZCL-HE-cellulose medium, Na acetate 25 mM (pH 4.5), 16 $g.l^{-1}$ agar - Amylase activity : 1 $g.l^{-1}$ AZCL-amylose medium, Na acetate 100 mM (pH 6), 16 $g.l^{-1}$ agar - Protease activity : 1 $g.l^{-1}$ AZCL-casein medium, Na phosphate 100 mM (pH 7), 16 $g.l^{-1}$ agar. Buffers and pH values were chosen using the manufacturer's suggestions (Megazyme International Ireland - Bray Business Park - Bray, Co. Wicklow, Ireland). Petri dishes were kept at 30°C for one day before reading the results. The principle of AZCL media is based on azurine cross-linked polymers (xylan, cellulose, starch and casein in this case). When an enzymatic activity appears, the corresponding polymer is degraded and azurine is released. Then, big blue stains can be observed on the agar plates [15].

V.3. Results

V.3.1. *Protist isolation and identification*

Bacteria contaminated the culture medium at a mean concentration of 10^8 Colony Forming $Units.ml^{-1}$. No colonies of molds were observed, and it was necessary to inhibit bacterial contamination. Minimal Inhibitory Concentrations (MIC) are presented in **Table 1**. The effect on bacteria and protists was almost always negative, except for ciprofloxacin, which was able to allow protist growth only. The action of ciprofloxacin consists in inhibiting

DNA gyrase, an enzyme which allows transcription and replication of DNA. Ciprofloxacin is effective on Gram-positive and Gram-negative bacteria [16]. Consequently, ciprofloxacin was used at a concentration of 32 $mg.l^{-1}$ in the culture medium and allowed to reach a bacterial contamination of $2\text{-}3.10^4$ $CFU.ml^{-1}$. Then, Medium 1 (see 2.4.) was tested and allowed to reach a bacterial concentration of less than 100 $CFU.ml^{-1}$ and was chosen as the culture medium. Ciprofloxacin led to the development of a unique protist, showing a circular shape and a size of about 10 µm (**Figure 1, A-B-C-D**).

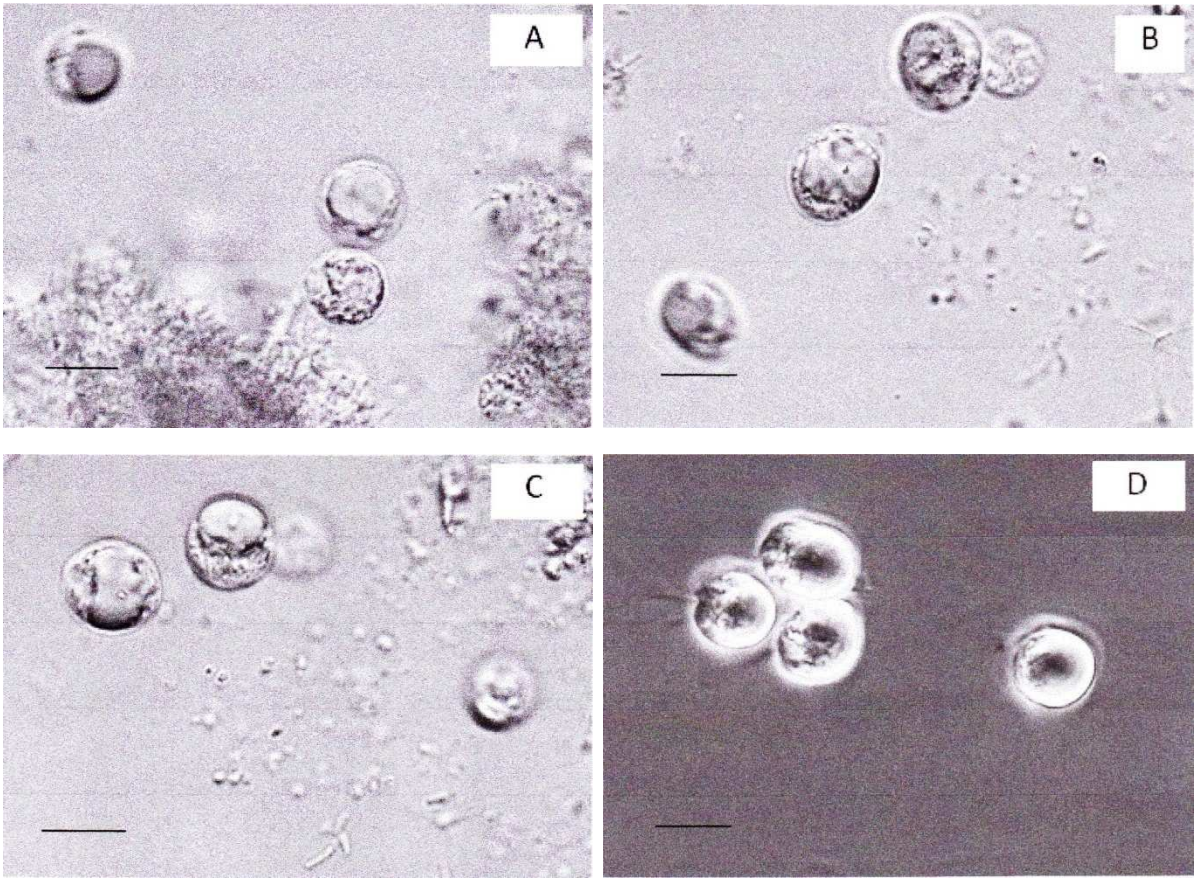

Figure 1 A-B-C-D. Protist isolated from the gut of *Reticulitermes santonensis* growing in the rice medium after addition of ciprofloxacin (pictures taken using an electron microscope, images obtained with an AxioCam camera, scale bar = 10 µm).

About 240 µg of DNA were extracted from 6 ml of a culture containing 5.10^6 cells/ml. The PCR amplification of the extracted DNA led to a unique band of about 1500 bp, suggesting amplification of 18S rDNA, using primers 1 and 2 specific to protozoa, and a unique band of about 800 bp, indicating amplification of 5.8S rDNA, using primers ITS1 and ITS4. The sequences are available on GenBank (5.8S rDNA GenBank ID : JQ409545; 18S rDNA GenBank ID : JQ409546). The sequences were compared with the sequences available on GenBank after aligning with the Vector NTI® program. The NCBI-BLAST program [17]

was used to identify the sequences. It appeared that the protist is related to *Poterioochromonas* (96% of sequence similarity for the 18S rDNA) and *Ochromonas* (96% of sequence similarity for the 18S rDNA) genera. The sequence of the 5.8S rDNA led to the identification of the genus *Poterioochromonas* (94% of sequence similarity for the 5.8S rDNA with *Poterioochromonas malhamensis* (Pringsheim)). Consequently, *Poterioochromonas* sp. (GenBank ID : AY699607.1) was found to be the most similar microorganism to the protist in the culture medium. The phylogenetic tree based on 18S rDNA sequences is presented in **Figure 2**.

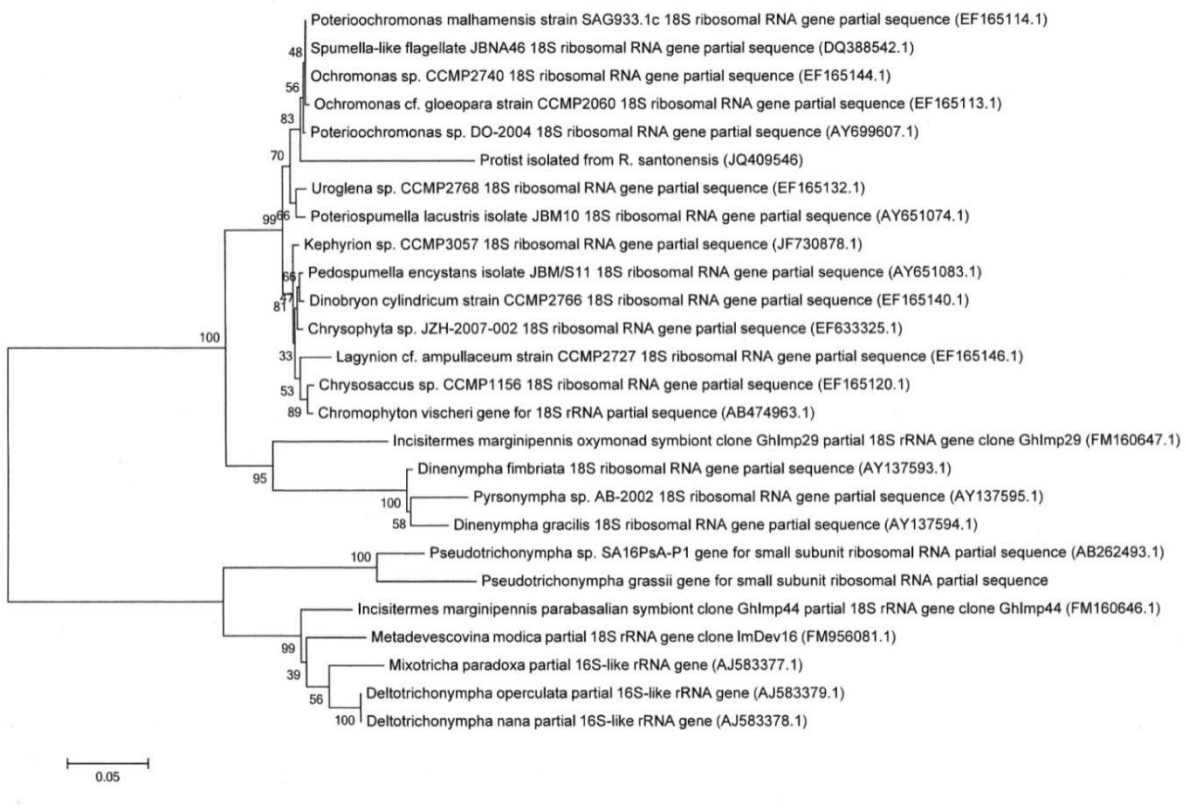

Figure 2. Phylogenetic tree showing the phylogenetic position of the protist (GenBank ID : JQ409546). Protists were isolated from termites and their ribosomal SSU DNA sequences were determined and included in the tree.

V.3.2. *Growth conditions of the protist*

Two different media were tested to make the growth curves : Medium 1 and Medium 2 (Medium 1 added with yeast extract, 4g.l^{-1}). In both media, the bacterial contamination was less than 100 CFU.ml^{-1}. Various parameters were measured during the growth of protists : cell concentrations, pH, glucose concentrations, maltose and maltotriose concentrations and, finally, starch concentrations. At pH 4 and 9, no growth was observed, the starch was not consumed and no glucose was produced. However, the protist was able to survive at pH 8.

The growth observed with yeast extract was significantly different from the growth observed without yeast extract ($p<0.05$), except for pH 7 (**Figure 3 A and B**).

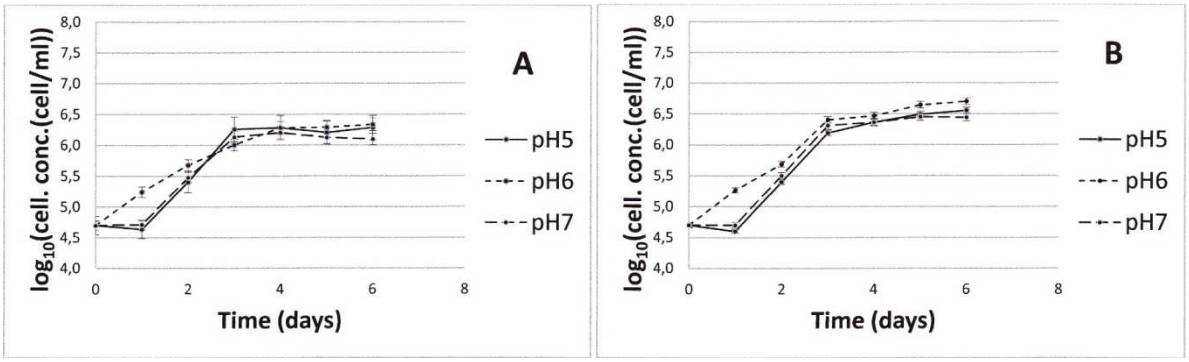

Figure 3. Growth curves of *Poterioochromonas* sp. in the culture media 1 **(A)** and 2 **(B)**.

There was also a significant difference between the final cell concentrations at pH 5, 6 and 7. Indeed, pH 6 allowed the highest cell concentration to be reached, which was significantly different from those obtained with pH values of 5 and 7. At a specific pH value (5, 6 or 7), yeast extract increases the final cell concentrations significantly. However, the pH of the culture media was not stable. For both media, the strongest increase of pH was observed for an initial pH of 6 (**Figure 4 A and B**).

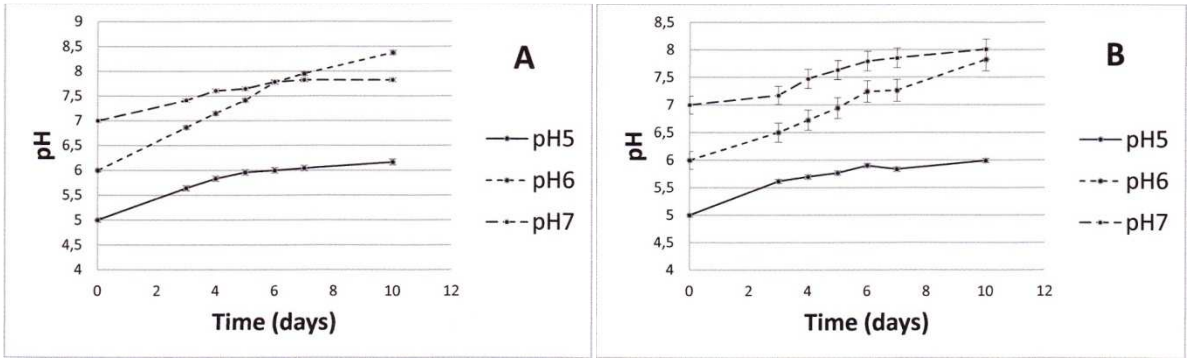

Figure 4. pH evolution in the cultures of *Poterioochromonas* sp. in the culture media 1 **(A)** and 2 **(B)**.

The dry matter was weighed at the end of five culture periods. Therefore, it was established that one gram of dry matter is equal to $6.1.10^9 \pm 10^9$ cells. Consequently, it was possible to calculate conversion rates (biomass vs. carbon source), considering glucose, maltose and maltotriose concentrations measured in the culture media. Conversion rates were 0.037 ± 0.001, 0.04 ± 0.001 and 0.0229 ± 0.0003 at pH 5, 6 and 7, respectively (Medium 1). These results show that preferential pH values for growth are at weakly acidic values. Those results were confirmed in Medium 2. Conversion rates were 0.103 ± 0.008, 0.11 ± 0.01 and

0.051 ± 0.006 at pH 5, 6 and 7, respectively. All these values were found to be significantly different.

V.3.3. *Hydrolysis products of starch*

Normality tests and tests for equal variances were done for each medium and each day, and were found to be positive ($p>0.05$). Neither of the culture media contained any glucose at the start. For Medium 1 (without yeast extract), the glucose concentration for pH 5 was significantly different ($p<0.05$) at pH 6 after 7 days. For Medium 2, there was a significant difference between the three pH values after day 3. In each case, pH 5 provided the highest glucose concentration (**Figure 5 A** and **B**).

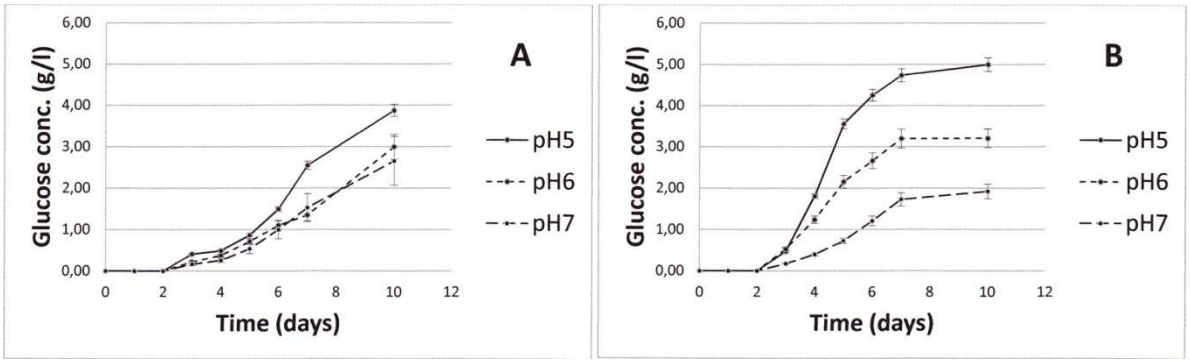

Figure 5. Evolution of glucose concentrations in the cultures of *Poterioochromonas* sp. in the culture media 1 **(A)** and 2 **(B)**.

The analysis of glucose oligomers was performed on Medium 2 (added with yeast extract), which provided the best results (**Figure 6 A** and **B**).

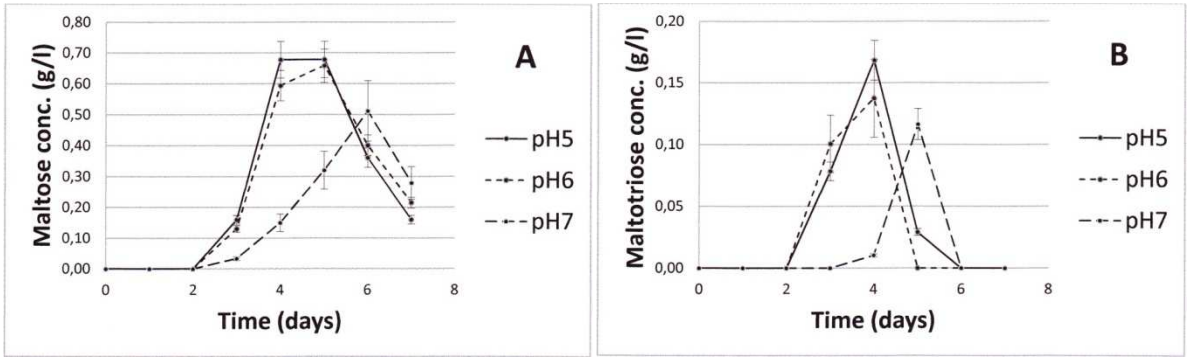

Figure 6. Evolution of maltose **(A)** and maltotriose concentrations **(B)** in the cultures of *Poterioochromonas* sp. determined in the Culture Medium 2.

Normality tests and tests for equal variances were achieved for each medium and each day and were positive ($p>0.05$). Very low concentrations of maltotetraose and maltopentose were detected, but were not quantifiable. The highest concentrations of maltose and maltotriose were found for pH values of 5 and 6. There were no significant differences

between the concentrations of these sugars for pH 5 and 6 (in both media). Mass spectrometry was used to identify a wide range of glucose oligomers. It was possible to detect oligomers up to a size of sixteen units of glucose. The mass spectrum was obtained from a sample of the Culture Medium 2 after 3 days at pH 5. Other spectra displayed lower peaks, although glucose oligomers were also detected (data not shown).

The starch concentrations were also measured (**Figure 7 A and B**). Each medium was submitted every single day to normality tests and tests for equal variances which proved positive ($p>0.05$). There were no significant differences between the starch concentrations in Medium 1 (without yeast extract) for different pH values. For Medium 2 (added with yeast extract), significant differences were observed for days 4, 5 and 6. Starch concentrations measured at pH 7 differed ($p<0.05$) from pH 5 and 6 and were higher (**Figure 7 B**). In both media, the starch completely disappeared, but this was significantly quicker in the medium containing yeast extract. The degradation of starch was also measured in uninoculated medium (pH 5, 6, 7 and 8) to check the potential degradation induced by the medium components, pH or temperature. No degradation was observed in such cases.

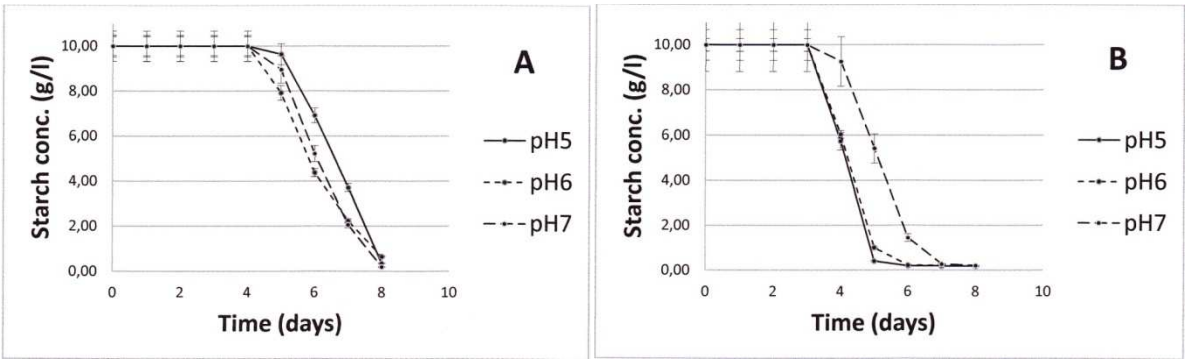

Figure 7. Evolution of starch concentrations in the cultures of *Poterioochromonas* sp. in the culture media 1 **(A)** and 2 **(B)**.

V.3.4. *Investigation of enzymatic activities*

Agar plates added with different media were tested to investigate the enzymatic activities of the protist : amylase, protease, cellulase and endo-1,4-β-D-xylanase. No protease, cellulase or endo-1,4-β-D-xylanase were detected. However, it was possible to confirm amylase activities. These assays were carried out on the protist growing in Medium 2 (added with yeast extract). Amylase activity was observed on Petri dishes, appeared on day 3 and remained in the culture medium until the end of the culture time (10 days). The contaminant bacteria were grown in the culture medium without ciprofloxacin. The protist was unable to grow without antibiotics because of the development of bacteria in the culture medium. Agar

plates were tested with samples collected from these cultures, and no amylase activity was detected.

Filtration of the culture medium was performed using a cellulose acetate membrane (0.45 µm of porosity) to remove the cells. Amylase activity was detected both in the crude and the membrane-filtered samples. The intensity of coloration depends on amylase activity and was found to be the same for both types of samples.

V.4. Discussion

V.4.1. *Protist identification*

The protists *Ochromonas* and *Poterioochromonas* sp. belong to the Phylum of *Chrysophyta*, which are unicellular microorganisms that are able to grow in fresh or salt water [18]. *Ochromonas* and *Poterioochromonas* sp. are closely related as chrysomonads [19]. The class of *Chrysophyceae* is described as a highly diversified group [20]; chrysophytes are heterokont cells (displaying flagella of unequal length), which are able to obtain energy from photosynthesis and heterotrophy [21]. The morphology of *Ochromonas danica* (Pringsheim), a typical chrysophyte, was previously described; typical cells of chrysophytes contain a nucleus, flagella, contractile vacuoles, Golgi bodies, chloroplasts, mitochondria and chrysolaminarin vesicles [22].

The bootstrap value observed in the phylogenetic tree is low. The most closely related protist was *Poterioochromonas sp*. (GenBank ID : AY699607.1) and the protist which was isolated was phylogenetically closer to *Poterioochromonas* than *Ochromonas* genus. *Poterioochromonas* and *Ochromonas* genera were also found to be morphologically alike [23], and the morphology of the present protist was also similar. However, it is generally accepted that a bootstrap value of 70-80% is enough to confirm a clade [24]. *Ochromonas* and *Poterioochromonas* species are very close phylogenetically. The phylogenetic tree obtained here can be compared with the phylogenetic trees reported before [20]. It was found that several species of *Ochromonas* had a polyphyletic relationship. Phylogenetic trees were designed with the genera *Poterioochromonas* and *Ochromonas*, and both were classified in clade C. *Ochromonas tuberculata* (Hibberd) was the only protist to be classified in a different clade. However, higher bootstrap values were calculated [20]. The phylogenetic tree was also built on the basis of protists that are found in termite guts. *Dinenympha* and *Pyrsonympha* genera belong to *Oxymonadidae*. *Pseudotrichonympha*, *Metadevescovina*, *Mixotricha* and *Deltotrichonympha* genera belong to the Class of *Parabasalia*. The phylogenetic tree clearly

shows two groups. Those groups are perfectly defined and characterized by a bootstrap value of 100. Consequently, it is possible to conclude that oxymonads are more closely related to chrysophytes (such as *Poterioochromonas* sp.) than *Parabasalia*. Oxymonads also have a particular cellular structure. They consist of a special group that does not include any energy-generating organelles such as mitochondria or hydrogenosomes, and their intracellular membrane system is poorly developed. Another aspect is how difficult their culture is [6]. *Oxymonadidae* comprise five families and include *Dinenympha*, *Pyrsonympha* and *Oxymonas* genera.

The cell morphology observed here is comparable with the descriptions given before [21]. The protist is related to species belonging to the *Ochromonas* and *Poterioochromonas* genera that are morphologically close. *Ochromonas* cells have a size of 2 to 30 µm with one long flagellum and one short flagellum, one or two chloroplasts and occasionally a stigma. *Poterioochromonas* cells display the same characteristics. They also have 2 flagella, but the short one is protected by a chitinous lorica at the end of a stalk [23]. Although the lorica is not visible on the protist that was isolated, the morphology corresponds to a description given previously [25]. In their study, the lorica structure was not observed on the strain of *Poterioochromonas* sp.

V.4.2. *Protist growth and effect of yeast extract*

Yeast extract allowed a better development in the culture medium, bringing more minerals, trace elements, peptides and vitamins, and also resulted in the faster consumption of the carbon source. A pH value of 6 seems to be optimal for the growth of protists. On the other hand, given that the pH shows a strong tendency to increase, pH 7 is also suitable, but varies too quickly. Growth curves with different pH values suggest that a weakly acidic pH is more conducive to the growth of protists than a weakly alkaline pH. The optimal pH for the growth of protozoa is generally found to be between 6 and 8 [26]. The production of ammonia by the planktonic protists was cited before [27], which can explain the increase of pH observed in the culture medium. The growth efficiency of phagotrophic protists is generally comprised between 10 and 40%, while the remaining amount of carbon source (90-60%) is lost in excretion or respiration [28]. However, the growth efficiency observed here was quite low (with a maximal biomass yield of 0.11).

Yeast extract had a significant effect on protist growth, biomass yields, glucose and starch concentrations. All these parameters are bound. An increase of glucose concentration is

the result of starch hydrolysis, caused by the secretion of an amylase by the protist. Starch is hydrolyzed, releasing glucose units that can be assimilated through a specific metabolism, leading to a specific biomass yield. The final cellular concentrations depend on the amount of starch that was consumed and glucose that was not assimilated by the protist. Yeast extract made it possible to reach 1.9 (pH 5), 2.3 (pH 6) and 2.2 (pH 7) times higher final cell concentrations. It also led to 2.8 (pH 5), 2.8 (pH 6) and 2.2 (pH 7) times higher biomass yields. The strongest effect was observed at pH 5 and 6. A pH value of 6 seems more suitable for the protist development in regard to cell concentrations and biomass yields. The evolution of glucose and starch concentrations is more representative of amylase activity, although it is also bound to cell growth. The evolution of glucose concentrations reveals a strongest amylase activity at pH 5, with or without yeast extract. The latter causes a faster production of glucose from starch as well as higher glucose concentrations. Consequently, *Poterioochromonas* sp. secretes an amylase for which the optimal pH is about 5. Trace elements matter a lot as well. These metals are often used to boost enzymatic activities of bacteria and molds [29, 30]. In this case, trace elements are provided by yeast extract. Vitamins can also play a role in the increase of amylase activities.

V.4.3. *Enzymatic activities of Poterioochromonas sp.*

Studies on amylase activities in chrysophytes are seldom. Two days were necessary to detect such amylase activity. The excretion of an amylase produced by *Ochromonas* sp. was previously studied [31]. It was observed that the protist was able to ingest and digest starch, casein, oil droplets and small organisms; it was also found that *Ochromonas* was able to grow in a medium containing glucose, sucrose, fructose, galactose or starch. The production of an amylase by *Poterioochromonas stipitata* (Scherffel) was suggested on the basis of the liquefaction of a starch gel [32]. Amylase activity was also revealed in the mid- and hindgut of the termite *Odontotermes obesus* (Rambur) [33]. The optimal pH for the enzyme was 5.8-6.6. The highest concentrations of glucose produced by the highest activities are found for an initial pH of 5 in this case. The maximal amylase activity was observed after 5 days at an initial pH of 5 (Culture Medium 2), which relates to a pH of 5.7-5.8, and sticks to what has been observed so far. Our strain produces an extracellular amylase, taking account of the results observed on AZCL-amylose medium inoculated with both crude and filtered culture samples. Moreover, the absence of an amylase activity produced by the contaminating bacteria leads to the conclusion that this activity comes only from the protist. Amylase activities can be calculated from the glucose concentrations. One katal (kat) of amylase

activity is defined as the amount of enzyme able to provide 1 mol of glucose in one second. Yeast extract boosted the secretion of amylase significantly. With yeast extract, the maximal amylase activity was detected after 5 days of culture, with values of 112 and 59 nkat.l^{-1} at pH 5 and 6, respectively. At pH 7, 7 days were necessary to reach a value of 34 nkat.l^{-1}. However, without yeast extract, the maximal amylase activity was measured after 7 days of culture, with values of 68 and 34 nkat.l^{-1}, at pH 5 and 7, respectively. At pH 6, a maximal activity of 35 nkat.l^{-1} was reached after 10 days.

The degradation of starch involves different types of amylases; α-amylase can hydrolyze internal α-(1-4)-linkages in amylose and amylopectin; β-amylase hydrolyzes α-(1,4)-linkages and releases maltose (inverting C-1 position during hydrolysis) and β-limit dextrins [34]; amyloglucosidase hydrolyzes α-(1,4)- and α-(1,6)-linkages of the starch extremities, and releases 95% of glucose [35], although it can completely hydrolyze starch [34]; and α-(1,6)-glucosidases are able to break the α-(1,6)-linkages to produce limited dextrins [36]. These starch-debranching enzymes are divided into two types : pullulanases (able to break α-(1,6)-linkages and release pullulan) and isoamylases (able to break α-(1,6)-linkages and release molecules other than pullulan). The action of these different activities is necessary for the complete degradation of starch [36]. The cultivated protist is able to produce glucose, maltose, maltotriose and some glucose oligomers of more than 3 units of glucose in low quantities. Consequently, the amylase activities developed by the protist should include amyloglucosidase, α-amylase and possibly β-amylase. Indeed, maltose should stem from a β-amylase activity or α-amylase activity hydrolyzing maltotetraose into two molecules of maltose. Maltose and maltotriose concentrations evolved in the same way. At pH 7, maltose and maltotriose needed more time to be detectable. Three days were necessary before detecting maltose and maltotriose. Maximal maltose concentrations were 0.68, 0.66 and 0.51 g.l^{-1} at pH 5, 6 and 7, respectively, while maximal maltotriose concentrations were 0.17, 0.14 and 0.12 g.l^{-1}. There were no significant differences between the results found at pH 5 and 6.

Starch hydrolysis may also release isomaltose (maltose isomer), panose and isopanose (maltotriose isomers). These molecules contain α-(1,6)-linkages and come from reticulated branches of starch. In this study, starch stemmed from potatoes, usually composed of 21% amylose and 79% amylopectin. Amylose is a linear chain that mainly consists of α-1,4-linked glucose residues, although α-1,6-linkages can be observed (about one branch per 1000 residues). Amylopectin is more branched. It is considered that approximately 5% of glucose residues have both α-1,4- and α-1,6-linkages [37]. Maltose (Degree of Polymerization : 2) and

maltotriose (DP : 3) were used as calibration oligomers. However, HPAEC-PAD did not allow separating isomers. Consequently, the peaks also contain signals coming from minority components : isomaltose, panose and isopanose.

As far as we know, this is the first time a chrysophyte has been extracted from the termite gut and subsequently cultivated. Mixotrophy gives the chrysophytes a real advantage relating to their ability to develop in a particular environment. There are different types of mixotrophy. *O. danica* and *P. malhamensis* can facultatively use phototrophy or phagotrophy and osmotrophy [38]. The conditions of the termite gut force the protist to produce its energy using heterotrophic respiration. The termite gut is not completely anoxic; there is an oxygen gradient between the oxic gut epithelium and the anoxic gut contents [39]. Although available in low quantities, oxygen can be used for respiration. The pH in the gut of *R. sanonensis* is close to neutrality (6.8 in the midgut and 6.4 in the paunch) [40]. To our knowledge, protists belonging to the genus *Poterioochromonas* are not able to grow at highly acidic pH, which contrasts with *Ochromonas* species [19]. It may sound strange to find amylolytic protists in termite guts. Moreover, this is the first time that a chrysophyte has been extracted from a termite gut. *Reticulitermes santonensis* was grown on poplar wood in the present study. Poplar wood is mainly composed of cellulose (45%), hemicelluloses (25%) and lignin (20%) [41]. The trees control their metabolism by sensing critical shortening of the photoperiod. When environmental conditions become too harsh, poplars alter their metabolic pathways and synthetize storage molecules, such as starch [42]. This could explain the presence of amylolytic protists inside termite guts. Another point is that the isolated chrysophyte can metabolize glucose, composing cellulose. Another explanation may be that *Poterioochromonas* sp. uses glucose resulting from cellulose hydrolysis by the cellulolytic microflora. The phylogentic tree also showed the relationship between chrysophytes and oxymonads, which are natural residents of lower termite guts.

V.5. Conclusion

Poterioochromonas sp. was isolated from the gut of the lower termite *R. santonensis*, which offers acceptable conditions for the development of protists. Chrysophytes, such as *Poteriochromonas* and *Ochromonas* genera, are mixotrophic, and it is the first case of isolation of such microorganisms from the gut of termites. *Poterioochromonas* sp. was found to be closer to oxymonads than parabasalids. The isolation method which was used in the present study may induce further experiments. It is true that isolating protists is particularly difficult, and antibiotic methods may be an efficient way of reducing or doing away with

bacterial contaminations. New techniques of isolation such as antibiotic treatments combined with low speed density gradient centrifugation could be tried out.

The protist grew at a pH close to neutrality and produced an extracellular amylase, which hydrolyzed starch in glucose and glucose oligomers. However, the activity was quite low. High-Performance Anion Exchange Chromatography and Mass Spectrometry identified oligomers as being progressively hydrolyzed before being metabolized. No other enzymatic activity was detected. Another study including research of amylase genes in the protist DNA should be complementary to this work. Then, on the basis of the sequences, it may be possible to confirm the type of amylase activities.

V.6. References

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CHAPITRE VI.

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Isolation and cultivation of xylanolytic and cellulolytic *Sarocladium kiliense* and *Trichoderma virens* from the gut of the termite *Reticulitermes flavipes*

Ce chapitre correspond à l'article intitulé "*Isolation and cultivation of xylanolytic and cellulolytic Sarocladium kiliense and Trichoderma virens from the gut of the termite Reticulitermes santonensis*" (Cédric Tarayre, Julien Bauwens, Catherine Brasseur, Christel Mattéotti, Catherine Millet, Pierre Alexandre Guiot, Jacqueline Destain, Micheline Vandenbol, Daniel Portetelle, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart) actuellement soumis à la revue **Environmental Science and Pollution Research**.

Les termites disséqués jusqu'à présent étaient soumis à une diète conventionnelle composée de bois de peuplier. Cette diète a inévitablement un effet sur la microflore constitutive de l'intestin du termite. Rappelons que, jusqu'à présent, des bactéries, une moisissure et un protiste non cellulolytique ont pu être isolés et cultivés. Il nous a paru intéressant de tester l'influence de diètes artificielles sur la composition de la microflore du termite. Au total, 5 diètes ont été testées : du xylane de hêtre, de la cellulose microcristalline (additionnée ou non de lignine) et de la cellulose microcristalline de type α (additionnée ou non de lignine).

Ce chapitre présente les résultats obtenus avec les diètes ayant mené à l'isolement de deux moisissures en particulier : *Trichoderma virens* CTGxAviL et *Sarocladium kiliense* CTGxxyl. La caractérisation des enzymes cellulolytiques et hémicellulolytiques qu'elles produisent fait l'objet principal de ce chapitre.

Résumé

Le but de ce travail était d'isoler et cultiver des microorganismes cellulolytiques et xylanolytiques extraits à partir du système digestif du termite inférieur *Reticulitermes santonensis*. De la cellulose microcristalline (avec ou sans lignine) et du xylane de hêtre ont été utilisés en tant que diètes à la place du bois de peuplier afin de sélectionner les mycètes capables de dégrader la cellulose et les hémicelluloses. La souche *Sarocladium kiliense* (*Acremonium kiliense*) CTGxxyl a été isolée des termites nourris de xylane, alors que la souche *Trichoderma virens* CTGxAviL a été isolée des termites nourris de cellulose (avec et sans lignine). Les deux moisissures ont été cultivées dans des milieux liquides contenant différents substrats : des résidus agricoles ou des polymères purifiés. *S. kiliense* a produit des activités β-glucosidase, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase et endo-1,4-β-D-xylanase maximales de 0.103, 3.99, 0.53 et 40.8 U.I./ml, respectivement. *T. virens* a produit des activités β-xylosidase, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase et endo-1,4-β-D-xylanase maximales de 0.38, 1.48, 0.69 et 426 U.I./ml. Les activités optimales des xylanases ont été observées à pH9-10, 60^{o}C pour *S. kiliense*, et à pH5-6, 50^{o}C pour *T. virens*. Les conditions optimales étaient différentes pour les cellulases : pH10, 70^{o}C pour *S. kiliense* et pH9, 70^{o}C pour *T. virens*. La zymographie a identifié différentes xylanases produites par les deux moisissures, et certaines tailles de fragments ont pu être mises en évidence : 35, 100 et 170 kDa pour *S. kiliense*, et 20, 40, 80 et 170 kDa pour *T. virens*. Dans les deux cas, les activités endo-1,4-β-D-xylanase ont été confirmées par spectrométrie de masse.

Mots-clés : Xylanase, Cellulase, *Reticulitermes flavipes*, *Sarocladium kiliense*, *Trichoderma virens*

Abstract

The purpose of this work was the isolation and cultivation of cellulolytic and xylanolytic microorganisms extracted from the gut of the lower termite *Reticulitermes santonensis*. Microcrystalline cellulose (with and without lignin) and beech wood xylan were used as diets instead of poplar wood in order to select cellulose and hemicellulose-degrading fungi. The strain *Sarocladium kiliense* (*Acremonium kiliense*) CTGxxyl was isolated from the termites fed on xylan, while the strain *Trichoderma virens* CTGxAviL was isolated from the termites fed on cellulose (with and without lignin). Both molds were cultivated in liquid media containing different substrates : agro-residues or purified polymers. *S. kiliense* produced maximal β-glucosidase, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and endo-1,4-β-D-xylanase activities of 0.103, 3.99, 0.53 and 40.8 I.U./ml, respectively. *T. virens* produced maximal β-xylosidase, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and endo-1,4-β-D-xylanase activities of 0.38, 1.48, 0.69 and 426 I.U./ml. Optimal activities of xylanases were observed at pH9-10, 60^{o}C for *S. kiliense*, and at pH5-6, 50^{o}C for *T. virens*. The optimal conditions were different for cellulases : pH10, 70^{o}C for *S. kiliense* and pH9, 70^{o}C for *T. virens*. Zymography identified different xylanases produced by both molds, and some fragment sizes were highlighted : 35, 100 and 170 kDa for *S. kiliense* and 20, 40, 80 and 170 kDa for *T. virens*. In both cases, endo-1,4-β-D-xylanase activities were confirmed through mass spectrometry.

Keywords : Xylanase, Cellulase, *Reticulitermes flavipes*, *Sarocladium kiliense*, *Trichoderma virens*

VI.1. Introduction

The degradation of vegetal structural polysaccharides requires an efficient enzymatic complex able to break the lignocellulosic matrix. Various enzymes are necessary to achieve this aim, more particularly cellulases and xylanases. These enzymes are able to hydrolyze cellulose and hemicelluloses, releasing fermentable sugars that can be exploited in biofuel production [1]. Microbial cellulases can degrade cellulose, which consists of a β-1,4-linked glucose polymer, through different modes of action. Endo-1,4-β-D-glucanases hydrolyze amorphous parts of cellulose, which are more easily accessible than crystalline regions. Exo-1,4-β-D-glucanases (or cellobiohydrolases) attack cellulose extremities, extracting cellobiose from crystalline or amorphous structures. Finally, β-glucosidases hydrolyze linkages binding the monomers constituting the cellooligosaccharides available in solution [2]. Hemicelluloses are also present in vegetal cell walls and their composition is more complex than cellulose that only contains glucose : xylose, arabinose, mannose, glucose, galactose and glucuronic acid. In those polymers, 5- and 6-carbon sugars and their uronic acids are β-1,4-linked [3, 4]. Xylan is the most common hemicellulose and consists of a main chain of β-1,4-xylose residues, linked to α-D-glucuronic acid, 4-O-methyl-α-D-glucuronic acid or α-L-arabinofuranose. Hemicelluloses are bound to lignin by ester and ether linkages [5]. Xylanolytic enzymes are mainly endo-1,4-β-D-xylanases, which cleave internal glycosidic bonds in the xylan backbone, and β-xylosidases, which hydrolyze small xylooligosaccharides and xylobiose. Acetylxylan esterase hydrolyzes the o-acetyl groups esterified on β-D-xylopyranosyl residues. Arabinase hydrolyzes the linkages between L-arabinose and β-D-xylopyranosyl residues. α-glucuronidase breaks the bonds between glucuronic acid and β-D-xylopyranosyl monomers of the backbone. Finally, ferulic acid esterase and p-coumaric acid esterase break ester linkages in xylan [6]. Other secondary xylanases, such as mannanases, could also be mentioned.

Many microorganisms can produce such enzymes. However, mycetes have particularly developed a real enzymatic arsenal. Many cases of cellulase- and xylanase-producing fungi were reported. Mycetes are able to produce cellulases (endo-1,4-β-D-glucanases, exo-1,4-β-D-glucanases and β-glucosidase) but also xylanases (endo-1,4-β-D-xylanase, β-xylosidase, mannanase, β-mannosidase, etc.) [7]. A few yeasts, such as *Aureobasidium pullulans*, *Cryptococcus albidus* and *Trichosporon cutaneum*, produce xylanases [8]. Some publications also report the production of cellulases by yeasts. *Candida peltata* was found to produce β-glucosidase [9]. *Trichosporon cutaneum* and *Trichosporon*

pullulans were also found to secrete cellulases [10]. However, filamentous fungi show more abilities in regard to enzyme production. The most studied cellulolytic molds are *Trichoderma*, *Humicola*, *Aspergillus* and *Penicillium* [11]. Many xylanase producers have also been quoted : *Aspergillus*, *Acrophialophora*, *Aureobasidium*, *Chaetomium*, *Fusarium*, *Myceliophtora*, *Penicillium*, *Trichoderma* and *Thermomyces* [6].

Some animals, like termites, earthworms, millipedes and collembola harbor a specialized microbial community inside their intestines [12-15]. More specifically, termites are symbiotically bound to bacteria, archaea, protists and mycetes. In lower termites, bacteria, mycetes and protists play a key role in lignocellulose digestion [16]. Interactions between mycetes and termites are particularly interesting. It has been suggested that filamentous fungi are not able to colonize the termite guts because of their cell size. Moreover, the structure of mycelium is quite fragile [12]. In some cases, termites have developed a close relationship with mycetes. The basidiomycete genus *Termitomyces* is cultivated in purpose-built chambers in the nest of higher termites [12]. However, filamentous fungi can also be found inside the termite guts [17].

The aim of this study was to isolate cellulolytic and hemicellulolytic fungi from the gut of the termite *Reticulitermes santonensis*. Artificial diets composed of microcrystalline cellulose and beech wood xylan were tried to select such strains. Then, a research of the potential cellulose- or hemicellulose-degrading fungi was achieved. Two molds, *Sarocladium kiliense* (*Acremonium kiliense*) CTGxxyl and *Trichoderma virens* CTGxAviL, showed interesting activities : β-glucosidase, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, β-xylosidase and endo-1,4-β-D-xylanase. The properties of the cellulases and xylanases were studied through quantitative assays (enzymatic activities, effect of pH and temperature), protein analysis (zymography and mass spectrometry) and mass spectrometry applied to the hydrolysis products.

VI.2. Materials and Methods

VI.2.1. *Organisms*

Reticulitermes santonensis Feytaud (*Rhinotermitidae*) was obtained from the Island of Oleron (France). The termites were grown in darkness at 27^{o}C with a relative humidity of 70%. The termites were first fed on pinewood, which was gradually replaced by poplar wood. Then, artificial diets were used to boost the xylanolytic and cellulolytic strains living inside

the termite guts. The diets were prepared in solid agar medium. Three diets were tried : Diet 1 (agar 15g/l, β-sitosterol 0.6g/l, microcrystalline cellulose 150g/l, lignin 50g/l), Diet 2 (agar 15g/l, β-sitosterol 0.6g/l, microcrystalline cellulose 150g/l) and Diet 3 (agar 15g/l, β-sitosterol 0.6g/l, beech wood xylan 200g/l).

VI.2.2. *Isolation and identification of the strains*

Twenty-five termites of each artificial diet were washed in ethanol, then water, and held with two dissection forceps. The digestive tracts were then extirpated and the hindgut sections were put in the corresponding liquid media. The basic composition for the media was derived from [18] : KH_2PO_4 2g/l, NH_4Cl 0.2g/l, $MgSO_4.7H_2O$ 1.54g/l, $CaCl_2.2H_2O$ 1.1g/l, H_3BO_3 2.86mg/l, $MnCl_2.4H_2O$ 1.81mg/l, $ZnSO_4.7H_2O$ 0.222mg/l, $NaMoO_4.2H_2O$ 0.39mg/l, $CuSO_4.5H_2O$ 0.079mg/l, $Co(NO_3)_2$ 0.031mg/l, pH7. Three different carbon sources were used : Medium 1 contained microcrystalline cellulose 7g/l and lignin 2.35g/l, Medium 2 contained microcrystalline cellulose 10g/l and Medium 3 contained beech wood xylan 11g/l. The flasks were agitated at 30°C for 3 days. Then, 100µl of different dilutions of both culture media were spread on agar plates containing potato dextrose agar added with chloramphenicol (0.5g/l). The Petri dishes were kept in darkness at 30°C. After 3 days, one strain from each culture medium was isolated. The identification was achieved on the basis of a morphological analysis by the specialists of the mycotheque of the University of Louvain-la-Neuve (Bâtiment Kellner 1er étage, Croix du Sud 2 bte L7.05.06, 1348 Louvain-la-Neuve, Belgium).

VI.2.3. *Culture conditions*

The molds were stored at -80°C. They were grown on PDA medium (chloramphenicol 0.5g/l) for 3 days. Then, the mycelium was collected by washing the plates with a peptone solution (casein peptone 1g/l, NaCl 5g/l, Tween 80 2ml/l), and the flasks were inoculated with 1 ml of a suspension showing an optical density of 0.7 (600 nm). Sterile peptone solution was used to achieve the dilutions. The two molds were grown at 30°C in flasks containing 100 ml of the following medium, derived from [19] : casein peptone 2.5g/l, KH_2PO_4 3g/l, $(NH_4)_2SO_4$ 2.6g/l, K_2HPO_4 6g/l, $MgSO_4.7H_2O$ 0.2g/l, NaCl 2g/l, $CaCl_2.2H_2O$ 2mg/l, $FeSO_4.7H2O$ 1.1mg/l, $ZnSO_4.7H2O$ 1.5mg/l, $MnCl_2.4H_2O$ 7.9mg/l, $CuSO_4.5H_2O$ 6.4mg/l, pH6. Six carbon sources were tried separately to improve enzyme production (40g/l) : Microcrystalline Cellulose (MC), Beech wood Xylan (X), α-Cellulose (αC), Wheat Bran (WB), Wheat Distiller's Grains (WDG) and Rapeseed Oil Cake (ROC). MC, X and αC are purified substrates, while WB, WDG and ROC are agricultural residues or wastes.

VI.2.4. *Quantitative enzymatic assays*

The culture samples were centrifuged for 10 minutes at 10,000g. Then, the supernatant was used to measure the enzymatic activities. Endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and endo-1,4-β-D-xylanase activities were measured by using dinitrosalicylic acid according to a method developed previously [20]. Endo-1,4-β-D-glucanase activity was established with carboxymethylcellulose (20g/l) as a substrate in phosphate buffer (0.1M, pH5.85). Exo-1,4-β-D-glucanase activity was measured with microcrystalline cellulose (10g/l) in phosphate buffer (0.1M, pH5.85). Endo-1,4-β-D-xylanase activity was determined with a solution of beech wood xylan (10g/l) prepared in a phosphate buffer (0.057M, pH6.5). Endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and endo-1,4-β-D-xylanase assays consisted in mixing the supernatant samples with the substrate solutions for 10, 30 and 5 minutes, respectively. The samples were then introduced into a water bath (100^{o}C, 5 minutes) and diluted with distilled water before measuring the absorbance at 550nm. The samples containing microcrystalline cellulose were centrifuged (5 minutes, 10000g) before being read. The activities were calculated on the basis of a calibration curve obtained by using glucose or xylose. β-glucosidase and β-xylosidase were measured in accordance with the method used by Ghorai et al. [21]. Paranitrophenyl β-D-glucopyranoside ($2.5x10^{-3}$M in phosphate buffer 0.1M, pH5.85) and paranitrophenyl β-D-xylopyranoside (1g/l in phosphate buffer 0.1M, pH6.5) were used to investigate β-glucosidase and β-xylosidase activities, respectively. The enzyme samples were mixed with substrates for 15 minutes before adding Na_2CO_3 (1M) to stop the reaction. The absorbance was read at 400nm after diluting the samples. The activities were determined with a calibration curve based on paranitrophenol. All the assays were made at 40^{o}C. Controls were also performed on the substrate solutions and enzyme samples considered separately. All the enzymatic activities were calculated in International Units corresponding to the amount of enzyme able to provide 1 µmol of glucose or xylose in one minute. The right hydrolysis times and enzyme dilutions were chosen according to enzymatic assays achieved with commercial enzymes (Sigma-Aldrich®). All the enzymatic activities measured in the cultures were analyzed with the MINITAB16® statistical software, and all the measurements were taken in triplicate. Normality tests and tests for equal variances were applied to all the series of data before achieving the variance analyses.

VI.2.5. *Effect of pH, temperature, pH and thermal stability*

The aim of these assays was to identify the optimal conditions and the stability of enzymes acting on cellulose and xylan with regards to pH and temperature. Consequently, beech wood xylan and microcrystalline cellulose were chosen as the substrates. Carboxymethylcellulose could have been chosen, but it is more easily degraded than microcrystalline cellulose. This latter type of cellulose is the most problematic regarding the hydrolysis process. Finally, β-glucosidase acts on cellobiose, a soluble component the hydrolysis of which is not considered as problematic. The optimal pH conditions were investigated at different pH values (4, 5, 6, 7, 8, 9, 10 and 10.8). Suspensions of xylan and microcrystalline cellulose (10 g/l) were prepared in a citrate-phosphate buffer (B1, 0.02M, pH3 to 5.5), phosphate buffer (B2, 0.02M, pH6 to 8) and carbonate-bicarbonate buffer (B3, 0.02M, pH9.2 to 10.8). Endo-1,4-β-D-xylanase and exo-1,4-β-D-glucanase activities were measured by the methods described before for each pH. The effect of pH on endo-1,4-β-D-xylanase and exo-1,4-β-D-glucanase activities was evaluated on samples of enzyme adjusted to pH values ranging from 3 to 10.8 with buffers B1, B2 and B3 at 0.2M. These samples were incubated for 4 hours at 25^{o}C. Then, the residual enzymatic activities were measured by the methods described before for each sample. The optimal conditions of temperature were investigated at temperature values ranging from 30 to 80^{o}C. Endo-1,4-β-D-xylanase and exo-1,4-β-D-glucanase activities were measured in accordance with the methods described before at the different temperature values. The effect of temperature was investigated using enzyme samples kept at different temperature values under agitation for 30 minutes at pH7 (phosphate buffer at 0.2M). Then, residual activities were measured in accordance with the method described before.

VI.2.6. *Zymographic assay and protein analysis*

Culture samples were centrifuged (5’ at 14000 rpm), and the pellets were resuspended in 100 mM Tris-HCl buffer pH8, followed by sonication, and 12 µg of protein (3.9 µl) were mixed with 7.8 µl Laemmli Sample Buffer (Bio-Rad). Each sample was divided into two fractions. The first fraction was heated for 3 minutes at 95^{o}C (300 rpm), while the other part was used without any thermal treatment. Electrophoresis was performed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 0.2% xylan from beech wood (Sigma-Aldrich) or 0.2% microcrystalline cellulose (Sigma-Aldrich). Zymography was done as previously [22] with the following slight modifications. PageRuler Prestained Protein Ladder

(Thermo Scientific) was added to check the molecular weight of the bands observed. To visualize enzymatic activity, the gel was rinsed twice for 30 min in 100 mM Tris-HCl buffer pH8, containing 1% Triton X-100 to remove SDS and then incubated for 2 h in 100 mM Tris-HCl buffer (pH6 for the sample from *T. virens* strain CTGxAviL on xylan; 9 for the samples from *S. kiliense* strain CTGxxyl and *T. virens* strain CTGxAviL on xylan and microcrystalline cellulose, respectively; 10 for the sample of *S. kiliense* strain CTGxxyl on microcrystalline cellulose) to maximize enzymatic activity. The pH values were chosen in accordance with the optimal pH conditions (see ***Results – Optimal pH and pH stability***). The substrate was then stained with Congo red (Sigma-Aldrich) for 30 minutes and destained with 1 M NaCl. Destained bands were visible and the gel was imaged under UV light. Other electrophoreses were achieved to identify total proteins with Coomassie Brilliant Blue R-250 (0.25% w/v) in acetic acid (10%). This assay was made on the samples stemming from both molds, with and without heating treatment (3 minutes at 95^{o}C (300 rpm)).

Picking of activity bands in accordance with their corresponding positions on Coomassie Brilliant Blue-stained gel was done manually. Gel pieces from destaining activity bands of zymograms were rinsed several times to minimize dye/protein ratio. Gel pieces were washed twice with 50 mM NH_4HCO_3 followed by 50 mM NH_4HCO_3 + 50% (v/v) acetonitrile. Proteins were reduced for 45 min with dithiothreitol (10 mM) at 56°C and alkylated for 1 h with iodoacetamide (55 mM) at 20^{o}C. Gel pieces were washed twice as previously and then dehydrated with pure acetonitrile. Proteins were digested for 4 h with 3 µl of NH_4HCO_3 (25 mM) containing 10 ng/µl trypsin and peptides were extracted with 1% (w/v) trifluoroacetic acid. Peptides were subjected to a MALDI-TOF-TOF analysis achieved by the GIGA Research Center (Avenue de l'Hôpital 1, 4000 Liège, Belgium). Peptide Mass Fingerprints and MS/MS data were used to query Swissprot database (all entries). Initial error tolerances were 100 ppm and 0.3 Da for PMF and MS/MS, respectively.

VI.2.7. *Detection of xylose and glucose oligomers*

Oligomers were measured with a mass spectrometer. A matrix of 2,5-dihydroxybenzoic acid (DHB) was used at 20 mg.ml^{-1} in acetonitrile/water 0.1% trifluoroacetic acid. Samples were prepared with a mixture of 1 µl hydrolysate solution and 1 µl matrix. Measurements were performed with a time-of-flight mass spectrometer, Ultraflex II TOF/TOF (Bruker Daltonics, Bremen Germany), equipped with a frequency-tripled Nd : YAG laser (355 nm). Calibration was performed with peptide calibration standard II solution

(Bruker). The spectra were recorded in positive reflectron ion mode, with an accelerating voltage of 25 kV and a laser shot rate of 100 Hz. The voltages of the electrodes 1 and 2 were set at 21.8 kV and 9.5 kV, respectively, to carry out the pulsed ion extraction. The time delay before the ion extraction was set at 30 ns. A total of 10,000 shots were accumulated for each mass spectrum. The acquisition m/z range started at 400 to exclude high intensity signals from matrix ions.

Crude enzymes samples stemming from the cultures of the isolated strains were used on microcrystalline cellulose and beech wood xylan. The reactions occurred at 40^{o}C for 24 hours before freezing the samples immediately (-20^{o}C) : 1 ml of enzyme was added to 1 ml of substrate suspension (20 g/l microcrystalline cellulose and beech wood xylan in phosphate citrate buffer 0.2 M, pH6). Different dilutions of enzymes were tested. Control samples were prepared with enzymes or substrates only, and were also heated at 40^{o}C for 24 hours before freezing the samples.

VI.3. Results and Discussion

VI.3.1. *Isolation and identification*

Two molds were isolated through our methodology. The strains were identified morphologically and named *Trichoderma virens* strain CTGxAviL (the same mold isolated from Media 1 and 2) and *Sarocladium kiliense* (also called *Acremonium kiliense*) strain CTGxxyl (isolated from Medium 3). Other studies report yeasts or molds isolated from termite guts. RAPD-PCR made it possible to identify some yeasts from the gut of the termites *Heterotermes indicola*, *Mastotermes darwiniensis,* a *Neotermes jouteli*-related termite, *Reticulitermes santonensis*, *Zootermopsis angusticollis*, and *Zootermopsis nevadensis* [23]. In two other studies, 13 species of yeasts were isolated from the guts of the termites *Zootermopsis angusticollis* and *Neotermes castaneus*. The genera *Candida*, *Cryptocercus*, *Debaryomyces*, *Pichia* and *Sporothrix* were identified [24, 25]. Molds were also isolated from termite guts : *Alternaria alternater*, *Aspergillus awamuri*, *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Cladosporium* sp., *Paecilomyces fusiporus*, *Rhizopus stolonifer* [26, 27]. Other molds were found in *Reticulitermes flavipes* : *Aspergillus nidulans*, *Neurospora crassa*, *Verticillium dahliae*, *Trichoderma viridae* and *Thermomyces lanuginosus*. However, those strains were not isolated but found to be present on the basis of glycoside hydrolase family 11 sequences [17]. To our knowledge, this is the first time *Sarocladium kiliense* has been isolated from the gut of a termite. The genus *Sarocladium* is

usually found in soil or grows on decaying organic matter [28]. Consequently, it is not surprising to find this microorganism inside the gut of a termite. *Trichoderma* species also come from soil, and are well-known molds that can be found all over the world. *Trichoderma* is one of the best known fungi and enzyme producers [29]. *Trichoderma viridae* was previously detected in the gut of *R. flavipes*, which can be considered as *R. santonensis* [30, 17]. However, the present work is the first study in which *Trichoderma virens* has been extracted from the gut of this termite.

VI.3.2. *Enzyme production by S. kiliense strain CTGxxyl and T. virens strain CTGxAviL*

Six different carbon sources were tested in the culture medium (MC – microcrystalline cellulose, X – beech wood xylan, αC - α-cellulose, WB – Wheat bran, WDG – Wheat Distiller's Grains, ROC – Rapeseed Oil Cake). All the measures were taken in triplicate. β-glucosidase, β-xylosidase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase and exo-1,4-β-D-glucanase activities were measured in the cultures of both molds. No β-xylosidase activity was detected for *S. kiliense*, while no β-glucosidase activity was observed for *T. virens*. The enzymatic activities are shown in **Figure 1** (**A-B-C-D-E-F-G-H**). Other cases of cellulase- and xylanase-producing fungal strains are presented in **Table 1** and **Table 2** (quantitative data), respectively.

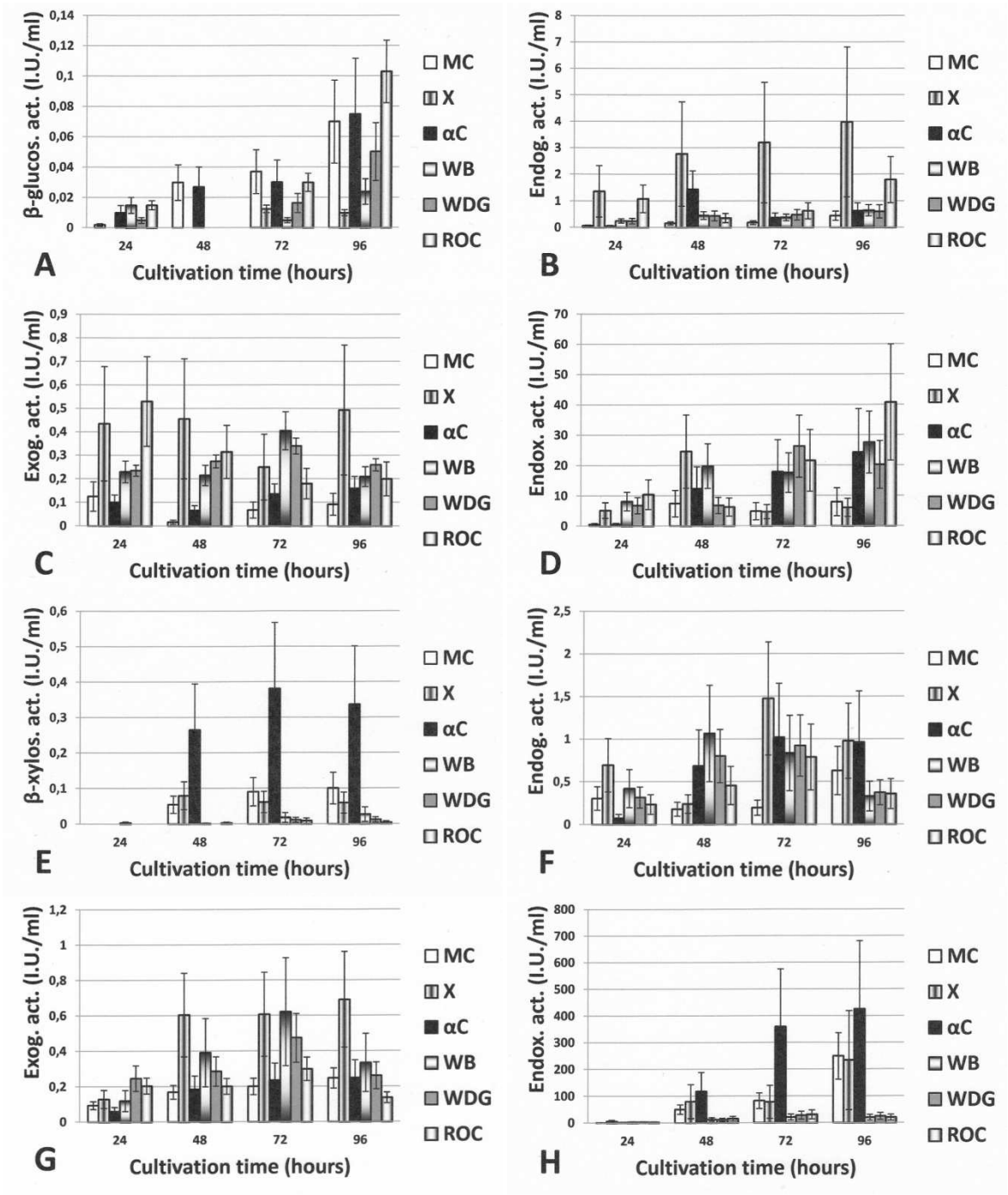

Figure 1. A – Evolution of β-glucosidase activity produced by *S. kiliense* strain CTGxxyl according to the carbon source of the culture medium – **B** – Evolution of endo-1,4-β-D-glucanase activity produced by *S. kiliense* strain CTGxxyl – **C** – Evolution of exo-1,4-β-D-glucanase activity produced by *S. kiliense* strain CTGxxyl – **D** – Evolution of endo-1,4-β-D-xylanase activity produced by *S. kiliense* strain CTGxxyl – **E** – Evolution of β-xylosidase activity produced by *T. virens* strain CTGxAviL – **F** – Evolution of endo-1,4-β-D-glucanase activity produced by *T. virens* strain CTGxAviL – **G** – Evolution of exo-1,4-β-D-glucanase activity produced by *T. virens* strain CTGxAviL – **H** – Evolution of endo-1,4-β-D-xylanase activity produced by *T. virens* strain CTGxAviL. MC = Microcrystalline Cellulose, X = Beech wood Xylan, αC = α-Cellulose, WB = Wheat Bran, WDG = Wheat Distiller's Grains, ROC = Rapeseed Oil Cake.

S. kiliense strain CTGxxyl produced the highest β-glucosidase activity on ROC (0.103 I.U./ml). Pure MC and αC also provided relatively good results, 0.07 and 0.075 I.U./ml, respectively. Xylan was the least effective carbon source regarding β-glucosidase production. This observation may seem logical because xylan contains xylose, which is not a cellulase inducer. Normality tests and tests for equal variances were done for each medium after 96h of culture (maximal values reached), and were found to be positive ($p>0.05$). Variance analyses did not show any significant differences between the various media as regards β-glucosidase production ($p>0.05$). However, xylan as a carbon source led to a relatively good production of endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase. Indeed, endo-1,4-β-D-glucanase activity reached a maximal value of 3.99 I.U./ml, while it was less than 2 I.U./ml using the other carbon sources after 4 days of culture. Normality tests and tests for equal variances were done for each medium after 96h of culture and were not found to be positive ($p<0.05$). Consequently, no significant difference should be highlighted. The evolution of exo-1,4-β-D-glucanase activity was chaotic and the maximal value was detected after 1 day of culture with ROC. No carbon source was significantly a better inducer than the other sources. Normality tests and tests for equal variances were achieved for each medium after 24h of culture and were not found to be positive ($p<0.05$). Therefore, no significant difference was shown. ROC also led to the highest xylanase production (40.8 I.U./ml), while MC provided the lowest activity. It seems that xylan is a good substrate to improve both endo-1,4-β-D-xylanase and cellulase (endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase) activities, while cellulose (MC and αC) does not lead to relatively good activities. Such a result may be due to the fact that endo-1,4-β-D-xylanase activity is the most important, releasing xylose and xylose oligomers that can be metabolized by the mold. This hypothesis is supported by the observation of cultures under a microscope, showing the best development with xylan (pictures not shown). This growth leads to a higher biomass and consequently more capacity of enzyme production whichever activity is considered. Normality tests and tests for equal variances were done for each medium after 96h of culture, and were found to be positive ($p>0.05$). However, it was not possible to show a significant difference between the endo-1,4-β-D-xylanase production of each medium ($p>0.05$). Those values can be compared to other strains reported in other studies (see **Table 1** and **Table 2**). Exo-1,4-β-D-glucanase activities are often very low, and the values reported here are comparable to those of other studies. This tendency is due to the difficulty in attacking MC, structured in a complex configuration which does offer few attackable sites. β-glucosidase activities were found to be very low too. As regards this, other studies report many molds producing much more β-glucosidase activity. Consequently, our

strain shows an incomplete cellulase complex, because the final β-1,4-glucopyranosyl-linkages of cellobiose are hardly hydrolyzed. Endo-1,4-β-D-glucanase activities, measured with CMC, were higher. CMC has an amorphous structure that increases the number of attackable sites compared to MC. The activities induced by our strain are also comparable to those mentioned in other publications (see **Table 1** and **Table 2**). Endo-1,4-β-D-xylanase activities were dominant and are comparable to the other reported strains. Surprisingly, the highest xylanase activity was reached with ROC as a substrate and not pure xylan. ROC is characterized by a protein content of 32%, which is the highest among the agro-residues tested in this study [31]. Consequently, ROC can be considered as the best source of amino-acids. It also contains 12% cellulose and 6% hemicelluloses, the hydrolysis of which releases metabolizable sugars [31].

Table 1. Cellulolytic activities produced by molds from other studies. Cellulase activities are expressed in International Units/ml or /mg of protein. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited. FP = FPcellulase activity, βG = β-glucosidase activity, EG = Endo-1,4-β-D-Glucanase activity, EXG = Exo-1,4-β-D-Glucanase activity.

Strain	Cellulase activity (IU/ml or /mg prot.)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Acremonium cellulo-	FP - 0,66 IU/mg	-	-	-	-	-	-
lyticus CF-2612 [7]	βG - 1,2 IU/mg	-	-	-	-	-	-
	EG - 4,52 IU/mg	-	-	-	-	-	-
	EXG - 0,26 IU/mg	-	-	-	-	-	-
Acremonium cellulo-	FP - 15,5 IU/ml	-	-	4,5	45	-	-
lyticus P-18508 [42]							
Acremonium persicinum	βG - 5,4 IU/mg	4,3	128	5,5	-	5-9,5	up to 50
QM107a [35]							(30 min)
Aspergillus sp. [43]	FP - 0,45 IU/ml	-	-	-	-	-	-
	βG - 63 IU/ml	-	-	-	-	-	-
	EG - 11,7 IU/ml	-	-	-	-	-	-
Aspergillus sydowii	βG - 83,3 IU/ml	-	95	5	50	4-6	up to 60
BTMFS55 [44]							
Chaetomium thermo-	βG - 7,55 IU/ml	6,7-4,9	39-480	4	-	-	-
philum MTCC4981 [45]		-4,3					
Humicola insolens	βG - 3,82 IU/ml	8,2-5,3	39-480	4	-	-	-
MTCC4520 [45]		-4,2					
Penicillium citrinum	FP - 1,72 IU/ml	-	-	6,5	60	-	-
MTCC6489 [46]	EG - 1,89 IU/ml	-	38-90	5,5 and 8	65	-	-
Penicillium	EG - 0,11 IU/ml	4,99	41,1	5-9	60	-	84% after 1h
echinulatum 9A02S1 [39]							at 70
Penicillium pinophilum	βG - 3,2 IU/ml	5,2	120	3,5	32	-	-
KMJ601 [47]							
Trichoderma	βG - 0,92 IU/ml	-	-	5,5 (growth)	28 (growth)	-	-
harzianum [48]	EG - 0,79 IU/ml	-	-	5,5 (growth)	28 (growth)	-	-
	EXG - 7,8 IU/ml	-	-	5,5 (growth)	28 (growth)	-	-
Trichoderma reesei	βG - 0,64 IU/ml	-	-	5,5	27	-	-
RUT-C30 and				(growth)	(growth)	-	-
Aspergillus phoenicis	FP - 1,54 IU/ml	-	-	5,5	27	-	-
QM329 [49]				(growth)	(growth)	-	-

Table 2. Xylanolytic activities produced by molds from other studies. Xylanase activities are expressed in International Units/ml. pI = Isoelectric Point, Mw = Molecular Weight (kDa). Optimal conditions of pH and temperature, thermal and pH stabilities are also cited. EX = Endo-1,4-β-D-xylanase activity, βX = β-xylosidase activity.

Strain	Xylanase Activity (IU/ml)	pI	Mw (kDa)	pH opt	T opt (°C)	Stab pH	Stab T (°C)
Aspergillus candidatus [50]	EX - 69 IU/ml	-	-	-	-	-	-
Aspergillus fumigatus AR1 [51]	EX - 30 IU/ml at pH9 EX - 135 IU/ml at pH5		212-253	6-6,5	60	5-9	up to 50
Aspergillus fumigatus M71 [34]	EX - 1040 IU/ml	-	-	4,5	60	5-10	50 % after 1h at 55
Aspergillus niger NII-08121 [52]	βX - 1400 IU/ml	-	120	4,8	70	-	90 % after 50 h at 50
Aspergillus niveus RS2 [53]	EX - 18,2 IU/ml	-	22,5	7	50	68,7 % at pH 9	70,9 % after 40 min at 50
Aspergillus terreus [33]	EX - 42,2 IU/ml	-	31 and 66,4	8-10	70	-	up to 60
Fusarium proliferatum NRRL 26517 [54]	EX - 4,7 IU/ml		22,4	5-5,5	55	5-7,5	up to 55
	βX - 0,02 IU/ml	-	-	-	-	-	-
Fusarium solani F7 [55]	EX - 94,7 IU/ml	-	89	5,5 (growth)	30 (growth)	-	-
Graphium putredinis [56]	EX - 153,22 IU/ml		32	6	50	-	1 day at 60
	βX - 12,99 IU/ml	-	-	-	-	-	-
Penicillium janczewskii [57]	EX - 15,19 IU/ml	-	-	-	-	-	-
	βX - 0,16 IU/ml	-	-	-	-	-	-
Penicillium oxalicum GZ-2 [58]	EX - 115,2 IU/ml	-	15-130	4	50	4-9	up to 50
	βX - 0,089 IU/ml	-	-	-	-	-	-
Penicillium sclerotiorum [59]	βX - 0,25 IU/ml	-	42 97	2,5	60	2-7	up to 60
Rhizopus oryzae ATCC9363 [60]	EX - 260 IU/ml	-	22	4,5	55	-	-
Thermomyces lanuginosus SSBP [61]	EX - 3580 IU/ml	-	24,7	6,5	70	5,5-9,5	up to 70
Trichoderma sp. [62]	EX - 107,22 IU/ml	-	-	5	50	-	40-70
Trichoderma harzianum [56]	EX - 148,35 IU/ml		46	5	50	-	2 days at 60
	βX - 8,31 IU/ml	-	-	-	-	-	-
Trichoderma harzanium 1073D3 [63]	EX - 480,1 IU/ml	-	-	5	60	3-7	-
Trichoderma reesei Rut C-30 [64]	EX - 261 IU/ml	-	21	6	60	-	up to 50

T. virens strain CTGxAviL has produced its highest β-xylosidase activity (0.38 I.U./ml) after 72h of culture on αC. The other substrates led to enzymatic activities lower than 0.1 I.U./ml. Normality tests and tests for equal variances were done for the different media after 72h of culture, and proved to be positive ($p<0.05$). Variance analysis highlighted a significant increase of activity caused by αC ($p<0.05$). However, the other substrates were not found to be significantly different when compared with each other ($p>0.05$). The endo-1,4-β-D-glucanase activities produced by *T. virens* strain CTGxAviL were lower than those of *S. kiliense* strain CTGxxyl. The highest activity was reached after 72h of culture with xylan as a substrate (1.48 I.U./ml). Normality tests and tests for equal variances were achieved for each medium after 72h of culture and were found to be positive ($p>0.05$). The endo-1,4-β-D-glucanase activity obtained with xylan was significantly the best. The enzymatic activities reached with ROC, WB, WDG and αC were significantly lower and the lowest activity was obtained with MC. The evolution of endo-1,4-β-D-glucanase activities showed the same evolution as before. Exo-1,4-β-D-glucanase activities produced by *T. virens* strain CTGxAviL were found to be higher than those of *S. kiliense* strain CTGxxyl. The highest exo-1,4-β-D-glucanase activity was reached with xylan (0.69 I.U./ml). Normality tests and tests for equal variances were done for each culture medium after 96h of culture and were found to be positive ($p>0.05$). Xylan led to the significantly highest activity ($p<0.05$), while no significant difference was observed between the five other substrates. Finally, the best endo-1,4-β-D-xylanase activity was reached with αC (426 I.U./ml). No statistical analysis was achieved because normality tests and tests for equal variances were found to be negative after 96h ($p<0.05$). β-xylosidase activities reported in other studies (**Table 2**) are distributed over a widespread range. Our strain produces β-xylosidase activity in low amounts. Exo-1,4-β-D-glucanase and endo-1,4-β-D-glucanase activities are quite low too (see **Table 1**). However, our strain of *Trichoderma virens* produced a high endo-1,4-β-D-xylanase activity, in comparison with the other strains reported in **Table 2**. In this case, the highest endo-1,4-β-D-xylanase activity was produced with αC. Surprisingly, the best β-xylosidase and endo-1,4-β-D-xylanase activities were obtained with αC. Some publications have already highlighted the presence of other polymers than cellulose in commercial αC, such as xylan and mannan [32]. However, the presence of xylan cannot explain such a difference because pure xylan leads to a lower activity, but other polymers can interfere. On the other hand, pure xylan led to the highest endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase activities. This observation proves that a specific activity is not unavoidably bound to its corresponding substrate.

VI.3.3. *Optimal pH and pH stability*

The optimal conditions of pH and the effect of pH are shown in **Figure 2**. The optimal conditions of pH and the effect of pH as regards the endo-1,4-β-D-xylanase produced by *S. kiliense* strain CTGxxyl show the same profile. The xylanase stands alkaline conditions better than acidic pH values. A pH value of 9-10 is ideal for the enzyme, while a pH of 4 reduces the activity by about 80%. Regarding the effect of pH, the enzyme keeps at least 75% of its original activity on a wide range of pH (6-11). Most molds show optimal enzymatic conditions at acidic pH values (see **Table 2**). Consequently, our strain has original properties among molds. A strain of *Aspergillus terreus* showed such properties [33]. As for exo-1,4-β-D-glucanase activity, the strain produced a cellulase showing some alkaline resistance, comparable to xylanase properties. Optimal pH for the enzyme was close to 10, while residual activities were higher at a pH of about 9. Consequently, the cellulase of *S. kiliense* strain CTGxxyl is alkaline-tolerant. Other cases of cellulase-producing molds are presented in **Table 1**. In most cases, molds produce cellulases (β-glucosidase, endo-1,4-β-D-glucanase and exo-1,4-β-D-glucanase more precisely) with acidic optimal pH values, and these enzymes also retain their stability in acidic pH rather than alkaline pH. Other molds, such as *Acremonium* (*Sarocladium*) *percisinum* QM107a, *Aspergillus fumigatus* M71 and *Myceliophtora thermophila* M77, also showed a good resistance to alkaline pH values. However, their optimal pH values were acidic [34, 35]. The isolation of alkaline enzyme-producing fungi from the gut of the termite *Reticulitermes santonensis* is surprising because the pH of the gut is close to neutrality (Ebert and Brune 1997). However, the pH can reach a value of 12.5 in the gut of some soil-feeding termites (Brune and Kühl 1996).

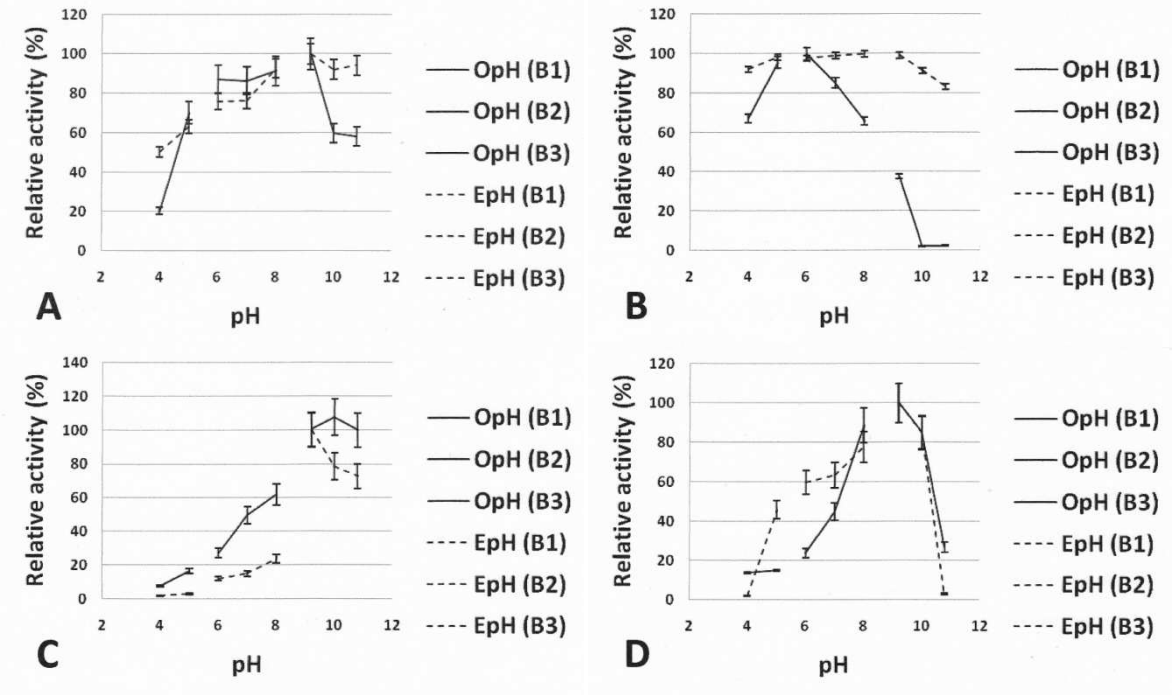

Figure 2. A – Optimal pH (time : 5 minutes, temperature : 40°C) and pH stability (samples incubated 4 hours at 25°C and pH values of 4, 5, 6, 7, 8, 9, 10 and 10.8 before the assay; time : 5 minutes, temperature : 40°C) of the xylanase produced by *S. kiliense* strain CTGxxyl; OpH = Optimal pH, EpH = Effect of pH. B1 refers to Buffer 1 (citrate-phosphate), B2 refers to Buffer 2 (phosphate) and B3 refers to Buffer 3 (carbonate-bicarbonate). The values are expressed as percentages of the maximal activities – **B** – Optimal pH and pH stability of the xylanase produced by *T. virens* strain CTGxAviL – **C** – Optimal pH (time : 30 minutes, temperature : 40°C) and pH stability (samples incubated 4 hours at 25°C and pH values of 4, 5, 6, 7, 8, 9, 10 and 10.8 before the assay; time : 30 minutes, temperature : 40°C) of the cellulase produced by *S. kiliense* strain CTGxxyl – **D** – Optimal pH and pH stability of the cellulase produced by *T. virens* strain CTGxAviL.

T. virens strain CTGxAviL produced more endo-1,4-β-D-xylanase activity than *S. kiliense* strain CTGxxyl, but its optimal pH is more common (5-6). Such a property is found in many molds. However, this xylanase showed a very good resistance on a wide range of pH (more than 90% of activity retained between pH4 and 10). A maximal endo-1,4-β-D-xylanase activity was observed at pH6, while less than 40% of that activity was observed at pH9. As regards exo-1,4-β-D-glucanase activity developed by our strain of *Trichoderma*, the enzyme is more stable at alkaline pH (8-10). The enzyme also shows an optimal pH of 9. Alkaline enzymes are much sought-after in the field of industrial processes, but another critical characteristic is their thermal resistance.

VI.3.4. *Optimal temperature and effect of temperature (thermal stability)*

The optimal conditions of temperature and the effect of temperature are shown in **Figure 3**. The endo-1,4-β-D-xylanase produced by the mold *S. kiliense* strain CTGxxyl shows

an excellent resistance to temperature. It conserved more than 90% of activity after 30 minutes at 80°C. Optimal temperature for enzyme functioning is about 60°C. The enzymatic activity decreases to 40% at 80°C. Then, the endo-1,4-β-D-xylanase produced shows satisfying properties as regards temperature. Other molds producing thermo-tolerant xylanases were reported (see **Table 2**). *Aspergillus brasiliensis* and *Aspergillus niger* were able to produce an endo-1,4-β-D-xylanase showing an optimal activity at 75°C [36]. Some molds have also been shown to synthesize xylanases particularly resistant to thermal denaturation. As an example, a strain of *Trichoderma harzianum* was able to produce a xylanase which retained 52% of activity after 4 hours of heating at 100°C [37]. However, in most cases, the effect of temperature on xylanases secreted by molds is comparable to our results. The cellulase of our strain of *Sarocladium* proved less tolerant in respect of temperature. Indeed, it conserved less than 50% of activity after 30 minutes at 50°C. However, it conserved 40% of activity after 30 minutes of heating at 80°C. The strain produces a cellulase showing a high optimal temperature (70°C). This observation makes our strain quite original. However, such a temperature causes denaturation if the heating time is too long. The enzyme observed in this study is quite satisfying regarding thermal denaturation. Other thermo-tolerant cellulases have been reported in previous studies. A cellulase produced by a strain of *Thermoascus aurantiacus* was shown to have an optimal temperature of 75°C as regards endo-1,4-β-D-glucanase activity [38]. Another mold has been shown to produce a cellulase the endo-1,4-β-D-glucanase activity of which is maximal at 70°C [34]. As regards thermal resistance, very little information is available. In most cases, the studies consider optimal temperatures, while the effect of temperature is not assessed. As an example, *Penicillium echinulatum* 9A02S1 kept 84% of activity after 1 hour of heating at 70°C [39]. However, the effect of temperature on mold cellulases is often comparable to the one observed of our study.

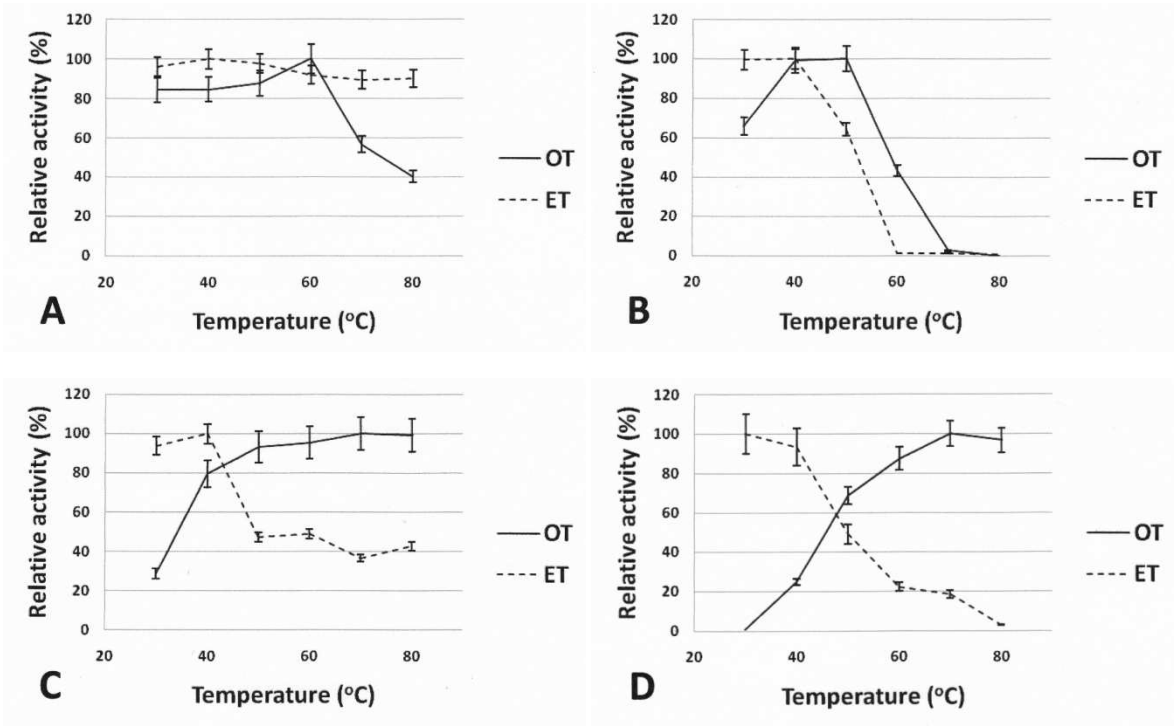

Figure 3. A – Optimal temperature (time : 5 minutes, pH : 7) and thermal stability (samples incubated 30 minutes at pH7 and temperatures of 30, 40, 50, 60, 70 and 80°C before the assay; time : 5 minutes, temperature : 40°C) of the xylanase produced by *S. kiliense* strain CTGxxyl; OT = Optimal Temperature, ET = Effect of Temperature (Thermal Stability). The values are expressed as percentages of the maximal activities – **B** – Optimal temperature and thermal stability of the xylanase produced by *T. virens* strain CTGxAviL – **C** – Optimal temperature (time : 30 minutes, pH : 7) and thermal stability (samples incubated 30 minutes at pH7 and temperatures of 30, 40, 50, 60, 70 and 80°C before the assay; time : 30 minutes, temperature : 40°C) of the cellulase produced by *S. kiliense* strain CTGxxyl – **D** – Optimal temperature and thermal stability of the cellulase produced by *T. virens* strain CTGxAviL.

The endo-1,4-β-D-xylanase produced by *T. virens* strain CTGxAviL was not as stable as the endo-1,4-β-D-xylanase secreted by our strain of *Sarocladium*. Its optimal temperature is lower (40-50°C). The enzyme only shows 40% of activity at 60°C while this temperature was shown to be optimal for the strain *Sarocladium*. The destructive effect of temperature is also stronger for the strain *Trichoderma*. The residual activity is about 60% after a heating time of 30 minutes at 50°C, while no activity remains when the temperature increases to 60°C. However, the cellulase from *Trichoderma* has been shown to be more resistant to temperature. The optimal temperature is 70°C. The enzyme keeps about 90, 50 and 20% of its activity after a heating duration of 30 minutes at 40, 50 and 60°C, respectively. The cellulases produced by our strains of *Sarocladium* and *Trichoderma* show close characteristics regarding the effect of temperature and optimal temperature conditions.

VI.3.5. *Zymographic assay and protein analysis*

Xylanases and cellulases were analyzed through zymography. Xylan and microcrystalline cellulose were tested in order to detect xylanase and cellulase activities. Samples were collected from cultures of *T. virens* strain CTGxAviL and *S. kiliense* strain CTGxxyl containing α-cellulose as a carbon source. Samples stemming from cultures containing agro-residues have also been tested but contained too many types of proteins. Cultures containing xylan would have been ideal because they enabled us to obtain good cellulase and xylanase activities (see **Figure 1**). However, the presence of fine xylan particles made the protein extraction very difficult. Cellulase activity was hardly detected by this method (data not shown), but many fragments were identified for both molds as regards xylanases. Electrophoresis gels are shown in **Figure 4**. The most outstanding bands were picked and six of them could be identified through mass spectrometry.

Some activity bands differ in apparent molecular weight, depending on the sample preparation. This is caused by a more efficient disruption of disulfide bonds and denaturation when sample is boiled, resulting in the alteration of the total protein migration pattern (see **Figure 4 B**). However, the renaturation and refolding of proteins after SDS-PAGE in order to show the enzymatic activities is more critical when the sample has been boiled [40]. Under such conditions, a loss of activity is possible.

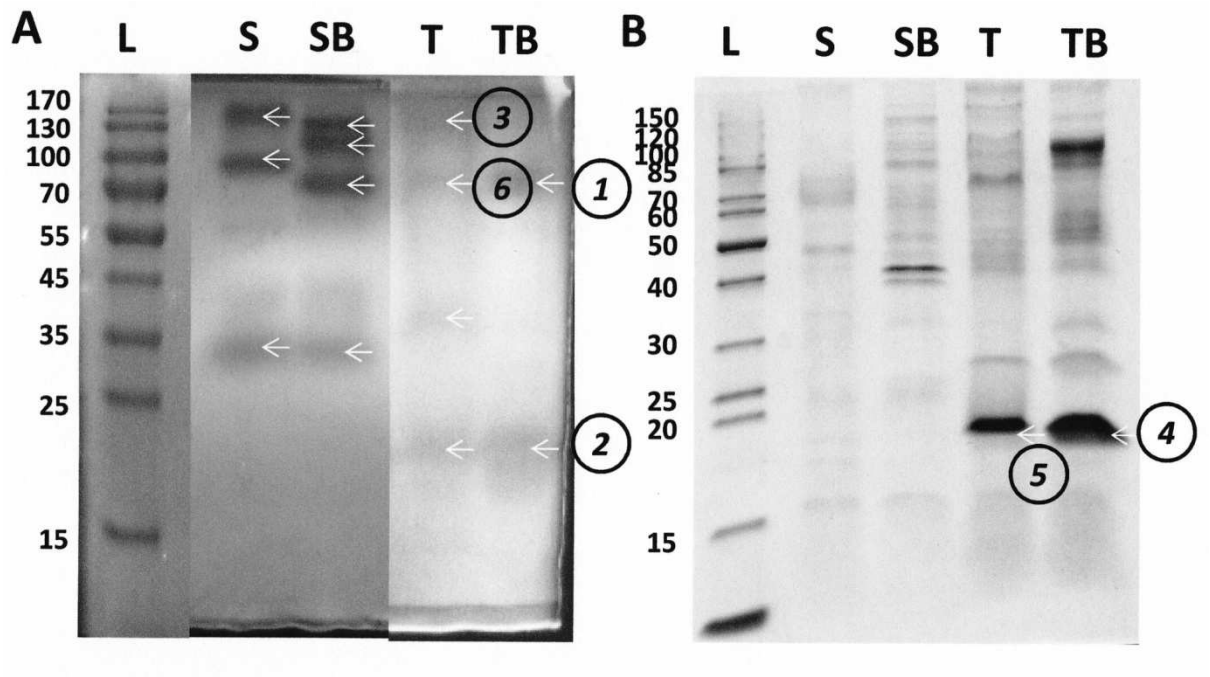

Figure 4. A – Zymogram of the xylanases produced by the molds *S. kiliense* strain CTGxxyl and *T. virens* strain CTGxAviL. Row L : molecular weight markers. Row S : Culture sample of *S. kiliense* strain CTGxxyl. Row SB : Culture sample of *S. kiliense* strain CTGxxyl after heating at 96°C (3 minutes) before migration. Row T : Culture sample of *T. virens* strain CTGxAviL. Row TB : Culture sample of *T. virens* strain CTGxAviL after heating at 96°C (3 minutes) before migration – **B** – Detection of total proteins in the samples by Coomassie Brilliant Blue. Row L : molecular weight markers. Row S : Culture sample of *S. kiliense* strain CTGxxyl. Row SB : Culture sample of *S. kiliense* strain CTGxxyl after heating at 96°C (3 minutes) before migration. Row T : Culture sample of *T. virens* strain CTGxAviL. Row TB : Culture sample of *T. virens* strain CTGxAviL after heating at 96°C (3 minutes) before migration. Arrows indicate activity or excised bands (all activity bands have not been excised). Numbers associated to arrows indicate identified protein (see **Table 3**).

Table 3. Protein identification from tandem mass spectrometry analysis (MALDI-TOF-TOF) of trypsin digested activity bands and corresponding Coomassie Brilliant Blue-stained bands. Attributed numbers (N°) are indicated on **Figure 4**. N = Number. Sc = Score, defined as $-10*\log_{10}(P)$, P is the probability that the observed match is a random event. Pep = number of masses matched to this protein/total number of masses observed for this band. Mw = Molecular Weight of the protein match (source : database where protein is identified), RMS = Root Mean Square, the square root of the mean square deviations (between the observed and theoretical masses), Cov = Coverage rate of matched peptide sequences compared to protein hit sequences. Peptide seq (MS/MS) = sequence(s) of peptides hits identified by the analysis of fragments from precursor ions.

N	Protein ID (PMF)	Sc	Pep	Mw	RMS (ppm)	Cov (%)	Peptide seq (MS/MS)	Sc
1	XYN_TRIHA: Endo-1,4-beta-xylanase of Trichoderma harzianum	54	4/38	20691	14	42	VNQPSIIGTATFYQYWSVR	99
2	XYN_TRIHA: Endo-1,4-beta-xylanase of Trichoderma harzianum	57	6/50	20691	10	35	VNQPSIIGTATFYQYWSVR	167
3	gi|2791278: beta-xylosidase of Hypocrea jecorina	38	8/78	87479	19	13	WLVGFDR LSIPIPVSALAR TLIHQIADIISTQAR	187
4	XYN_TRIHA: Endo-1,4-beta-xylanase of Trichoderma harzianum	53	5/41	20691	11	34	VNQPSIIGTATFYQYWSVR	142
5	XYN_TRIHA: Endo-1,4-beta-xylanase of Trichoderma harzianum	59	4/28	20691	15	42	/	
6	XYN_TRIHA: Endo-1,4-beta-xylanase of Trichoderma harzianum	61	4/51	20691	13	42	VNQPSIIGTATFYQYWSVR	119

No protein from *Sarocladium* could be correctly identified, probably owing to the lower characterization of this genus in contrast to *Trichoderma* (respectively 33 and 49243 results for protein sequences in NCBI). Activity bands 1 and 6 were identified as the same endo-1,4-β-D-xylanase as the one in bands 2, 4 and 5 though the observed molecular weight does not correspond to this enzyme (**Figure 4 A and B**). A multimer formation or a strong interaction with other proteins could be the cause of such an observation. However, a peptide from MS/MS data (common in bands 1, 2, 4 and 6) could be retrieved in many xylanases, varying in size and taxonomy, after BLAST analysis. Adjustment of Mascot search parameters did not identify xylanases according to the apparent molecular weight. Such adjustments (database : NCBInr Fungi; peptide mass error tolerance : 35 ppm) were used to analyze band 3 and allowed a non-significant identification of a β-xylosidase from *Trichoderma*. This identification was though confirmed by MS/MS data. Band 3 also showed a slight activity on another zymogram using microcrystalline cellulose as a substrate (data not shown).

VI.3.6. *Hydrolysis products of beech wood xylan and microcrystalline cellulose*

The following samples were tested : 4-day culture samples from *S. kiliense* strain CTGxxyl grown in the medium containing ROC and *T. virens* strain CTGxAviL grown in the medium containing αC. Those substrates were chosen because they led to the highest enzymatic activities. Different dilutions were tried, and the enzymatic activities relating to the different samples were determined by the methods described before. The samples tested on cellulose were not exploitable because the amounts of oligosaccharides were too weak. However, it has been possible to detect many fragments of xylo-oligosaccharides (**Figure 5**).

Xylan extracted from beech wood is a polymer of ortho-acetyl-(4-ortho-methylglucurono)-xylan. These molecules contain 4-ortho-methylglucuronic acid substitutes for approximately every 15 xylose residues [41]. The masses of the fragments detected relate to fragments of xylose oligosaccharides containing 1 4-ortho-methylglucuronic acid substitute. Those masses appear above the peaks with the number of xylose units in each fragment. The absence of fragments corresponding to oligomers without substitute leads to the conclusion that no glucuronidase activity is produced by the molds. Such an observation was also made in a previous article studying a xylanolytic *Bacillus subtilis* strain ABGx under the same conditions [31]. In that study, it was possible to observe a distribution of the fragments under the form of a Gaussian curve. In our present study, it was also possible to show such a distribution.

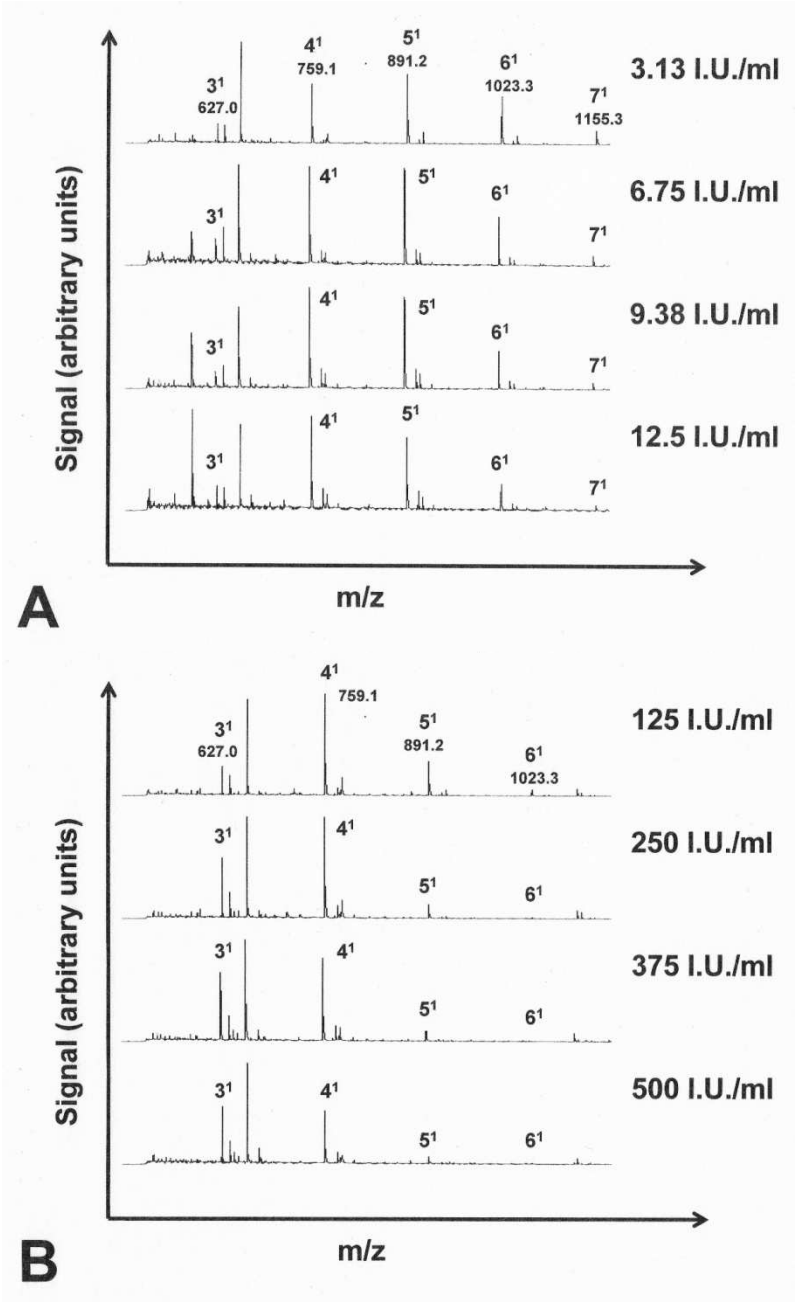

Figure 5. A – Mass spectrum obtained after the hydrolysis of beech xylan by the enzyme of *S. kiliense* strain CTGxxyl at different dilutions (3.13 I.U./ml, 6.75 I.U./ml, 9.38 I.U./ml, 12.5 I.U./ml). The exponents represent the number of methylglucuronic acids substituting the oligomers. The masses and numbers of xylose residues are indicated above each peak – **B** – Mass spectrum obtained after the hydrolysis of beech xylan by the enzyme of *T. virens* strain CTGxAviL at different dilutions (125 I.U./ml, 250 I.U./ml, 375 I.U./ml, 500 I.U./ml). The exponents represent the number of methylglucuronic acids substituting the oligomers. The masses and numbers of xylose residues are indicated above each peak.

Our two strains *S. kiliense* strain CTGxxyl and *T. virens* strain CTGxAviL show the same enzymatic profile using the method of mass spectrometry. Indeed, an endo-1,4-β-D-xylanase activity can be highlighted. The sample stemming from the culture of the strain *Trichoderma* showed a stronger activity, leading to a higher hydrolysis and consequently

smaller fragments of xylo-oligosaccharides. However, the spectrum relating to the sample from *Sarocladium* is comparable to the spectrum observed in the study of *Bacillus subtilis* strain ABGx [31]. In both studies, an endo-1,4-β-D-xylanase activity was clearly shown by the method used, while we confirmed that no glucuronidase was produced by our strains.

VI.4. Conclusion

Our work led to the following observations :

- It was possible to extract molds from the gut of the termite *R. santonensis*. Artificial diets affect the consortia living in the intestines because each strain was isolated from a specific diet.
- The molds which were isolated, *S. kiliense* strain CTGxxyl and *T. virens* strain CTGxAviL, showed different enzymatic activities : α-amylase, β-glucosidase, endo-1,4-β-D-glucanase, endo-1,3-β-D-glucanase, FPcellulase activity and endo-1,4-β-D-xylanase.
- Agro-residues and purified polymers had a specific effect on enzyme production. Surprisingly, the substrates which were used as carbon sources did not lead to an increase of the corresponding enzymatic activity.
- Although cellulase activities were determined in both molds, the main enzymatic activity was endo-1,4-β-D-xylanase. Maximal activities were 40.8 and 426 I.U./ml for *S. kiliense* strain CTGxxyl and *T. virens* strain CTGxAviL, respectively.
- On the basis of the substrates that were used, xylanase and cellulase activities proved to be interesting. Xylanases and cellulases were found to be mostly alkaline and showed a very good thermal resistance. Such properties are important as regards industrial applications.
- Zymography revealed the existence of several fragment sizes as regards xylanases for both molds, but some of those fragments proved to contain a partial specific sequence.
- Mass spectrometry has confirmed the presence of endo-1,4-β-D-xylanase activity and the absence of glucuronidase activity for each mold.

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VI.6. References

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CHAPITRE VII.

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Multiple analyses of microbial communities applied to the gut of the wood-feeding termite *Reticulitermes flavipes* fed on artificial diets

Ce chapitre correspond à l'article intitulé *"Multiple analyses of microbial communities applied to the gut of the wood-feeding termite Reticulitermes flavipes fed on artificial diets"* (Cédric Tarayre*, Julien Bauwens*, Christel Mattéotti, Catherine Brasseur, Catherine Millet, Jacqueline Destain, Micheline Vandenbol, Edwin De Pauw, Éric Haubruge, Frédéric Francis, Philippe Thonart, Daniel Portetelle) actuellement soumis à la revue **Symbiosis**.

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Le concept de diète artificielle a déjà été introduit dans le chapitre précédent (**Chapitre VI**). Celui-ci s'est focalisé sur l'étude complète de deux moisissures. Toutefois, une approche multidisciplinaire peut être appliquée à l'étude de l'effet des diètes artificielles. C'est l'objet du présent chapitre, qui peut être décomposé en deux aspects :

- Il présente d'abord les divergences observées entre les populations microbiennes issues des différentes diètes artificielles. Ces modifications ont été étudiées d'un point de vue visuel au microscope, par une approche métagénomique, protéomique et à travers l'analyse différentielle du métabolisme des microflores à l'aide de microplaques commerciales.
- Ensuite, il présente les différentes souches isolées à partir des diètes artificielles ainsi que leurs activités enzymatiques.

Résumé

Le but de ce travail était d'observer les différences entre les communautés microbiennes vivant dans l'intestin des termites *Reticulitermes flavipes* nourris à l'aide de différentes diètes. Les termites ont été nourris de bois de peuplier (diète originale) et de diètes artificielles composées de cellulose cristalline (avec ou sans lignine), d'α-cellulose (avec ou sans lignine) et de xylane. Ensuite, les termites ont été disséqués et les communautés de protistes ont été analysées par microscopie, menant à la conclusion que les espèces de protistes présentes sont fortement influencées par les diètes. Les microplaques BIOLOG ECO Microplate® ont été employées pour évaluer les propriétés métaboliques des différents types de consortia, mettant en évidence de fortes différences sur base d'une analyse en composantes principales et du calcul de taux de similitude. Les microflores ont été cultivées en milieux liquides correspondant aux diètes artificielles avant d'être caractérisées par métagénomique et protéomique. La métagénomique a identifié 7 phyla principaux : *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia* et *Planctomycetes*, et il a été montré que les proportions ont été très influencées par les diètes. La protéomique a été appliquée selon deux méthodes différentes, et chaque technique a identifié des divergences entre les microflores. Deux bases de données ont été testées : la base de données générale du NCBI (bacteria) et une base de données spécialement créée sur base des informations propres aux intestins de termites. Il a été possible d'isoler plusieurs souches microbiennes à partir des milieux liquides, et une bactérie et plusieurs moisissures se sont révélées produire d'intéressantes activités enzymatiques. La bactérie *Chryseobacterium* sp. XAvLW a produit de l'α-amylase, de la β-glucosidase, de l'endo-1,4-β-D-glucanase, de l'endo-1,4-β-D-xylanase et de la filter paper-cellulase, alors que les moisissures *Sarocladium kiliense* CTGxxyl et *Trichoderma virens* CTGxAviL ont produit les memes activités en plus de l'endo-1,3-β-D-glucanase.

Mots-clés : Biolog, diètes artificielles, métagénomique, protéomique, *Reticulitermes flavipes*

Abstract

The purpose of this work was the observation of the differences between the microbial communities living in the gut of the termite *Reticulitermes flavipes* fed on different diets. The termites were fed on poplar wood (original diet) and artificial diets consisting of crystalline cellulose (with and without lignin), α-cellulose (with and without lignin) and xylan. The termites were then dissected and the protist communities were analyzed through microscopy, leading to the conclusion that protist species are strongly influenced by diets. BIOLOG ECO Microplates® were used to assess the metabolic properties of the different types of consortia, highlighting strong differences on the basis of principal component analysis and calculation of similarity rates. The microflora were cultivated in liquid media corresponding to the artificial diets before being characterized through metagenomics and proteomics. Metagenomics identified 7 main phyla : *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes*, and the proportions were found to depend strongly on the diets. Proteomics was applied according to two different methods, and each technique identified strong differences between the microflora. Two databases were tested : the general database of NCBI (bacteria) and a homemade database created on the basis of data specific to termite guts only. It was possible to isolate several strains from the liquid media, and one bacterium and several fungi were found to produce interesting enzymatic activities. The bacterium *Chryseobacterium* sp. XAvLW produced α-amylase, β-glucosidase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase and filter paper-cellulase, while the fungi *Sarocladium kiliense* CTGxxyl and *Trichoderma virens* CTGxAviL generated the same activities added with endo-1,3-β-D-glucanase.

Keywords : Biolog, artificial diets, metagenomics, proteomics, *Reticulitermes flavipes*

VII.1. Introduction

Lignocellulose, the main component of plant biomass, has become a capital raw material as regards bio-fuel production [1]. Plant biomass is commonly composed of 45% of cellulose, 25% of hemicelluloses, 25% of lignin and 5% of other components [2]. In wood, the concentrations in cellulose, hemicelluloses and lignin reach variable values of 38-50%, 17-32% and 15-30%, respectively [3]. Lignin is a problematic component because it acts as a barrier against the release of fermentable sugars from biomass. These sugars can be used to produce second generation bio-ethanol [4, 5]. The structure of lignocellulose causes problems because of its high stability. Bio-ethanol production requires the hydrolysis of cellulose and hemicelluloses, but these macromolecules are included in a complex structure which makes them inaccessible to chemical or enzymatic treatments. Indeed, lignin, cellulose, hemicelluloses and other molecules are closely associated in a three-dimensional structure, acting as a fortress against chemical and enzymatic agents. This molecular structure is a significant limiting factor in the field of bio-ethanol production [6]. Consequently, it is necessary to increase the hydrolysis efficiency of cellulose and hemicelluloses. The research of new enzymes could be a solution to that problem, but the improvement of pretreatment techniques is also required.

Some insects are able to degrade the lignocellulosic complex. More specifically, *Blattodea* (such as termites), *Coleoptera* and *Orthoptera* have been shown to degrade carboxymethylcellulose. Crystalline cellulose is less easily attackable and was shown to be degraded by *Coleoptera*, *Hymenoptera*, *Lepidoptera* and *Orthoptera* [7]. Wood-feeding termites can digest up to 85 and 83% of glucosyl and xylosyl residues from lignocellulose, respectively [8]. This high hydrolytic potential is mainly due to the microbial community (bacteria, archaea, mycetes and protists) living in the digestive tract of termites. Two different types of termites are described. Higher termites harbor bacteria, archaea and mycetes, while lower termites also harbor protists. Some studies were achieved to characterize the composition of the termite gut microflora, but also to understand the relationship between the different actors. Among them, Todaka et al. [9] used metatranscriptomics and sequenced cDNA stemming from protists living in the termite *Reticulitermes speratus*. This analysis highlighted many cellulase and hemicellulase sequences some of which were involved in crystalline cellulose degradation [9]. It has been established that protists produce GHF7 exoglucanases and cellobiohydrolases, while the termite itself produces GHF9 endoglucanases [10]. A metagenomic analysis was also applied to the prokaryotic microflora

of the termite *Nasutitermes* [11]. The study identified 700 unique glycoside hydrolase catalytic domains from 45 carbohydrate active gene families, but other genes implicated in other activities, such as nitrogen fixation and hydrogen synthesis, were also found [11]. Tartar et al. [6] studied the hydrolytic potential in *Reticulitermes flavipes* on the basis of two cDNA libraries obtained from the host and the hindgut symbionts. Glycoside hydrolase gene families were found, acting on cellulose, hemicelluloses, alpha carbohydrates and chitin. Cellulases were identified in both cDNA libraries, while hemicellulases were rather identified in the symbiont cDNA library. The termite itself was also found to produce detoxification enzymes : laccase, catalase, peroxidase, superoxide dismutase, carboxylesterase and cytochrome P450. Phenoloxidase activity was produced by both symbionts and host tissues [6].

The termite *Reticulitermes flavipes*, a lower termite, can be considered as similar with *R. santonensis* [12]. This insect produces endogenous enzymes the action of which is combined with the one of microbial enzymes. Their actions are complementary but occur in specific locations, and lead to the destruction of the lignocellulolytic complex [6]. It is considered that the host and symbiont-derived enzymes account for about 1/3 and 2/3 of lignocellulose degradation in this termite, respectively [13]. Boucias et al. [14] studied the hindgut lumen microflora through pyrosequencing of 16S V5-V6 amplicons. This methodology led to 99.9% of bacterial sequences, while 0.11% of the sequences were found to belong to archaea. The main bacterial phyla were *Spirochaetes* (24.9%), *Elusimicrobia* (19.8%), *Firmicutes* (17.8%), *Bacteroidetes* (14.1%), *Proteobacteria* (11.4%), *Fibrobacteres* (5.8%), *Verrucomicrobia* (2.0%), *Actinobacteria* (1.4%) and *Tenericutes* (1.3%). The study also highlighted the effect of environment on the microflora composition [14].

The objectives of the present study were multiple. Artificial diets were tried on *Reticulitermes santonensis*. Our first purpose was to identify the effect of those diets (microcrystalline cellulose, microcrystalline cellulose and lignin, α-cellulose, α-cellulose and lignin, xylan) on the original symbiotic protist community. We also wanted to evaluate the effect on bacterial and fungal communities. Then, we assessed the modifications of metabolic properties developed by the bacterial and fungal microflora by using BIOLOG ECO Microplates®. We also tried to cultivate the different consortia, taking account of the fact that this step induces a selection among the original communities, since most microorganisms are not cultivable. We dissected termites grown on the different diets and cultivated their microflora in the corresponding liquid media. Then, proteomics and genomics were used on the different cultures to highlight the differences of microflora between the different samples.

Finally, we also tried to isolate bacterial and fungal strains from our cultures and assessed their enzymatic activities.

VII.2. Methods

VII.2.1. *Organisms*

Reticulitermes flavipes (Kollar) (*Rhinotermitidae*) was obtained from the Island of Oleron (France). The termites were grown in darkness at 27°C with a relative humidity of 70%. The termites were first fed on pinewood, gradually replaced by poplar wood. Artificial diets were then used to boost the xylanolytic and cellulolytic strains living inside the termite guts. They were prepared in solid agar medium. Five artificial diets were tried : Diet 1 (agar 15g/l, β-sitosterol 0.6g/l, microcrystalline cellulose 200g/l, notation : **Av**), Diet 2 (agar 15g/l, β-sitosterol 0.6g/l, microcrystalline cellulose 150g/l, lignin 50g/l, notation : **AvL**), Diet 3 (agar 15g/l, β-sitosterol 0.6g/l, α-cellulose 200g/l, notation : **α**), Diet 4 (agar 15g/l, β-sitosterol 0.6g/l, α-cellulose 150g/l, lignin 50g/l, notation : **αL**) and Diet 5 (agar 15g/l, β-sitosterol 0.6g/l, beech wood xylan 200g/l, notation : **X**). The original diet (poplar wood) is noted **W**.

VII.2.2. *Observation of protist communities*

The hindguts of termites fed for 6 weeks on artificial diets were dissected after being chilled on ice, torn in a drop of solution U [15], and observed under microscope until 400X magnification. For each diet replication, 6 termites were observed. Relative abundance of protist species was estimated after 10 minutes of observation.

VII.2.3. *Metabolism assessment of the different microbial communities by BIOLOG ECO Microplates®*

25 termites of each diet (original and artificial) were washed in ethanol, then water and held with two dissection forceps. The digestive tracts were then extirpated and the hindgut sections were put in tubes containing 20ml of sterile NaCl solution (9g/l), about 100 autoclaved glass beads (1mm) and 8.5g of cationic resin (DOWEX, 20-50 mesh). The tubes were agitated for 2 hours at 4°C before centrifuging the suspensions at 800g for 2 minutes. A first supernatant was obtained. The pellets were then added with 30ml of sterile NaCl solution (9g/l). The resulting suspensions were re-agitated for 1 hour at 4°C before centrifuging the tube contents at 800g for 2 minutes. Those second supernatants were added to the first fractions. The BIOLOG ECO Microplates® were inoculated with the six supernatants containing the different microflora in aerobic conditions. Each well was filled with 130µl from the sample. Only the negative controls were not : those were filled with a sterile NaCl

solution. Each plate is composed of 96 wells, containing three repetitions of 32 wells. Each well contains a substrate combined with tetrazolium blue the reduction of which causes a violet colouring. The intensity depends on the respiration of the carbon sources [16]. Three microplates were used for one microflora, which involves 9 repetitions for each sample. The evolution of the optical densities was determined for each substrate, taking account of the negative controls. Next, a mean curve was calculated with all substrates for each repetition. These values are called "AWCD" ("Average Well Color Development"). The data relating to a mean optical density of 0.3 were chosen for further analysis. This value was selected because it relates to the start of the growth and the consumption of the substrate inside the wells. After that, the optical densities relating to all substrates of each repetition of sample were divided by the corresponding AWCD. The aim of that step is to normalize data [17]. The values were further analyzed through two methods. The MINITAB® program was used to perform all statistical analyses.

Principal Component Analysis is a statistical transformation of possibly correlated variables into a set of artificial variables without linear correlation. With this method, each repetition of sample is considered independent. The values of the principal components are calculated by the following expression.

$$C_i^k = a_1^k X_{i1} + \cdots + a_j^k X_{ij} + \cdots + a_m^k X_{im}$$

C_i^k is the component k relating to the sample repetition i, a_m^k is one of the m parameters of the linear combination calculated (one specific parameter for each AWCD) and X_{im} is the AWCD of the repetition i for the susbtrate m. This method is visual. It leads to graphs showing the relation between the main principal components (1st component versus 2nd component, 1st component versus 3rd component, etc.). A graph showing a specific position for each group of samples (**Av**, **AvL**, **α**, **αL**, **X** and **W**) means that a specific diet leads to a specific metabolism.

The second method consists in calculating similarity rates. Variance analysis requires respecting application conditions. Consequently, a mathematic transformation of data is necessary. A logarithmic variable transformation allows respecting normality tests and tests for equal variances ($p>0.05$). A variance analysis was applied to the 6 diets (6 factors) for each substrate. The Least Significant Difference Test was used to investigate the significant differences between the diets for each substrate. A similarity rate of 100% between 2 diets

means that there were no significant differences between the transformed data of all substrates ($p>0.05$ for all substrates between those two diets).

VII.2.4. *Cultivation of the consortia stemming from the termites fed on artificial diets*

25 termites of each diet (original and artificial) were washed in ethanol, then water before extirpating the digestive tracts. The hindgut sections were put in flasks containing the following liquid medium : KH_2PO_4 2g/l, NH_4Cl 0.2g/l, $MgSO_4.7H_2O$ 1.54g/l, $CaCl_2.2H_2O$ 1.1g/l, H_3BO_3 2.86mg/l, $MnCl_2.4H_2O$ 1.81mg/l, $ZnSO_4.7H_2O$ 0.222mg/l, $NaMoO_4.2H_2O$ 0.39mg/l, $CuSO_4.5H_2O$ 0.079mg/l, $Co(NO_3)_2$ 0.031mg/l, pH 7 (derived from [18]). Each flask also contained a specific carbon source, corresponding to the diets : **Medium 1**, microcrystalline cellulose 10g/l - **Medium 2**, microcrystalline cellulose 7g/l, lignin 2.35g/l - **Medium 3**, α-cellulose 10g/l - **Medium 4**, α-cellulose 7g/l, lignin 2.35 g/l - **Medium 5**, beech wood xylan 11g/l - **Medium 6**, pinewood sawdust 10g/l. The flasks were agitated in darkness at 30°C and the culture medium was replaced each week for 4 months. Those flasks were used as enrichment cultures. All experiments were achieved in aerobic conditions.

VII.2.5. *Proteomic analysis*

Culture samples were added with CHAPS (0,1%) and protease inhibitor (Mini-Complete, Roche) prior to sonication and centrifugation. Proteins collected from the supernatant were extracted using 2D Clean-up kit (GE Healthcare) and were re-suspended in rehydration buffer (8 M Urea, 2 M Thiourea, 0.5% (wt : vol) CHAPS). Protein content was quantified using RC DC protein assay kit (Bio-Rad).

The samples were digested by trypsin as follows : protein pellet corresponding to 10 µg of total proteins was re-suspended in 100 µl of NH_4CO_3 50 mM. Reduction was carried out by adding 5 µl of dithiothreitol (DTT) 200 mM, NH_4CO_3 100 mM and heating at 100°C for 10 minutes. Next, 4 µl of alkylation solution (iodoacetamide 1M, NH_4CO_3 100 mM) were added and the sample was incubated for one hour in darkness. Alkylation reaction was stopped by adding 20 µl of reduction solution. Digestion by trypsin was performed for 18 hours at 37°C. Trypsic peptides were analyzed by tandem mass spectrometry, using a LC (nano Ultimate 3000, Dionex) ESI-Ion Trap (AmaZon Speed ETD, Bruker Daltonics), in positive ion mode. Database searches were performed using Mascot server and Protein Scape (Bruker) on NCBI non-redundant (NR) “Bacteria” database. Protein is considered identified when matching at least 2 peptides (scores above 15) including one peptide with score above Mascot identity score. This filtering method allows working protein by protein. In order to

adopt a more global approach in parallel, homology-scored trypsic peptides were blasted against NCBI NR "Bacteria" database. The results of these BLASTs were analyzed with MeGAn 5 using default algorithm (lowest common ancestor, LCA) parameters. Only the minimal support, i.e. the number of reads needed to consider the taxon present in the sample was not. This parameter was set at 1 instead of 5, due to the low number of hits compared to metagenomic analyses. The results were used to compare cultivated communities from a taxonomic and functional point of view. A termite specific database was designed including all proteins matching the keyword "termite" in NCBI protein database.

VII.2.6. *Metagenomics applied to bacterial communities*

The genomic analysis was achieved by Progenus® (Rue des Praules 2, 5030 Sauvenière, Belgium) on the different culture samples. Total DNA was extracted with the Nucleomag 96 Trace Kit (Macherey-Nagel) by using a Kingfisher 96 (Thermo Scientific) apparatus. Tag PCR was achieved with the following primers : bact337 FwTn combined with bact533 RkTn (with n = 1 to 6). The following PCR programme was used : 2 minutes at 94^{o}C, 30 cycles consisting of 30 seconds at 94^{o}C (denaturation step), 30 seconds at 48^{o}C (annealing step), 2 minutes at 68^{o}C (elongation step), and a final elongation of 35 minutes at 68^{o}C. The different samples were diluted to obtain a DNA concentration of 20ng/µl before mixing them in an eppendorf. The mix was purified with the High Pure PCR Product Purification (HP040) Kit. The amplicons were end-repaired and purified with the Ion Plus Fragment Library (IPFL003) and Agencourt AMPure XP Kits. The method was derived from the Short Amplicon Prep Ion Plus Fragment Library Kit. The same kits were used for the ligation of adapters, nick-repair, amplification and purification steps. The preparation before sequencing was achieved with the OT2-001 Kit in accordance with the method described in the ION PGM template OT2 200 Kit. Ion semiconductor sequencing was used to identify the sequences. DNA fragments were fixed to Dynabeads® MyOne™ Streptavidin C1. The method is the one described in the Ion PGM template OT 200 Kit. The sequencing reaction was done with the SV2-001 Kit by using the method described in the ION PGM Sequencing 200 Kit v2, adapted for Ion 316 v2 chips. The raw reads obtained from the high-throughput sequencing step were processed through two different filters in order to retain only the reads with the highest quality, i.e. the ones with a low rate of sequencing error. Firstly, reads lacking a valid pool tag sequence were discarded. The tag sequences were then removed from the reads and, on the basis of the tag sequence, each read was assigned to the corresponding sample. Secondly, reads shorter than 150 bp were disregarded. After the filtering step, the

corrected sequences were assigned with the RDP Classifier programme [19]. The number of sequences corresponding to each identified rank was divided by the total number of sequences retained in the sample after filtering and multiplied by 100 to obtain a relative abundance expressed as a percentage.

VII.2.7. *Isolation and identification of the strains*

The identification of the molds was achieved on the basis of a morphological analysis by the specialists of the mycotheque of the University of Louvain-la-Neuve (Bâtiment Kellner 1er étage, Croix du Sud 2 bte L7.05.06, 1348 Louvain-la-Neuve, Belgium). Bacteria were identified through 16S rDNA sequencing. The universal primers SP0 (5'-GAAGAGTTTGATCCTGGCTCAG-3') and SP6 (5'-CTACGGCTACCTTGTTACGA-3') were used to amplify the sequences. The amplification step consisted of 5 min of denaturation (94^{o}C), 35 cycles of amplification composed of 30 s at 94^{o}C, 60 s at 50^{o}C, 90 s min at 72^{o}C, and a final extension of 10 min at 72^{o}C. PCR products were used for the sequencing reaction. Sequencing was achieved by Progenus® (Rue des Praules 2, 5030 Sauvenière, Belgium) with a Genetic Analyzer 3130 designed by Applied Biosystems®. The sequences were aligned with the Vector NTI® program and the homologous sequences present in the GenBank database were identified using the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) BlastN program [20].

VII.2.8. *Investigation of enzymatic activities on agar plates*

The following enzymatic activities were tested : endo-1,4-β-D-xylanase, α-amylase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, filter paper-cellulase activity and β-glucosidase. The following basic medium, derived from [21], was prepared for all the assays except β-glucosidase : agar 17g/l, $NaNO_3$ 2g/l, $MgSO_4$ 0.5g/l, KCl 0.5 g/l, casein peptone 0.2g/l, H_3BO_3 2.86mg/l, $MnCl_2.4H_2O$ 1.81mg/l, $ZnSO_4.7H_2O$ 0.222mg/l, $NaMoO_4.2H_2O$ 0.39mg/l, $CuSO_4.5H_2O$ 0.079mg/l, $Co(NO_3)_2$ 0.031mg/l. Two series of experiments were achieved : pH5 and pH7 (with phosphate buffer at 50 mM). Different substrates were used in accordance with the enzymatic activity considered : AZCL-amylose medium 0.5g/l, (Megazyme, for detection of α-amylase), carboxymethylcellulose 2g/l (for detection of endo-1,4-β-D-glucanase), AZCL-curdlan medium 0.5g/l (for detection of endo-1,3-β-D-glucanase), mixed tork paper 2.2g/l (filter paper-cellulase activity), AZCL-xylan medium 0.5g/l (Megazyme, for detection of endo-1,4-β-D-xylanase). The following medium was used to detect β-glucosidase activity : casein peptone 8g/l, esculin sesquihydrate 1g/l, ammonium ferric citrate 1g/l, agar 17g/l, pH 7.4. First, the bacteria and molds were respectively cultivated

in rich liquid medium (glucose 20g/l, casein peptone 10g/l, yeast extract 10g/l) and potato dextrose broth for 24 hours. Then, 60µl of culture were dropped on each agar medium (3 repetitions). Filtered commercial enzymes were used as positive controls, and filtered distilled water was used as a negative control. The plates were incubated at 30°C in darkness. The plates were revealed after 3 days. The revelation of the plates containing tork paper and carboxymethylcellulose was made by flooding the plates with Gram's iodine, in accordance with the method described by Kasana et al. [21].

VII.3. Results and Discussion

VII.3.1. *Effect of artificial diets on protist communities*

Microscopic observation of termite hindgut revealed alteration of the flagellate populations balances. Influence of the polymerization degree of cellulose on the flagellates' populations in the gut of *Coptotermes formosanus* (Shiraki) has previously been described by Tanaka and colleagues [22]. Effect of lignin in an artificial diet was also investigated in a previous study [23]. Here we described modifications of populations balance on a more complex community, depending on multiple carbon sources (**Table 1**). As previously observed, presence of free lignin in the diets reduced the populations of Pyrsonympha species. Hemicelluloses seemed to favor small flagellates as *Spironympha kofoidi* and other species (*Trichomitus trypanoides*, *Microjoenia fallax* and *Monocercomonas sp*.) in contrast to larger species. A previous study [24] involving *Trichonympha agilis* in cellulose digestion is supported by our observations : *T. agilis* population was strongly reduced or disappeared only when termites were fed on xylan diet and this species was the only one to show such a strong effect. In general, xylan, or more precisely the absence of cellulose in the diet, resulted in loss or strong decrease of several flagellate species. By contrast, non-spirochaete bacterial presence seemed increased further to the xylan diet.

Table 1. Relative abundance of flagellates species in the gut of differentially-fed termites (/ : absence or very low abundance, + to +++ : low to high abundance, respectively). Av = Diet 1 (microcrystalline cellulose), AvL = Diet 2 (microcrystalline cellulose and lignin), a = Diet 3 (α-cellulose), al = Diet 4 (α-cellulose and lignin), X = Diet 5 (xylan), W = Diet 6 (poplar wood). During 10 minutes of observation, the live preparation was observed under a microscope and punctuated by 40 "stops", corresponding to an observation point. If at least one representative of a species was observed from 51% to 100% of the stops, this species was considered as abundant (+++). The "+" and "++" indications refer to species with at least 1 representative observed from 5 to 25% and from 26 to 50% during the stops, respectively. When a species was observed no more than twice, it was considered to be very low abundant or absent and noted "/".

Flagellates	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Dinenympha fimbriata	++	++	+++	++	/	+++
Dinenympha gracilis	+	+	+	++	+	+
Spirotrichonympha flagellata	++	++	+	+++	++	++
Trichonympha agilis	+++	+++	++	+++	+	+++
Spironympha kofoidi	+	+	+	+	+++	+++
Holomastigotes elangatum	/	+	+	++	++	++
Pyrsonympha vertens	+++	+	+++	++	/	+++
Pyrsonympha major	+++	/	+++	+	/	+++

VII.3.2. *Investigation of metabolic activities developed by the consortia*

The graphs showing the principal component analysis applied to the consortia living inside the termite guts are shown in Figure **1A**, **B** and **C**. **Figure 1A** represents the first two principal components. The groups relating to the consortia extracted from the termites fed on Diets 1 and 3 are dispersed on the graph. This observation is due to a lower cell concentration in the samples. Indeed, termites fed on microcrystalline cellulose and α-cellulose had smaller digestive tracts than termites fed on the other diets. Consequently, repeatability is not as good as in the other samples. However, it is possible to highlight some groups in the graph. The principal components corresponding to the termites fed on wood are concentrated by the coordinates (-2.5; 5.5), while the principal components corresponding to xylan, microcrystalline cellulose added with lignin and α-cellulose added with lignin are concentrated by the coordinates (1; 5).

These results highlight a strong difference of metabolism between the original consortium and the consortia corresponding to Diets 2, 4 and 5. Surprisingly, those 3 diets show the same profile of metabolic activities, according to this first principal component analysis.

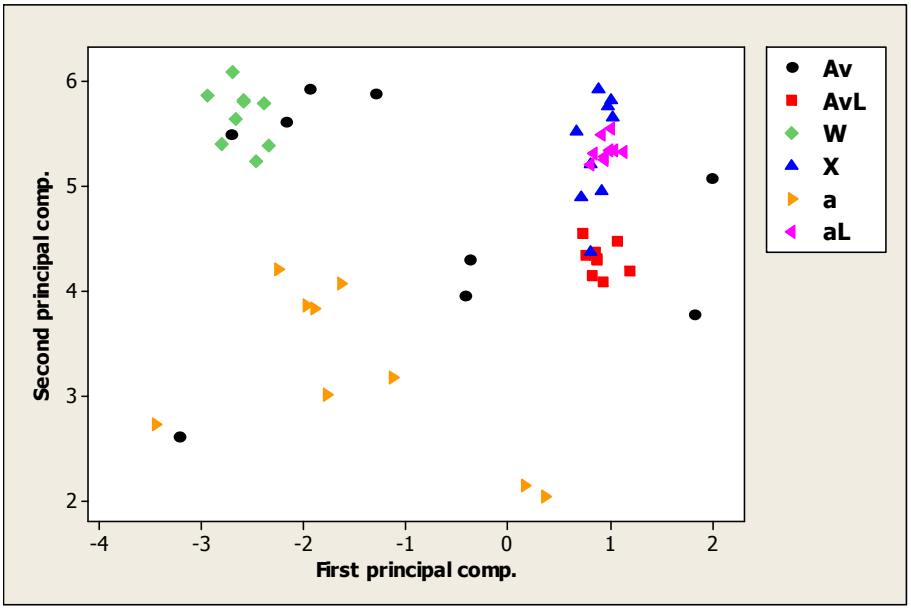

Figure 1A. Graph showing the first two principal components applied to the data of all microflora. Av = Diet 1 (microcrystalline cellulose), AvL = Diet 2 (microcrystalline cellulose and lignin), W = Diet 6 (poplar wood), X = Diet 5 (xylan), a = Diet 3 (α-cellulose), al = Diet 4 (α-cellulose and lignin).

Another matrix was established without considering the values of Diets 1 and 3. The aim of this step is to delete the data which bring no information and focus on data relating to Diets 2, 4, 5 and 6. The graph is shown in **Figure 1B**. The wood diet still remains separated from the other diets. It is possible to observe a difference in the repartition of those diets. The samples stemming from Diet 2 (microcrystalline cellulose and lignin) correspond to the coordinates (-0.5; 3.5), while the samples of Diet 4 (α-cellulose and lignin) correspond to the coordinates (-0.5; 5). The xylan diet seems to be halfway between Diets 2 and 4, and the distribution of the samples is more variable. The removal of data corresponding to Diets 1 and 3 enabled us to highlight the differences between Diets 2, 4 and 5.

This graph also shows the strong difference between the metabolic pathways of the original consortium and the artificial consortia. Poplar wood is composed of a lignocellulosic complex, while the other substrates are purified polymers. Consequently, the accessibility to the substrate is different and can explain this heterogeneity. Moreover, poplar wood is a natural wood and contains other components than cellulose or xylan. Free lignin (in Diets 2 and 4) can have a toxic effect on the original microflora, and this molecule is less accessible in natural wood. Those differences can affect the composition of the microflora, leading to the modification of the metabolic pathways which are observed here. Surprisingly, xylan seems to lead to metabolic properties which are very close to those corresponding to Diets 2 and 4, containing free lignin. A significant difference can also be observed between Diets 2 and 4.

Both contain lignin and cellulose, but the type of cellulose is not the same (crystalline cellulose in Diet 2 and α-cellulose in Diet 4). Commercial α-cellulose usually contains cellulose and other polymers, which may affect the composition of the microflora.

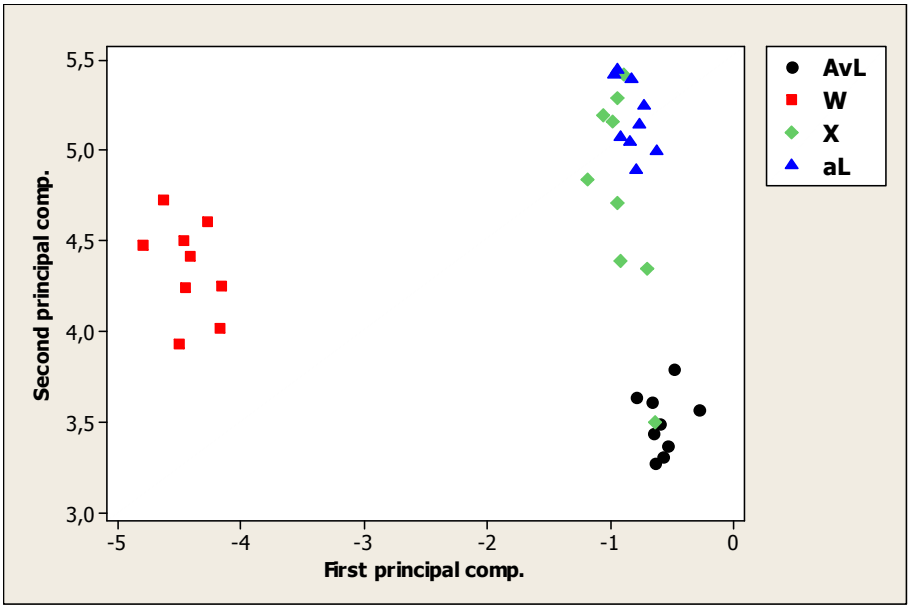

Figure 1B. Graph showing the first two principal components applied to the data of the microflora after removing the Diets 1 and 3. AvL = Diet 2 (microcrystalline cellulose and lignin), W = Diet 6 (poplar wood), X = Diet 5 (xylan), al = Diet 4 (α-cellulose and lignin).

The first and third principal components can be used as well to highlight differences. The first, second and third components are the most significant and include the biggest fraction of variability between the samples. **Figure 1C** shows the values of the first and third principal components without considering the results obtained with Diets 1 and 3. One more time, it is possible to show the specificity of the samples corresponding to Diet 6 (wood). However, the other three diets are more changeable than before. This seems logical because the first and the second components are the most important and represent the main fraction of variability. In conclusion, our principal component analysis suggests the existence of differences between the consortia stemming from each diet. The analysis of variances followed by the calculation of similarity rates enables to investigate deeper into these differences (see **Table 2**).

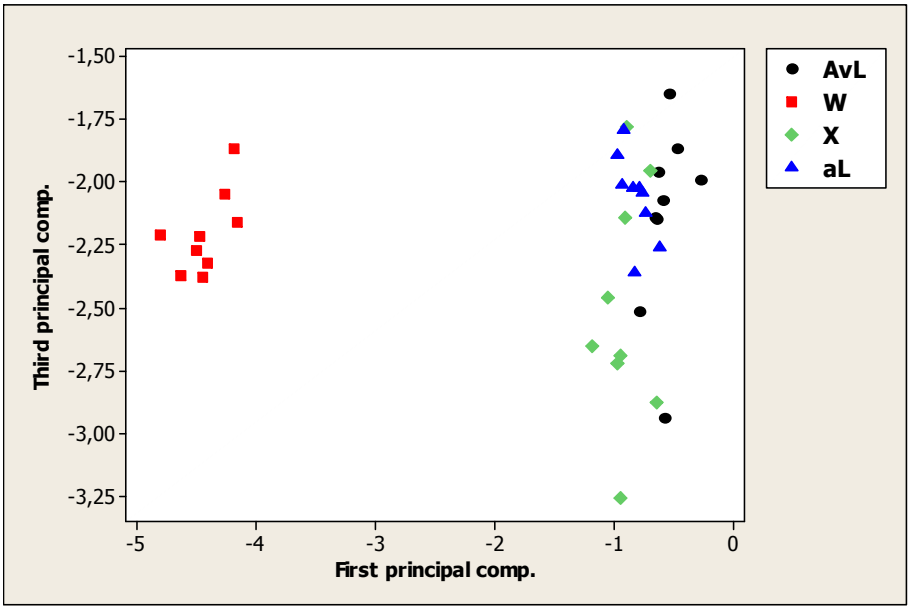

Figure 1C. Graph showing the first and third principal components applied to the data of the microflora after removing the Diets 1 and 3. AvL = Diet 2 (microcrystalline cellulose and lignin), W = Diet 6 (poplar wood), X = Diet 5 (xylan), al = Diet 4 (α-cellulose and lignin).

Table 2. Similarity rates calculated between the microflora obtained by the different diets applied to termites. Diet 1 = microcrystalline cellulose, Diet 2 = microcrystalline cellulose and lignin, Diet 3 = α-cellulose, Diet 4 = α-cellulose and lignin, Diet 5 = beech wood xylan, Diet 6 = poplar wood.

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**
Diet 1	100.0	19.4	51.6	58.1	64.5	35.5
Diet 2	19.4	100.0	48.4	74.2	77.4	25.8
Diet 3	51.6	48.4	100.0	64.5	64.5	29.0
Diet 4	58.1	74.2	64.5	100.0	83.9	25.8
Diet 5	64.5	77.4	64.5	83.9	100.0	25.8
Diet 6	35.5	25.8	29.0	25.8	25.8	100.0

The figures show the strong differences between the original diet (poplar wood) and the artificial diets. This observation was also made with the principal component analysis. However, similarity rates are based on variance analysis, while principal component analysis is based on a data transformation. A strong difference exists between Diets with and without lignin, such as Diet 1/Diet 2 and Diet 3/Diet 4. It is also possible to show the difference between the metabolic properties of the consortia stemming from Diets 1 and 3. Similarity rates allow demonstrating the significant differences between the microflora, but it is possible to analyze the metabolism of each substrate, one by one. Some of them provide useful information about the degradation of cellulose and other molecules.

BIOLOG ECO Microplates® contain 31 substrates corresponding to nitrogen and carbon sources : β-methyl-D-glucoside, D-galactonic acid (γ-lactone), L-arginine, pyruvic acid methyl ester, D-xylose, D-galacturonic acid, L-asparagine, Tween 40, i-erythritol, 2-hydroxybenzoic acid, L-phenylalanine, Tween 80, D-mannitol, 4-hydroxybenzoic acid, L-serine, α-cyclodextrin, N-acetyl-D-glucosamine, γ-hydroxybutyric acid, L-threonine, glycogen, D-glucosaminic acid, itaconic acid, glycyl-L-glutamic acid, D-cellobiose, glucose-1-phosphate, α-ketobutyric acid, phenylethylamine, α-D-lactose, D,L-α-glycerol phosphate, D-malic acid and putrescin. There were no significant differences between the optical densities measured on the following substrates : β-methyl-D-glucoside, D-galacturonic acid, 4-hydroxybenzoic acid and D,L-α-glycerol phosphate. Consequently, the artificial diets had no significant effect on the global metabolism of those carbon sources. β-methyl-D-glucoside is a substrate which can be used to detect β-glucosidase activity. The hydrolysis is based on a double displacement mechanism involving two carboxylic acids [25]. In our case, β-glucosidase activity on this substrate was not altered by the artificial diets, and the enzymatic activity was high because the optical densities were close to 3-4 (for an AWCD of 0.3). This observation seems logical as regards microfloras coming from the gut of termites, able to digest wood and cellulose very efficiently. In this case, β-glucosidase activity does not seem to be affected by Diet 5 (xylan), although this substrate does not contain cellulose. D-galacturonic acid is a monomer found in pectins and can be metabolized by prokaryotes through three metabolic pathways [26]. Optical densities were also high for this substrate, which means that D-galacturonic acid is easily metabolized by all microfloras. In contrast, the metabolism of 4-hydroxybenzoic acid was very low, with optical densities of about 0.3. This molecule is an aromatic compound mostly degraded in aerobic conditions by both bacteria and archaea [27]. Inside the gut of *R. santonensis*, the results show that this substrate is not metabolized efficiently. The degradation of monohydroxybenzoic acids also provides an indication of the degradation of aromatic hydrocarbons, aromatic dicarboxylic acids and phenolic compounds such as lignin [28]. As regards this substrate, the results show that pure lignin (in Diets 2 and 4) does not stimulate the ability to degrade aromatic molecules. Glycerol-phosphate is very important to bacteria and archeae because it is used in the synthesis of the lipids constituting the cell membranes [29]. This molecule is metabolized by most microorganisms, and it is not surprising to observe high optical densities (1.5-2) coupled with strong substrate consumption for an AWCD of 0.3.

Significant differences were observed between the other substrates according to the diets. Among those substrates, some are interesting to analyze. D-xylose is the main component of hemicelluloses. Xylan, resulting from the polymerization of xylose, is more easily attackable by enzymes than cellulose. Bacteria can metabolize D-xylose through the pentose phosphate pathway [30]. Optical densities (AWCD : 0.3) were higher for diets 2, 5 and 6 (about 0.5) than the other diets. Xylose metabolism, initially present in the initial microflora, was conserved with xylan and microcrystalline cellulose added with lignin. However, the ability significantly decreased with the other substrates, meaning that the metabolism was altered by those diets. The metabolism of D-cellobiose is quite interesting too because this molecule is a product of hydrolysis of cellulose. This disaccharide can be hydrolyzed by extracellular β-glucosidase, but it can also be transported by permeases. The glycosidic bond is hydrolyzed, leading to glucose-phosphate and glucose. Those common sugars can be metabolized by most microorganisms [29]. In our study, D-cellobiose was an excellent carbon source for all consortia. The metabolism was improved by using crystalline cellulose, while it decreased by using the other diets. This high degradation seems logical because termites fed on lignocellulosic materials which contain cellulose. Optical densities (AWCD : 0.3) reached maximal values of 4. Crystalline cellulose seems to be a good substrate to stimulate β-glucosidase activity, although this effect was not observed with β-methyl-D-glucoside. Glucose-1-phosphate is another important substrate. Surprisingly, the metabolism of glucose-phosphate is less strong than the one observed with D-cellobiose, probably because of an inhibiting effect of glucose. Indeed, D-cellobiose leads to a progressive hydrolysis, which decreases the free glucose concentration in solution. Another explanation could be the transportation of cellobiose by the permeases, which would be more efficient than the direct use of extracellular glucose-1-phosphate as a carbon source. In this study, the metabolism of glucose-1-phosphate significantly decreased with the artificial diets, even with crystalline cellulose. Glucose-1-phosphate is usually converted into glucose-6-phosphate before being metabolized by the Entner-Doudoroff metabolic pathway and the pentose phosphate pathway [31]. The case of α-D-lactose can be compared with glucose because this disaccharide is hydrolyzed into galactose and glucose. Galactose can easily be turned into glucose-1-phosphate, followed by glucose-6-phosphate [31]. The metabolism of α-D-lactose was improved by the artificial Diets 1, 2, 4 and 5, but no improvement was observed with α-cellulose as a diet. A considerable heterogeneity was observable with the detergents Tween40® (polyoxyethylene sorbitan monopalmitate) and Tween80® (polyoxyethylene sorbitan monooleate). Lignin and α-cellulose improved the metabolism of

those molecules. This effect may be due to the presence of other polymers in commercial α-cellulose, such as xylan and mannan [32], while lignin may have a positive effect coming from its hydrophobicity. The case of 2-hydroxybenzoic can be compared to 4-hydroxybenzoic acid. Both substrates provide information about the metabolism of aromatic compounds like lignin, and their metabolism is totally different according to the diets. Artificial diets 1, 2, 4 and 5 increased the ability to degrade 2-hydroxybenzoic acid, while no significant difference was observed with 4-hydroxybenzoic acid. This observation shows the importance of the molecular structure and the relative position of the carboxyl and hydroxyl groups. Crystalline cellulose also led to a better metabolism than α-cellulose. However, the optical densities were relatively low (0.1-0.3), and the metabolism of this substrate was limited. The case of α-cyclodextrin is interesting because this type of component can only be hydrolyzed by cyclomaltodextrinases. They are composed of 6 α(1,4)-connected glucosyl units. Cyclodextrins are characterized by a strong resistance to a multitude of glycosyl hydrolases [33]. The metabolism of this carbon source was significantly improved by the artificial diets. Lignin also improved that metabolism because the optical densities were significantly higher in the consortium corresponding to Diet 2 than in the one corresponding to Diet 1.

The other substrates are mostly molecules containing both carbon and nitrogen, such as amino-acids. Some of them contain aromatic cycles (like lignin), such as L-phenylalanine and phenylethylamine. The metabolism of L-phenylalanine was significantly improved by using the Diets 2, 3, 4 and 5, while no difference was observed with crystalline cellulose. Phenylethylamine can be metabolized into phenylacetic acid, which can be used by many bacteria [34]. Phenylethylamine also contains an aromatic cycle like phenylalanine. The metabolism of that subtstrate was also significantly improved by the artificial diets.

Those observations show that the artificial diets modified the metabolism of the original microflora significantly. The degradation of D-cellobiose depends on β-glucosidase activity, and was significantly improved by using a diet containing crystalline cellulose, while α-cellulose and xylan had a negative effect on that metabolism. The metabolism of β-methyl-D-glucoside, another substrate the hydrolysis of which depends on β-glucosidase activity, was not altered by the artificial diets, meaning that the molecular structure of the substrate is very important. The metabolism of xylose, main component of xylan, was conserved with the diets composed of xylan and crystalline cellulose/lignin. This shows the complexity and the interconnections between the different metabolic pathways. It also shows that the artificial diets may be used as inducers of specific enzymatic activities.

VII.3.3. *Metagenomic and proteomic analyses on microbial communities after the enrichment step*

The method employed here to identify the microorganisms growing in the artificial culture media cannot be considered as metagenomics stricto sensu. Indeed, metagenomics is defined as the analysis of genetic materials directly from environmental samples without any culture segregation [35]. In our case, we have chosen to apply a metagenomic method to cultures in order to identify the differences between the resulting consortia, taking account of the effect of cultivation.

The total number of sequences which was retained was different for each diet : 66,000; 31,000; 126,000; 96,000; 94,000 and 82,000 for Diets 1, 2, 3, 4, 5 and 6, respectively. The number of sequences corresponding to the different phyla is listed in **Table 3**. Seven main phyla were identified in the different cultures and common to the different diets : *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes*.

Table 3. Total number of sequences assigned to the different phyla according to the cultures coming from the different diets. Diet 1 = microcrystalline cellulose, Diet 2 = microcrystalline cellulose and lignin, Diet 3 = α-cellulose, Diet 4 = α-cellulose and lignin, Diet 5 = beech wood xylan, Diet 6 = poplar wood.

Phylum	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**
Bacteroidetes	28080	11905	102527	12172	82063	7050
Proteobacteria	28898	18038	21890	83870	10222	74155
Firmicutes	6580	91	80	89	106	142
Actinobacteria	2355	14	968	15	712	278
Acidobacteria	5	646	-	1	448	121
Verrucomicrobia	60	1	12	16	361	178
Planctomycetes	36	3	57	7	2	221
Others	21	-	79	4	24	24

In *Reticulitermes* species, it has been established that the bacterial diversity is distributed over more than 15 phyla. The main phyla are *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, and the Termite Group 1 phylum comprising together about 80% of the total bacterial community [5]. The study of Boucias et al. [14] was based on a 16S rRNA sequencing like the present study. That work concluded that the bacterial microflora in the hindgut lumen of *R. flavipes* was mainly composed of *Spirochaetes*, *Elusimicrobia*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fibrobacteres*, *Verrucomicrobia*, *Actinobacteria*

and *Tenericutes* including 98.5% of the total bacterial diversity. Fisher et al. [36] analyzed 16S rRNA sequences extracted from *R. santonensis* and identified six major bacterial phyla : *Proteobacteria*, *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Endomicrobia*, corresponding to the former Termite Group 1 phylum. However, 16% of the sequences belonged to bacteria sharing less than 90% of identity with known bacteria recorded in GenBank. Those sequences may come from non-described bacterial genera [36]. Yang et al. [37] achieved an analysis of 16S rRNA from different microbial fractions extracted from four different compartments in *R. santonensis* : midgut, protozoa, hindgut fluid and hindgut wall. In the hindgut fluid fraction, they found sequences belonging to the phyla *Firmicutes* (31.6%), Termite Group 1 (27.8%), *Bacteroidetes* (20.3%), *Spirochaetes* (11.3%), *Proteobacteria* (7.5%) and TM7 (1.5%). In our work, we have identified cultivable bacteria belonging to the phyla *Bacteroidetes*, *Proteobacteria*, *Firmicutes* and *Actinobacteria*, previsously described in the other studies. We have also found bacteria which belong to the phyla *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes*, not found by the direct sequencing (16S rRNA) method described before [5, 14, 36, 37]. Finally, we have also identified minor phyla in our cultures, cited in the previous studies : *Elusimicrobia* (11 sequences all consortia considered together), *Spirochaetes* (2 sequences), *Fibrobacteres* (2 sequences), *Tenericutes* (6 sequences), and TM7 (2 sequences). However, no sequence of the phylum TG1 was identified. This observation sounds logical because the phylum TG1 (or *Endomicrobia*) consists of a majority of bacteria living inside the flagellates in the digestive tracts of termites and wood-feeding cockroaches. However, a recent study identified the phylum in other environments (rice soil, forest soil, river sediment, cow rumen, etc.) [38].

The distribution of the different phyla corresponding to the microflora obtained from the different diets is presented in **Figure 2**. The **Figures 3**, **4** and **5** show the distribution of the different classes belonging to the phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes* according to the diets. The percentages were obtained by dividing the number of sequences in each class by the total number of sequences corresponding to one specific microflora. In our study, the main phylum identified in the consortium resulting from the original diet was *Proteobacteria*, with about 90% of the total sequences. This observation was also made for the consortia stemming from Diets 2 and 4, containing cellulose and lignin. This combination of substrates in the artificial diets is the most similar to wood, containing cellulose, lignin and hemicelluloses. This could explain the conservation of *Proteobacteria* as the dominant phylum. Lignin is in the form of a fine powder in our cultures. However, its availability is

much lower in wood because it is bound to cellulose and hemicelluloses in the lignocellulosic complex. Our cultures contain lignin which can select lignin-degrading bacteria resisting to its toxic effects. Indeed, lignin has a negative effect on most bacteria because of the release of phenolic compounds. However, most microorganisms able to hydrolyze lignin are fungi, not bacteria [39]. Some bacterial genera have been shown to degrade lignin, such as *Aeromonas*, *Arthrobacterium*, *Flavobacterium*, *Xanthomonas*, *Corallina*, *Torula*, *Nocardia* and *Pseudomonas*, considered as the best lignin degrader. Some actinomycetes and cyanobacteria can also hydrolyze lignin [40]. Other genera have been found to degrade lignin more recently : *Bacillus*, *Paenibacillus* and *Aneurinibacillus*. The lignin-degrading bacteria generally belong to three main classes : actinomycetes, α-proteobacteria and γ-proteobacteria [41]. Surprisingly, the cultures containing lignin did not increase the bacterial community of actinomycetes, while crystalline cellulose, α-cellulose and xylan had a positive effect compared with the original diet. Free lignin contained in our cultures may have a toxic effect because of too high concentration and availability. Another point is that bacteria able to hydrolyze lignin usually solubilize it to get an access to cellulose and hemicelluloses, which are their main substrates. Consequently, lignin cannot be considered as a carbon source but rather as a compound to remove [29]. In the cultures containing α-cellulose and xylan, the dominant phylum is *Bacteroidetes*. The diet composed of crystalline cellulose and lignin led to the highest concentration in *Acidobacteria*, while bacteria belonging to the phylum *Firmicutes* were mostly found in the culture containing crystalline cellulose.

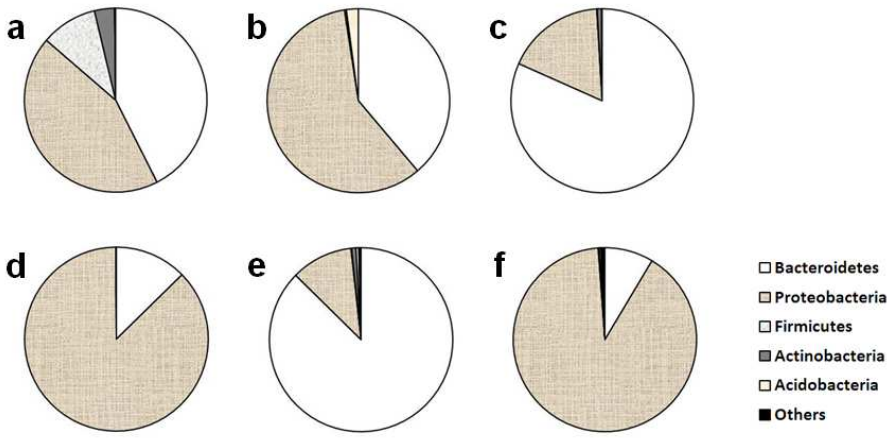

Figure 2. Distribution of the different phyla according to the cultures obtained from the termites grown on the different diets. a = Diet 1 (microcrystalline cellulose), b = Diet 2 (microcrystalline cellulose and lignin), c = Diet 3 (α-cellulose), d = Diet 4 (α-cellulose and lignin), e = Diet 5 (beech wood xylan), f = Diet 6 (poplar wood).

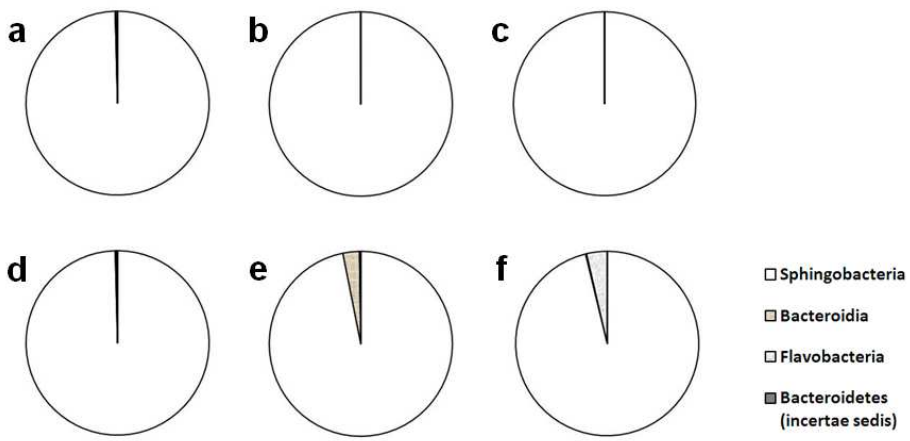

Figure 3. Distribution of the different classes belonging to the phylum *Bacteroidetes* according to the cultures obtained from the termites grown on the different diets. a = Diet 1 (microcrystalline cellulose), b = Diet 2 (microcrystalline cellulose and lignin), c = Diet 3 (α-cellulose), d = Diet 4 (α-cellulose and lignin), e = Diet 5 (beech wood xylan), f = Diet 6 (poplar wood).

The phylum *Bacteroidetes* was found in all microflora obtained from the cultures. *Sphingobacteria* was the dominant class (more than 96% of the total sequences in all samples). The other classes were hardly detected. Bacteria belonging to the class *Bacteroidia* were stimulated with xylan, while bacteria belonging to the class *Flavobacteria* were found in the culture stemming from the original diet. *Sphingobacteria* are aerobic or facultatively anaerobic chemoorganotrophic heterotrophs, common in water and soils. They include the genus *Cytophaga*, able to hydrolyze cellulose and chitin [42]. *Bacteroidia* are well studied, and abound in in the gastrointestinal tract of warm-blooded animals. *Flavobacteria* are necessarily aerobic microorganisms found in marine and freshwater environments. Taxonomy of *Flavobacteria* has been modified over the past decade, and many of them were transferred to the genera *Chryseobacterium* and *Empedobacter* [43]. *Sphingobacteria* were not identified in the study of Boucias et al. [14], suggesting that they are more easily cultivated than *Bacteroidia* and *Flavobacteria*. In our study, *Bacteroidia* are marginal, while they were identified in 6.2% of the total sequences in the study of Boucias et al. [14].

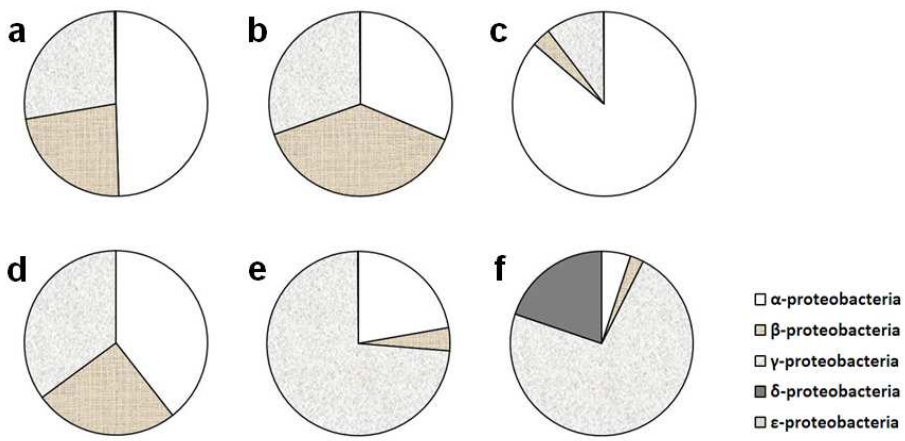

Figure 4. Distribution of the different classes belonging to the phylum *Proteobacteria* according to the cultures obtained from the termites grown on the different diets. a = Diet 1 (microcrystalline cellulose), b = Diet 2 (microcrystalline cellulose and lignin), c = Diet 3 (α-cellulose), d = Diet 4 (α-cellulose and lignin), e = Diet 5 (beech wood xylan), f = Diet 6 (poplar wood).

Proteobacteria were also present (α-, β-, γ- and δ-*Proteobacteria*), but ε-*Proteobacteria* were not detected. The same observation was made in the study of Boucias et al. [14]. α-, β-, γ- and δ-*Proteobacteria* were detected in concentrations of 7.0, 1.8, 1.4 and 1.2%, respectively. The study of Yang et al. [37] also detected *Proteobacteria* in the hindgut fluid and wall of *R. santonensis*. However, α- and β-*Proteobacteria* were the main classes, while δ- and ε-*Proteobacteria* were found in lower concentrations. The class of γ-*Proteobacteria* was not detected. In our study, the culture resulting from the original diet was mainly composed of γ-*Proteobacteria* (73.7%), while the other classes were less represented (22.2% α-*Proteobacteria*, 4,0% β-*Proteobacteria* and 0.1% δ-*Proteobacteria*). The percentage of δ-*Proteobacteria* decreased to less than 0.3% in the cultures resulting from the artificial diets. The concentration in γ-*Proteobacteria* was conserved with xylan, but decreased with all the other diets. The cultures obtained from Diets 1, 2 and 4 had a positive effect on β-*Proteobacteria*. α- and γ-*Proteobacteria* have been described as lignin degraders. In our study, lignin caused an increase of α- and β-*Proteobacteria*, while it reduced the bacterial concentration of γ-*Proteobacteria*. Some bacteria belonging to the phylum of *Proteobacteria* are considered as representative hemicellulase producers. The genera *Caulobacter* and *Agrobacterium* are α-*Proteobacteria* involved in the production of xylanase, xylosidase, arabinofuranosidase/arabinanase, α-glucuronosidase and acetyl xylan esterase. The genera *Cellvibrio* and *Xanthomonas* are γ-*Proteobacteria* which generate xylanase, xylosidase, arabinofuranosidase/arabinanase, α-glucuronidase and mannanase [44].

Proteobacteria have also been shown to produce cellulase, such as the genera *Caulobacter*, *Rhizobium* (α-*Proteobacteria*) and *Erwinia* (γ-*Proteobacteria*) [45-47].

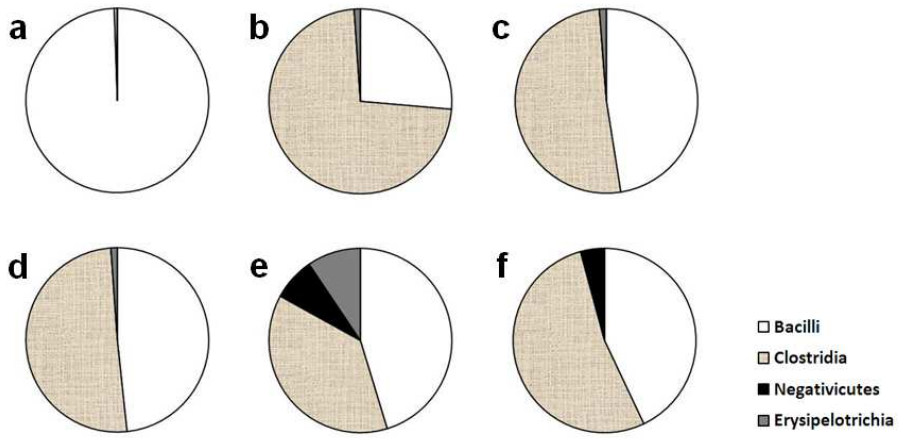

Figure 5. Distribution of the different classes belonging to the phylum *Firmicutes* according to the cultures obtained from the termites grown on the different diets. a = Diet 1 (microcrystalline cellulose), b = Diet 2 (microcrystalline cellulose and lignin), c = Diet 3 (α-cellulose), d = Diet 4 (α-cellulose and lignin), e = Diet 5 (beech wood xylan), f = Diet 6 (poplar wood).

As regards the phylum *Firmicutes*, the two main classes growing in the cultures were *Bacilli* and *Clostridia*. A small fraction of *Negativicutes* was observed in the cultures coming from the original diet and containing xylan. Bacteria belonging to the class *Erysipelotrichia* were hardly observed. *Bacilli* and *Clostridia* were also cited in the study of Boucias et al. [14], while the classes *Negativicutes* and *Erysipelotrichia* were not detected. In the study of Yang et al. [37], the phylum *Firmicutes* was shown in 27% of the assigned clones composing the clone libraries. Almost two-thirds of those bacteria belonged to the class *Clostridia*. There are many cases of enzyme-producing strains in the phylum *Firmicutes*. The genera *Bacillus* and *Clostridium* have been shown to produce cellulases and hemicellulases. The genus *Geobacillus* is also able to produce [44, 47] hemicellulases (xylanase, xylosidase, arabinofuranosidase/arabinanase, α-glucuronidase and acetyl xylan esterase), while the genera *Ruminococcus* and *Acetivibrio* hydrolyze cellulose.

Strictly identified proteins allowed identifying proteins from the main phyla. These "protein by protein" identifications are shown in **Table 4**. Taxonomic and functional analyses will be discussed later with homology-scored assignments.

Table 4. Proteins identified by at least 2 peptides scoring above 15, at least one of which above identity score, using the NCBI nr Bacteria database. Diet 1 = microcrystalline cellulose, Diet 2 = microcrystalline cellulose and lignin, Diet 3 = α-cellulose, Diet 4 = α-cellulose and lignin, Diet 5 = beech wood xylan, Diet 6 = poplar wood.

Diet	Accession	Protein	Score	n pept	Seq Cov	RMS90
1	**gi|295688897**	Translation elongation factor Tu (Caulobacter segnis)	166	3	10,6	64,89
	gi|148273804	elongation factor Tu (Clavibacter michiganensis)	153	3	10,3	129,3
	gi|16264946	Putative sugar uptake ABC transporter periplasmic solute-binding protein precursor (Sinorhizobium meliloti)	102	3	12,2	81,07
	gi|311104908	outer membrane protein A (Achromobacter xylosoxydans)	95,1	2	9,8	80,82
	gi|6649599	methanol: NDMA oxidoreductase (Amycolatopsis methanolica)	87,7	2	5,8	138,9
2	**gi|218892059**	quinoprotein alcohol dehydrogenase (Pseudomonas aeruginosa)	204	5	12	101,5
	gi|37704628	OprF (Pseudomonas sp)	162	3	14,9	59,45
	gi|307294972	translation elongation factor Tu (Sphingobium chlorophenolicum)	109	3	9,8	180,6
	gi|162148428	60 kDa chaperonin (Gluconacetobacter diazotrophicus)	137	2	5,4	25,67
	gi|294012111	chaperonin GroEL (Sphingobium japonicum)	117	2	4,7	131,9
3	**gi|295688897**	Translation elongation factor Tu (Caulobacter segnis)	151	3	10,9	181,7
	gi|110639133	DNA-binding protein HU-beta (Cytophaga hutchinsonii)	146	2	25,6	148,7
	gi|110639543	50S ribosomal protein L7/L12 (Cytophaga hutchinsonii)	124	2	16,3	100,4
	gi|254520283	ATP synthase F1 sector subunit beta (Clostridium sp.)	101	2	8,4	125
4	**gi|307294285**	OmpA/MotB domain protein (Sphingobium chlorophenolicum)	180	3	9,2	107
	gi|294010611	GTPase - translation elongation factor (Sphingobium japonicum)	170	3	9,8	122,6
	gi|23014093	COG0050: GTPases - translation elongation factors (Magnetospirillum magnetotacticum)	157	2	10,4	156,7
	gi|37704628	OprF (Pseudomonas sp)	122	2	11,5	28,39
	gi|90415618	TonB-dependent receptor, putative (marine gamma proteobacterium)	116	2	2,7	40,52
5	**gi|148272818**	glutamine synthetase I (Clavibacter michiganensis)	276	5	12,9	94,7
	gi|255326643	chaperonin GroL (Rothia mucilaginosa)	190	4	4,3	101,9
	gi|170780597	xylose isomerase (Clavibacter michiganensis)	197	3	9,3	116,4
	gi|148273804	elongation factor Tu (Clavibacter michiganensis)	180	3	9,3	81,5
	gi|88855326	ATP synthase subunit B (marine actinobacterium)	170	3	6,9	103,1
	gi|256372713	chaperone protein DnaK (Acidimicrobium ferrooxidans)	136	2	5,9	79,49
6	**gi|288549451**	hypothetical protein ENTCAN (Enterobacter cancerogenus)	305	6	17,9	82,61

gi|295095401	maltooligosaccharide-binding protein (Enterobacter cloacae)	220	5	20,6	57,78
gi|148546728	flagellin (Pseudomonas putida)	272	4	21,2	70,24
gi|288550137	maltoporin (Enterobacter cancerogenus)	225	4	11,3	83,76
gi|307315700	protein synthesis factor GTP-binding (Escherichia coli)	143	4	18,7	127,6
gi|295095403	maltoporin (phage lambda and maltose receptor) (Enterobacter cloacae)	204	3	16,1	43,11
gi|157836810	Chain A, structure of the periplasmic glucose galactose receptor (Salmonella thyphimurium)	153	3	12,4	62,54
gi|311279377	histone family protein nucleoid-structuring protein H-NS (Enterobacter cloacae)	127	3	20	94,18
gi|238753641	Major outer membrane lipoprotein (Yersinia ruckeri)	146	2	41,9	98,9
gi|70734100	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein (Pseudomonas fluorescens)	113	2	8,7	86,3
gi|167645571	Elongation factor Tu (Caulobacter sp)	110	2	7,1	72,36
gi|167035479	Chaperonin GroEL (Pseudomonas putida)	98,2	2	6	58,89

Alternatively to the general NCBI nr Bacteria database, a termite specific homemade database was used to highlight peptides known from termite gut community. This restricted search allowed less protein identification (**Table 5**) than the search in NCBI nr Bacteria database.

Table 5. Proteins identified by at least 2 peptides scoring above 15, at least one of which above identity score, using the termite specific nr database. Diet 1 = microcrystalline cellulose, Diet 2 = microcrystalline cellulose and lignin, Diet 3 = α-cellulose, Diet 4 = α-cellulose and lignin, Diet 5 = beech wood xylan, Diet 6 = poplar wood.

Diet	Accession	Protein	Score	n pept
1	**gi|334106552**	translation elongation factor Tu [Isoptericola variabilis 225]	83	2
	gi|212548608	translation elongation factor Tu [Candidatus Azobacteroides pseudotrichonymphae genomovar. CFP2]	47	2
2	**gi|329747997**	ABC transporter related protein [Sphaerochaeta coccoides DSM 17374]	35	2
3	**gi|333737448**	glyceraldehyde-3-phosphate dehydrogenase, type I [Treponema azotonutricium ZAS-9]	71	3
	gi|329749179	Xenobiotic-transporting ATPase [Sphaerochaeta coccoides DSM 17374]	40	1
	gi|212548608	translation elongation factor Tu [Candidatus Azobacteroides pseudotrichonymphae genomovar. CFP2]	38	1
	gi|337274294	acriflavin resistance protein [Acetonema longum DSM 6540]	34	1
4	**gi|474480185**	translation elongation factor TU [Clostridium termitidis CT1112]	59	2
	gi|337275392	ATPase associated with various cellular activities AAA_5 [Acetonema longum DSM 6540]	45	1
5	**gi|334108586**	Chaperone protein dnaK [Isoptericola variabilis 225]	122	4
	gi|334108129	ATP synthase subunit beta [Isoptericola variabilis 225]	121	2
	gi|334108423	60 kDa chaperonin [Isoptericola variabilis 225]	62	2
	gi|334108131	ATP synthase subunit alpha [Isoptericola variabilis 225]	45	3
	gi|391221689	peptide chain release factor 2 [Opitutaceae bacterium TAV1]	38	3

Blast analysis of homology-scored peptides revealed as expected higher diversity than the analysis of strictly identified proteins. The identification pattern was globally conserved : the main or single phyla identified via blast analysis of single peptides are the same as those identified « protein by protein ». Proteomic results poorly reflect those of genomic analysis of cultivated consortia because of the comparatively small amount of data generated. A complementary explanation is the tendency to identify most abundant peptides, which may be conserved among several phyla. BLAST analysis of homology-scored peptides generated relatively few data, with 336 unique reads, among which 237 could be assigned to any taxonomic level. The benefit from this approach is the assignment of conserved peptides to higher taxonomic levels. Identifications of *Actinobacteria* (genus *Isoptericola*) using both databases correspond to samples (Diets 1 and 5) exhibiting most homology-scored peptides for this phylum. *Proteobacteria* is one of the prevalent phyla in Diet 1. *Actinobacteria* and *Bacteroidetes* are also present but in different proportions. In the consortium from Diet 2,

Proteobacteria and *Bacteroidetes* are the main phyla, but the phylum *Proteobacteria* was still overrepresented, while *Bacteroidetes* was underrepresented. For Diet 3, we observed a reversed imbalance between *Proteobacteria* and *Bacteroidetes* in both experiments. *Firmicutes* represents the second main phylum from this diet, while there were no or very few representatives following the genomic analysis. Proteomic analysis of consortium from Diet 4 is closer to the 16S analysis results. *Proteobacteria* represent about 85% of taxonomic assignments. *Bacteroidetes* and *Firmicutes* share the 15% left in the results from the proteomic analysis, while no *Firmicutes* are found after genomic analysis. From all results of proteomic analysis, the consortium from Diet 5 is the only one in which *Proteobacteria* is not the major phylum, while this was also the case for Diet 3 as regards the genomic analysis. The major phylum is *Actinobacteria* and a few *Firmicutes* are also identified. In Diet 6, *Proteobacteria* is the most represented phylum in both experiments, but the second one is *Bacteroidetes* following genomics while the proteomic analysis suggests *Firmicutes*. The results of this analysis are shown in **Figure 6**. Some taxa were previously identified directly from wood-fed termite gut via 2DE-MALDI-TOF MS analysis in a previous study [48], as the following genera : *Achromobacter*, *Burkholderia*, *Pseudomonas*, *Enterobacter*, *Salmonella*, *Enterococcus*, *Clostridium*, *Corynebacterium*, *Streptomyces* and *Croceibacter*.

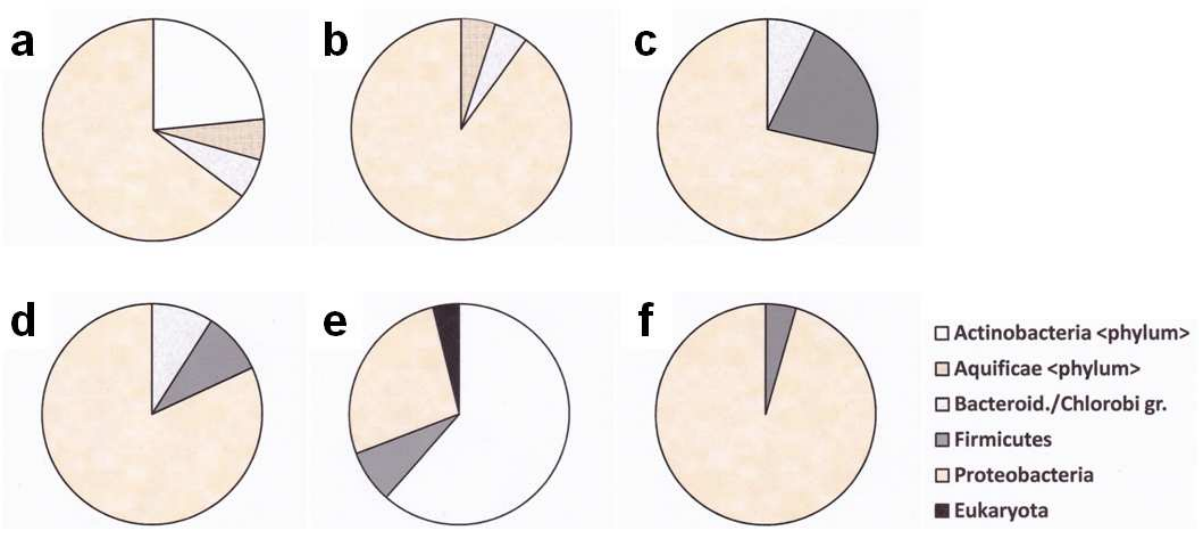

Figure 6. Taxonomic assignments of homology-scored peptides. a = Diet 1 (microcrystalline cellulose), b = Diet 2 (microcrystalline cellulose and lignin), c = Diet 3 (α-cellulose), d = Diet 4 (α-cellulose and lignin), e = Diet 5 (beech wood xylan), f = Diet 6 (poplar wood).

SEED analysis revealed that protein metabolism appears to be prevalent in all consortia. For wood-fed termites, identifications related to carbohydrates metabolism and

motility/chemotaxis are in the same range. Some taxonomic and/or functional assignments are pertinent regarding the BIOLOG ECO Microplates® experiment and results from genomic analysis. Peptides related to the metabolism of aromatic compounds were identified only in termites fed with wood diet. Proteins from *Pseudomonas* and *Caulobacter* genera were identified in the consortium cultivated from Diet 6, supporting their possible implication in aromatic compounds metabolism. Degradation of 2-hydroxybenzoic acid has been observed in *Pseudomonas* which was identified by OprF in Diets 2 and 4 and by a Quinoprotein alcohol dehydrogenase in Diet 2. These results support the implication of *Pseudomonas* in the degradation of aromatic compounds in consortia cultivated from termite gut, as both Diets 2 and 4 contain lignin. Xylose isomerase was identified in the metaproteome of the consortium from Diet 5 as 2 peptides from the pentose phosphate pathway. However, peptides related to a periplasmic xylose-binding protein were also identified in the consortium from Diet 1 (crystalline cellulose). As for the metabolism of lactose, a glucose galactose-binding periplasmic protein was identified from Diet 6. *Sinorhizobium* was identified in Diet 1 (putative sugar uptake ABC transporter periplasmic solute-binding protein precursor) among many proteobacteria in general. After cultivating the consortia, about 75 % of the homology-scored peptides were found to belong to *Proteobacteria*. The results are shown in **Figure 7**.

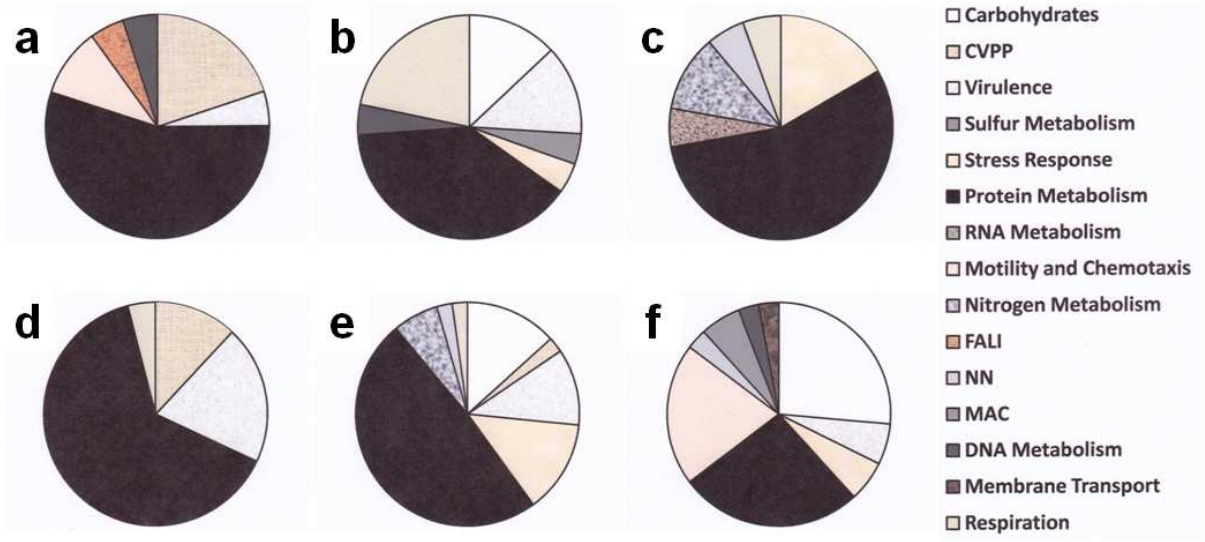

Figure 7. Functional assignments (SEED analysis) of homology-scored peptides. a = Diet 1 (microcrystalline cellulose), b = Diet 2 (microcrystalline cellulose and lignin), c = Diet 3 (α-cellulose), d = Diet 4 (α-cellulose and lignin), e = Diet 5 (beech wood xylan), f = Diet 6 (poplar wood). Legend – CVPP = Cofactors, Vitamins, Prosthetic groups, Pigments, FALI = Fatty Acids, Lipids and Isoprenoids, NN = Nucleosides and Nucleotides, MAC = Metabolism of Aromatic Compounds.

VII.3.4. *Isolates and enzymatic activities*

It was possible to isolate several bacteria and molds from the enrichment cultures. Three bacteria were isolated from **Media 2** (strain BAvL1), **5** (BX1) and **6** (BW1). However, a microscopic morphological analysis followed by enzymatic assays confirmed that the strains BAvL1, BX1 and BW1 were found to be a unique bacterium. The strain was found to belong to the genus *Chryseobacterium* (GenBank ID : KJ425110), and the strain was given the name *Chryseobacterium* sp. strain XAvLW. Several molds were also isolated from Media 1 (strains MAv1 and MAv2), 2 (strains MAvL1 and MAvL2), 3 (strains MαC1 and MαC2), 4 (strain MαCL1) and 5 (strains MX1, MX2, MX3 and MX4). Some of those molds were identified on the basis of a morphological analysis. The strains MAv1 and MAvL1 were identical. The mold was identified and given the name *Trichoderma virens* CTGxAviL. The strains MAv2 and MAvL2 were also found to be a unique microorganism, which was called *Fusarium solani* strain AvL. The strain MX3 was named *Sarocladium kiliense* CTGxxyl after identification.

To our knowledge, cases of isolation of *Chryseobacterium* sp. from termite guts were not reported. However, *Chryseobacterium* sp. was isolated from the cockroach *Periplaneta americana* [49]. Some molds, such as *Alternaria alternater*, *Aspergillus awamuri*, *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus nidulans, Cladosporium* sp., *Paecilomyces fusiporus* and *Rhizopus stolonifer*, have already been isolated from the gut of termites [50, 51]. However, to our knowledge, this is the first time that molds belonging to the genera *Sarocladium* (*Acremonium*), *Fusarium* and *Trichoderma* have been isolated from termite guts and cultivated.

Chryseobacterium sp. belongs to the Kingdom *Bacteria*, Phylum *Bacteroidetes*, Class *Flavobacteria*, Order *Flavobacteriales*, and Family *Flavobacteriaceae*. *Flavobacteria* were identified through genomic analysis in all enrichment cultures (**Media 1**, **2**, **3**, **4**, **5** and **6**). Bacteria belonging to the Phylum *Bacteroidetes* were also detected through proteomics. However, all diets were not found to contain bacteria of this Phylum. Those bacteria were found to be present in **Media 1**, **2**, **3** and **4**, although *Chryseobacterium* sp. was isolated from **Media 2**, **5** and **6**. This observation highlights the fact that protein translation brings additive information in comparison with genomic data.

Qualitative enzymatic tests were achieved on the isolated bacteria and molds. The results are indicated in **Table 6**. The production of enzymatic activities depends on pH value.

Such an observation was made on the molds MαC2 and MαCL1. All strains were found to secrete endo-1,4-β-D-xylanase, α-amylase, endo-1,4-β-D-glucanase, Filter Paper-cellulase activity and β-glucosidase. The molds *Trichoderma virens* CTGxAviL (MAv1/MAvL1 in **Table 6**), *Fusarium solani* strain AvL (MAv2/MAvL2 in **Table 6**) and *Sarocladium kiliense* CTGxxyl (MX3 in **Table 6**) were also found to produce endo-1,3-β-D-glucanase. As regards bacteria, very little information is available concerning the genus *Chryseobacterium* and the secretion of cellulases and xylanases. However, *Flavobacterium*, belonging to the same family, has been shown to produce xylanase and peroxidase [52, 53]. As regards molds, there are very few studies of *Sarocladium* strains in literature. De Almeida et al. [54] described two strains of *Sarocladium* able to produce Filter Paper-cellulase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, β-glucosidase, α-arabinofuranosidase and α-galactosidase. In another study, a strain of *Sarocladium fuci* was found to produce α-amylase, lipase, protease and cellulase but no β-glucosidase [55]. However, no case of endo-1,3-β-D-glucanase-producing strain of *Sarocladium kiliense* was reported before. Many studies of enzyme-producing strains of *Trichoderma* could be cited. That mold has been shown to produce xylanases, cellulases, endo-1,3-β-D-glucanases and chitinases [56-58]. Finally, xylanases and cellulases have been reported before for the molds *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium oxysporum* [59-62]. However, fungi belonging to this genus can be pathogenic with humans and plants. Its use as an enzyme producer is not applicable. *Trichoderma virens* qualifies for enzyme production, and many cases of cellulase and xylanase production were reported.

Table 6. Enzymatic activities detected in the isolated strains. Bacteria are indicated with the letter "B", while molds are indicated with the letter "M". E-XYL 5 = qualitative assay of endo-1,4-β-D-xylanase at pH 5, E-XYL 7 = endo-1,4-β-D-xylanase at pH 7, α-AM 5 = α-amylase at pH 5, α-AM 7 = α-amylase at pH 7, E-1,3-βG 5 = endo-1,3-β-D-glucanase at pH 5, E-1,3-βG 7 = endo-1,3-β-D-glucanase at pH 7, E-1,4-βG 5 = endo-1,4-β-D-glucanase at pH 5, E-1,4-βG 7 = endo-1,4-β-D-glucanase at pH 7, FPase 5 = Filter Paper-cellulase at pH 5, FPase 7 = Filter Paper-cellulase at pH 7, βGL = β-glucosidase at pH 7.4.

Strain/ Activity	BX1 BAvL1 BW1	MX1	MX2	MX3	MX4	MAv1 MAvL1	MAv2 MAvL2	MαC1	MαC2	MαCL1
E-XYL 5	+	+	+	+	+	+	+	+	+	+
E-XYL 7	+	+	+	+	+	+	+	+	+	+
α-AM 5	+	+	+	+	+	+	+	-	-	-
α-AM 7	+	+	+	+	+	+	+	-	+	+
E-1,3-βG 5	-	-	-	+	-	+	+	-	-	-
E-1,3-βG 7	-	-	-	+	-	+	+	-	-	-
E-1,4-βG 5	+	+	+	+	+	+	+	+	+	+
E-1,4-βG 7	+	+	+	+	+	+	+	+	+	+
FPase 5	+	+	+	+	+	+	+	+	+	+
FPase 7	+	+	+	+	+	+	+	+	+	+
βGL	+	+	+	+	+	+	+	+	+	+

VII.4. Conclusion

This work assessed the microflora modified by artificial diets provided to the termites they were living in. Microscopic observation of flagellate populations and the BIOLOG ECO Microplates® experiment showed that the hindgut microbial community was altered after different feeding treatments. The effect of the artificial diets was different according to the flagellate species. BIOLOG ECO Microplates® highlighted strong differences between the bacterial and fungal communities stemming from the artificial diets, but the protist communities were not considered in that experiment because the flagellates could not grow in the microplate wells. The cellulase activities were demonstrated on the basis of the degradation of β-methyl-D-glucoside and D-cellobiose.

Protein identification did not reflect all results from genomic analysis but supported some hypotheses from metabolism assessment. General enrichment in *Proteobacteria* was also observed by proteomic analysis. Protein metabolism was prevalent in most consortia. Microcrystalline cellulose and wood diets allowed identifying more peptides related to general carbohydrate metabolism. The metagenomic analysis identified 7 main phyla :

Bacteroidetes, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes*. The phyla *Bacteroidetes* and *Proteobacteria* were dominant, but the proportions were variable according to the diets.

The liquid cultures led to the isolation of several bacterial and fungal strains. The most interesting microorganisms were identified as the bacterium *Chryseobacterium* sp. and the fungi *Sarocladium kiliense* CTGxxyl and *Trichoderma virens* CTGxAviL. Those strains were found to produce diversified enzymatic activities (α-amylase, β-glucosidase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase and filter paper-cellulase) exploitable in various fields.

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CHAPITRE VIII.

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Discussion générale

VIII.1. Introduction

Le but de ce travail était d'isoler des microorganismes (bactéries, mycètes et protistes) capables de produire des enzymes utilisables pour hydrolyser le complexe lignocellulosique. Le termite, insecte social abritant une microflore complexe au sein de son tube digestif, est capable d'hydrolyser les composants de la lignocellulose à l'aide d'enzymes provenant de ses propres cellules et dont l'action est combinée à celle du consortium microbien intestinal. Les enzymes impliquées dans la dégradation de la lignocellulose agissent soit sur la lignine, soit sur la cellulose, soit sur les hémicelluloses. Cette thèse se concentre essentiellement sur l'hydrolyse des hémicelluloses et de la cellulose car ces polymères libèrent des sucres fermentescibles, ce qui n'est pas le cas de la lignine. Cette dernière agit plutôt comme une barrière à l'hydrolyse de la cellulose et des hémicelluloses et est majoritairement dégradée par les mycètes par des mécanismes d'oxydation.

Les travaux menés dans les différents chapitres ont clairement démontré que le termite est une source indéniable de cellulases et d'hémicellulases. Chaque microorganisme isolé a démontré des activités enzymatiques bien distinctes en plus de la capacité à hydrolyser la cellulose et les hémicelluloses. Toutes les souches productrices d'enzymes qui ont été identifiées sont reprises dans le tableau suivant (**Tableau 1**).

Cette discussion générale est subdivisée en 7 parties :

- La présente partie introductive,
- Une partie relative au **Chapitre III** discutant les résultats obtenus sur les souches isolées en atmosphères contrôlées (*Streptomyces* sp. ABGxAviA1, *Pseudomonas* sp. ABGxCellA, *Bacillus subtilis* ABGx, *Aspergillus fumigatus* ABGxAviA2),
- Une partie relative au **Chapitre IV** discutant les résultats de l'étude du bacille xylanolytique *Bacillus subtilis* ABGx,
- Une partie relative au **Chapitre V** discutant les résultats obtenus sur le protiste producteur d'amylase (*Poterioochromonas* sp.),
- Une partie relative au **Chapitre VI** discutant les résultats obtenus sur les moisissures cellulolytiques et hémicellulolytiques (*Sarocladium kiliense* CTGxxyl et *Trichoderma virens* CTGxAviL),
- Une partie relative au **Chapitre VII** discutant les résultats obtenus sur les termites élevés sur les diètes artificielles ainsi que les cultures de consortia microbiens qui en ont résulté,

- Une partie relative à différents aspects concernés par l'ensemble des chapitres expérimentaux.

Tableau 1. Bilan des souches isolées du termite *R. flavipes* et de leurs activités enzymatiques.

MICROORGANISME	**ACTIVITÉS ENZYMATIQUES**
BACTÉRIES	
Bacillus subtilis ABGx (**Chap. III et IV**)	α-amylase, endo-1,4-β-D-xylanase
Chryseobacterium sp. XAvLW (**Chap. VII**)	α-amylase, β-glucosidase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, filter paper-cellulase
Pseudomonas sp. ABGxCellA (**Chap. III**)	β-glucosidase, α-amylase
Streptomyces sp. ABGxAviA1 (**Chap. III**)	α-amylase
MOISISSURES	
Aspergillus fumigatus ABGxAviA2 (**Chap. III**)	α-amylase, β-glucosidase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase
Fusarium solani AvL (**Chap. VII**)	α-amylase, β-glucosidase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, filter paper-cellulase
Sarocladium kiliense CTGxxyl (**Chap. VI et VII**)	α-amylase, β-glucosidase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, filter paper-cellulase
Trichoderma virens CTGxAviL (**Chap. VI et VII**)	α-amylase, β-glucosidase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, filter paper-cellulase
PROTISTES	
Poterioochromonas sp. (**Chap. V**)	α-amylase

VIII.2. Utilisation des atmosphères contrôlées pour isoler les producteurs d'enzymes

Le Chapitre III décrit l'isolement de bactéries et d'une moisissure à l'aide de milieux liquides et solides contenant de la carboxyméthylcellulose, de la cellulose microcristalline et du cellobiose. Le travail s'est concentré sur les souches productrices d'enzymes (soit 3 bactéries et une moisissure), mais 12 souches ont été isolées au départ. Tous ces microorganismes ont été isolés en conditions micro-aérobies (atmosphère composée de 16% de CO_2 et 84% d'air), alors que les atmosphères anaérobies (CO_2 100% et H_2 85%/CO_2 15%) n'ont favorisé aucune croissance de souches microbiennes sur les milieux spécifiques.

Le dioxyde de carbone a un effet direct sur les organismes vivants. Il est nécessaire à la croissance de toute cellule, mais un apport de CO_2 en excès a des effets négatifs sur les activités métaboliques. Les causes de ces effets peuvent être liées à une diminution intracellulaire du pH, à une inhibition ou une stimulation d'activités enzymatiques par la modification de la synthèse de ces enzymes, ou à des effets de dégradation induits au niveau de la membrane de la cellule. Le CO_2 et le bicarbonate en phase aqueuse sont tous deux susceptibles d'avoir des effets sur les fonctions des protéines membranaires ainsi que des enzymes cytoplasmiques. Chaque microorganisme démontre une résistance spécifique à ces différents effets. Les plus sensibles sont ceux dont les activités enzymatiques sont facilement perturbées. L'effet du CO_2 sur les bactéries, levures ou moisissures dépend de son aptitude à traverser les parois, puis à modifier le pH intracellulaire, affectant directement les fonctions enzymatiques. L'effet globalement négatif du CO_2 sur les cellules résulte des différents effets présentés ci-dessus. Chaque bactérie, levure et moisissure possède son propre type de paroi et son arsenal enzymatique spécifique, ce qui a un effet direct sur la tolérance au dioxyde de carbone [1]. Dans certains cas, le carbonate, dont la présence est liée à celle du CO_2 et de HCO_3^-, peut donner lieu à la formation de radicaux carbonate $CO_3^{\cdot-}$, par exemple sous l'action de la superoxyde dismutase ou de la xanthine oxydase. Ce radical est un puissant agent oxydant. Il peut initier des réactions radicalaires sur les lipides des cellules ainsi que sur les protéines. Certains acides aminés y sont particulièrement sensibles, tels que le tryptophane, la cystéine, la tyrosine, la méthionine et l'histidine. Les liaisons peptidiques elles-mêmes peuvent aussi être le siège de réactions radicalaires. Les ions métalliques contenus dans les métalloprotéines peuvent aussi participer à ces réactions [2]. Le radical carbonate peut aussi oxyder les sucres et les acides nucléiques. Dans ce dernier cas, les dommages occasionnés sont importants et irréversibles. La guanine est la base nucléique la plus sensible à ces réactions radicalaires [3].

Les bactéries appartenant au genre *Pseudomonas* et les moisissures qui se développent sur les denrées alimentaires sont particulièrement sensibles au dioxyde de carbone, et leur croissance est significativement réduite sous une pression partielle de 0.1 atm en CO_2. A l'inverse, la levure *Saccharomyces cerevisiae* est plus tolérante et sa croissance n'est affectée qu'à partir de 0.5 atm de CO_2. *Lactobacillus* peut supporter une pression de 1 atm, et *Lactococcus lactis* peut supporter une pression de 8.6 atm en CO_2 avec un taux de croissance spécifique réduit à 50%. Il est important de noter que les bactéries anaérobies ont globalement une tolérance au CO_2 inférieure à celle des bactéries aérobies [1]. Cette tendance pourrait expliquer en partie l'absence de croissance de souches anaérobies sur les milieux soumis à une atmosphère riche en CO_2. Les colonies étaient directement soumises à l'influence du CO_2 sous forme gazeuse étant donné que les souches ont été étalées sur les milieux solides.

D'autre part, le CO_2 n'a pas pu avoir un effet acidifiant très poussé au niveau du milieu gélosé étant donné la présence de tampon MOPS (acide 3-(N-morpholino)propane-sulfonique) présent dans le milieu GBG à la concentration de 12 mM [4]. Les milieux solides étaient ajustés à un pH neutre, l'acide 3-(N-morpholino)propane-sulfonique) possédant un pKa de 7.2. Au niveau des transferts gazeux, la concentration en CO_2 est théoriquement dépendante de la loi de Henry et est directement proportionnelle à la pression partielle en dioxyde de carbone [5]. On a :

$$S = k_H.P$$

Où : S est la solubilité du gaz dissous exprimée en mol/l, k_H est le coefficient de Henry ($3{,}4.10^{-2}$ mol/l.atm pour le CO_2 à 25°C), et P est la pression partielle du gaz en atm.

Cette loi rend compte de la concentration à l'équilibre, mais le facteur temps n'y apparaît pas. Cependant, le transfert du CO_2, même dans l'eau pure, est un processus très lent [6]. Dans le cas présent, il s'agit d'un milieu gélosé de composition commune à tous les milieux solides utilisés sous les différentes atmosphères. Les composés du milieu (principalement l'agar, augmentant considérablement la viscosité) ont vraisemblablement ralenti les transferts gazeux de l'atmosphère vers le milieu de culture, et l'effet sur le pH a été contrôlé par le tampon MOPS. Il est donc très probable que l'effet du dioxyde de carbone sur les bactéries et mycètes provienne majoritairement de l'atmosphère elle-même, et non de l'acidification du milieu. Il est utile de rappeler ici que le dioxyde de carbone était présent dans toutes les atmosphères artificielles utilisées mais à des pressions partielles variables.

Bien que le CO_2 ait un effet négatif sur de nombreux microorganismes à forte concentration, l'absence de souches sur les milieux gélosés en atmosphères anaérobies peut aussi provenir de la difficulté d'isoler les souches anaérobies. Les différents genres bactériens isolés à partir du tractus intestinal de termites et présentés dans le **Chapitre II**, sur base des études précédentes, sont majoritairement aérobies ou anaérobies facultatifs (*Acinetobacter*, *Bacillus*, *Brevibacillus*, *Brucella*, *Cellulomonas*, *Cellulosimicrobium*, *Chryseobacterium*, *Citrobacter*, *Comamonas*, *Dyella*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Kocuria*, *Lactococcus*, *Microbacterium*, *Micromonospora*, *Ochrobactrum*, *Paenibacillus*, *Pilibacter*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Sphingomonas*, *Staphylococcus*, *Streptomyces*, *Zymomonas*). Le genre *Clostridium* est le seul à être strictement anaérobie [7]. En ce qui concerne les moisissures, *Aspergillus fumigatus* peut croître en aérobiose ou en anaérobiose. Dans ce dernier cas, la souche tire son énergie de la fermentation, considérablement moins avantageuse que la respiration du point de vue énergétique. La moisissure n'atteint alors qu'un rendement en biomasse réduit, environ égal à 10% de la valeur en aérobiose [8]. Les bactéries anaérobies strictes, elles, ne tolèrent pas la présence d'oxygène, ce qui complique la mise en œuvre des cultures et des méthodes d'isolement. Bien que les techniques aient été considérablement améliorées et mieux maîtrisées ces dernières années, le métabolisme des bactéries anaérobies n'en reste pas moins très lent, et ces souches présentent aussi une cultivabilité moins bonne que celle des souches aérobies ou anaérobies facultatives.

Un enrichissement des souches microbiennes anaérobies à partir de l'intestin du termite *R. flavipes* requiert un milieu anaérobie (reproduit par des atmosphères artificielles) contenant des substrats tels que la cellulose et des hémicelluloses, mais les processus de biodégradation anaérobies requièrent un temps considérable [9]. Par conséquent, une autre explication de l'absence de souches en atmosphères anaérobies peut être un temps insuffisant laissé aux cultures. Sur un plan plus pratique, ces raisons font des souches aérobies et anaérobies facultatives de meilleurs candidats à la production d'enzymes, sous la condition que ces enzymes montrent des activités satisfaisantes. Cet aspect ne pose pas de problème par rapport au termite étant donné que le tractus intestinal possède une portion volumique considérable contenant de l'oxygène. Cet environnement favorise les souches aérobies, anaérobies facultatives, voire micro-aérophiles [10]. Chez les termites inférieurs, la portion centrale du hindgut est soumise à de fortes concentrations en hydrogène et une absence d'oxygène, ce qui favorise la croissance des protistes anaérobies. Ces eucaryotes digèrent les

particules de bois ainsi que les bactéries, libérant ainsi des oligosaccharides et autres molécules accessibles pour la flore aérobie et anaérobie facultative [11].

La moisissure *Aspergillus fumigatus* isolée dans le cadre de ce travail présente les activités enzymatiques les plus nombreuses (α-amylase, β-glucosidase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase). Cependant, ce mycète est repris dans la classe II des pathogènes de l'Homme selon l'Institut Scientifique de Santé Publique. Un microorganisme de classe II est susceptible de provoquer une maladie chez l'Homme, mais toute transmission dans la collectivité est improbable, à l'inverse des microorganismes de classe III. La moisissure *A. fumigatus* doit donc être manipulée avec précaution. Certaines espèces du genre *Pseudomonas* sont aussi pathogènes de l'Homme : *P. aeruginosa* (classe II), *P. mallei* (classe III) et *P. pseudomallei* (classe III). L'utilisation de la souche *Pseudomonas* aurait nécessité une identification génétique si elle avait présenté des activités enzymatiques intéressantes (endoglucanase ou xylanase). La souche de *Streptomyces* ne produit que de l'amylase. Le bacille dégradant le xylane a donc été désigné comme le microorganisme le plus prometteur et a été étudié plus en détail dans le **Chapitre IV**.

VIII.3. Étude du bacille xylanolytique *Bacillus subtilis* ABGx

Parmi les souches isolées et présentées dans le **Chapitre III**, la bactérie *Bacillus subtilis* ABGx est celle qui présente le plus d'intérêt dans le cadre de ce travail. En effet, elle présente une activité enzymatique très intéressante du point de vue industriel : la xylanase. Les bactéries du genre *Bacillus* présentent de nombreuses propriétés intéressantes. Les bacilles produisent la plupart des protéases commerciales, actives à des pH neutres ou alcalins dans la majorité des cas, mais généralement caractérisées par une résistance à la température assez faible [12]. Ils produisent également de nombreuses autres enzymes, dont les cellulases et les hémicellulases. *B. arseniciselenatis*, *B. cereus*, *B. circulans*, *B. halodurans*, *B. licheniformis*, *B. mojavensis*, *B. polymixa*, *B. pumilus*, *B. stearothermophilus*, *B. subtilis*, *B. thermantarcticus* et *B. trypoxylicola* ont été rapportés comme producteurs de xylanases (voir **Chapitre IV**). En plus des enzymes, ils sécrètent diverses molécules telles que des antibiotiques, des molécules exploitables dans le secteur alimentaire ainsi que des insecticides [13]. Dans le cas de *Bacillus subtilis*, on a démontré l'existence de plus de 24 antibiotiques de structures très diversifiées. Parmi ceux-ci, les lipopeptides, composés d'acides aminés et d'acides gras combinés, sont très courants chez les bacilles et ont de nombreuses applications [14]. Enfin, ces bactéries montrent des propriétés de biodégradation intéressantes ; des souches ont démontré une activité sur certains polymères industriels tels que l'acide poly-

lactique, le PHBV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate) et le polyuréthane [15]. Ces différentes propriétés caractérisant les bacilles en font des microorganismes bien étudiés.

Dans le cas présent, la souche *Bacillus subtilis* ABGx produit de la xylanase (endo-1,4-β-D-xylanase) et de l'α-amylase. L'étude s'est portée sur la xylanase, étant donné que l'amylase n'intervient pas dans l'hydrolyse du complexe lignocellulosique. Un dosage des lipopeptides dans le surnageant a également été appliqué au bacille cultivé en milieu riche ; une concentration très faible en surfactine a été détectée. Cette concentration était fortement inférieure aux valeurs rapportées dans la littérature. La souche a montré une activité maximale de type endo-1,4-β-D-xylanase de 44 U.I./ml. Différents substrats lignocellulosiques ont été testés et le son de blé s'est révélé le stimulateur d'activité enzymatique le plus efficace (voir **Chapitre IV**).

Les applications des xylanases sont nombreuses. Tout comme les autres enzymes, elles possèdent une caractéristique fondamentale que n'ont pas les produits chimiques courants : la sélectivité. Les xylanases bactériennes sont souvent préférées aux autres car elles présentent une bonne stabilité à la température et peuvent être utilisées sur de larges gammes de pH. Dans tout procédé, l'enzyme doit être utilisée de manière à obtenir les rendements d'hydrolyse les plus élevés, ce qui implique une optimalisation du temps d'action, de la concentration en enzyme et en substrat, de la température et du pH. Les xylanases sont tout d'abord exploitées comme moyen de bioconversion du xylane en produits à haute valeur ajoutée. Le xylane est hydrolysé, puis les hydrolysats sont utilisés soit comme source de carbone pour les cultures de microorganismes, soit comme composés de base à la synthèse de produits chimiques. Dans ce dernier cas, des étapes de séparation et de purification sont nécessaires. Le xylane hydrolysé peut servir de base à la production de bioéthanol de seconde génération. Des souches microbiennes modifiées ont été créées pour convertir des mélanges de sucres fermentescibles en éthanol, telles que *E. coli* K011, *E. coli* SL40, *E. coli* FBR3, *Zymomonas* CP4 et *Saccharomyces* 1400. La fermentation du xylose provenant de l'hydrolyse des hémicelluloses est en effet problématique. Le xylose, tout comme l'arabinose, fait partie des pentoses. Ceux-ci ne sont pas fermentés par les souches utilisées industriellement sur les hexoses, à savoir principalement *Saccharomyces cerevisiae* et *Zymomonas mobilis*. Les hexoses sont majoritairement le glucose (provenant de l'hydrolyse de la cellulose) et d'autres sucres minoritaires issus des hémicelluloses (fucose, mannose, galactose, rhamnose). Seules les bactéries entériques et certaines levures peuvent fermenter le xylose et l'arabinose en éthanol. Cependant, ces organismes sont sensibles à l'éthanol même à de faibles

concentrations, et les rendements sont moins satisfaisants que ceux qui sont observés sur les autres sucres [16]. A l'heure actuelle, la recherche se focalise sur l'amélioration du rendement en bioéthanol. La fermentation des pentoses est l'une des clés pour atteindre ce but, et le rendement en bioéthanol pourrait théoriquement être doublé [17]. La souche bactérienne recombinante *E. coli* K011 précitée a fourni de très bons résultats sur le glucose et le xylose (103-106% du rendement théorique en éthanol). Le xylose semble aussi avoir eu un effet positif sur la croissance de la souche. Une souche modifiée de *Zymomonas mobilis* a également conduit à des résultats très prometteurs en termes de rendement de la fermentation du glucose et du xylose en bioéthanol, tout comme une version modifiée de la bactérie *Klebsiella planticola* ATCC 33531 [18]. La fermentation du xylose en bioéthanol est donc un domaine en cours de développement.

Le xylitol est issu du xylose et constitue une autre application très courante dans le secteur alimentaire. Cependant, les xylanases trouvent leur principale application dans l'industrie textile. Le traitement enzymatique des fibres est nécessaire pour supprimer les hémicelluloses, considérées comme impuretés et constituant un frein à l'absorption de l'eau lors du procédé industriel du filage. L'industrie papetière est aussi demandeuse de xylanases, nécessaires à la purification de la cellulose, constituant principal du papier. Dans ces conditions, les xylanases ne doivent en aucun cas être accompagnées d'activités cellulase interférentes, susceptibles de provoquer une diminution de la qualité de la pulpe. Le pré-blanchiment enzymatique de cette pulpe améliore l'efficacité des opérations suivantes du procédé. Les xylanases trouvent une application supplémentaire dans le recyclage du papier. Celui-ci s'effectue en deux étapes : la réduction sous forme de pulpe et le raffinage. La xylanase intervient sur la pulpe, et sert à dégrader les hémicelluloses restantes après la fabrication du papier. De cette manière, les fibres de cellulose se désolidarisent beaucoup plus facilement, ce qui facilite le recyclage. Par la suite, l'enzyme est désactivée par une hausse de la température. L'alimentation animale constitue une autre application des xylanases qui augmentent la valeur nutritionnelle des aliments. A ces utilisations principales peuvent être ajoutés les secteurs suivants : la boulangerie (amélioration de la tenue de la pâte à pain), la clarification des jus sucrés, les détergents, etc. [19] Toutes ces applications sont des valorisations potentielles pour la présente xylanase de *Bacillus subtilis* ABGx.

Le **Tableau 1** présenté dans le **Chapitre I** (p.5) contient les caractéristiques de quelques xylanases utilisées à l'échelle industrielle (données provenant des fournisseurs). La tendance générale qui apparaît est que ces enzymes possèdent des activités relativement

variables, de 5.10^3 à 2.10^6 UI/g. Il est nécessaire de rappeler qu'une unité d'activité xylanase est définie comme la quantité d'enzyme capable de produire 1 µmol de xylose par minute, mais les conditions opératoires varient d'une expérience à l'autre, et il n'existe actuellement pas de standardisation des méthodes de mesure. L'enzyme du bacille isolé dans le cadre de ce travail fournit une activité de 1800 UI/g, mais cette valeur est calculée sur la matière sèche totale contenue dans le surnageant de la culture. Il est impossible de calculer l'activité de la xylanase de *B. subtilis* ABGx par gramme d'enzyme purifiée. Par ailleurs, la purification est très coûteuse d'un point de vue industriel.

De manière globale, les moisissures sont les principaux producteurs d'enzymes industrielles en raison de leur excellente capacité à produire des protéines extracellulaires [20]. Le **Tableau 1** du **Chapitre I** montre une tendance générale : les xylanases industrielles ont souvent des pH optimaux acides ou proches de la neutralité. Or, les xylanases alcalines possèdent un avantage considérable. A pH alcalin, le xylane est hautement soluble, ce qui facilite considérablement l'action des xylanases. Cette considération explique l'intérêt de la mise en œuvre des xylanases alcalines dans le monde industriel [21]. Les enzymes provenant des mycètes montrent dans la plupart des cas des pH optimaux acides, conditions dans lesquelles l'accessibilité du xylane est mauvaise [22]. Les bactéries, elles, sont plus couramment capables de sécréter des enzymes alcalines, ce qui est le cas de la souche isolée dans la cadre de ce travail (conditions optimales de pH : 8-10, voir **Chapitre IV**). La bactérie *Bacillus subtilis* ABGx possède un pH optimal alcalin, ce qui constitue un avantage par rapport aux enzymes industrielles courantes. La tenue à la température de l'enzyme est aussi satisfaisante, la température optimale étant de 50^oC, bien que l'enzyme conserve 90% de son activité maximale à 70^oC. Le paramètre à améliorer est la production de l'enzyme elle-même. Le caractère extracellulaire de la xylanase a pu être montré par l'analyse de la séquence ADN, mettant en évidence une séquence de peptide signal (voir **Chapitre IV**). Les techniques actuelles de biotechnologie rapportent par ailleurs de nombreux cas d'insertion de gènes codant pour des enzymes dans des souches réceptrices dont le but est de produire l'enzyme en quantités supérieures. A titre d'exemples, des études antérieures rapportent la transformation d'une souche d'*E. coli* par une xylanase de *B. subtilis* [23], la transformation d'une souche de *B. subtilis* par une xylanase de *Bacillus* sp. [24], la transformation de souches d'*E. coli* et *Streptomyces lividans* par une xylanase de *Thermomonospora fusca* [25], ou encore la transformation de *B. subtilis* par une xylanase de *Clostridium thermocellum* [26]. Cette voie

constitue un moyen d'amélioration de la production de l'enzyme dans le cas de *Bacillus subtilis* ABGx.

VIII.4. Étude et isolement des protistes du termite *R. flavipes* (ex. *santonensis*)

Les protistes des termites inférieurs sont les microorganismes les plus difficiles à obtenir en culture pure à partir du contenu intestinal. Il n'existe en réalité que 3 espèces de protistes ayant pu être isolées et cultivées en conditions in vitro : *Trichomitopsis termopsidis*, *Trichonympha spaerica* et *Trichomitus trypanoides*. Le protiste *Trichomitus trypanoides*, appartenant à l'ordre des Trichomonades, a été cultivé en conditions anaérobies dans un milieu liquide composé d'extrait de levure, de vitamines et de minéraux. Il a été isolé du termite *Reticulitermes santonensis*. Des bactéries vivantes, également présentes dans le milieu de culture, étaient consommées par le protiste et utilisées comme source nutritive [27]. Le cas de *Trichomitopsis termopsidis*, isolé du termite *Zootermopsis angusticollis*, est particulièrement intéressant. Ce protiste possède une population méthanogène endo-symbiotique. Du bromo-éthane-sulfonate a été utilisé pour supprimer cette microflore, agissant comme répresseur de la méthanogenèse. Dans ces conditions, le protiste a montré un taux de croissance réduit d'un facteur 8 par rapport à la situation initiale. En revanche, l'introduction de bactéries tuées (*Bacteroides* sp.) a permis de réobtenir un taux de croissance normal [28]. D'autre part, la culture de ce protiste requiert, en plus de bactéries, de la cellulose, le bois ne permettant pas d'atteindre un développement satisfaisant. Cette information permet de mettre en évidence l'importance de la forme du substrat. Il a aussi été montré que les bactéries utilisées comme source de nutriments ont plus d'efficacité si elles proviennent du termite. Les cellules bactériennes semblent apporter aux protistes les acides aminés qu'ils sont incapables de synthétiser eux-mêmes. Le protiste *T. termopsidis* a montré des activités enzymatiques de type endo-1,4-β-D-glucanase et β-glucosidase, mais le xylane, l'amidon et les protéines ont aussi pu être dégradés par les enzymes du protiste [29]. Le cas de *Trichonympha sphaerica* est différent. Ce protiste, classé dans les Hypermastigides, a pu être obtenu en culture pure. Il a pu être démontré que ce protiste n'abrite pas de bactéries endogènes et montre une activité enzymatique sur la cellulose [30]. Les cas de succès d'isolement de protistes à partir des termites sont très rares.

Les protistes flagellés qui se développent au sein du tube digestif des termites inférieurs ont tendance à se concentrer dans la partie centrale. Cette zone est anaérobie et contient la concentration en hydrogène la plus élevée. Les protistes y phagocytent les bactéries ainsi que les particules de bois ingérées par le termite, et ils constituent eux-mêmes

un habitat spécifique pour des bactéries ecto- et endo-symbiotiques [11]. Ces conditions expliquent en partie la difficulté d'isolement de ces microorganismes. De nombreux protistes sont liés à des bactéries à travers des relations symbiotiques, et toute tentative d'élimination de ces bactéries afin d'obtenir des cultures pures est susceptible de briser les symbioses, empêchant ainsi la survie du protiste. Cette observation limite considérablement les possibilités d'isolement. Certaines informations sont toutefois connues et acceptées sur cette population eucaryote : les protistes sont capables d'hydrolyser la cellulose en conditions anaérobies, principalement métabolisée en acétate, CO_2 et H_2. L'isolement de protistes demande donc théoriquement des conditions anaérobies [29]. Paradoxalement, il a été montré que certains protistes, comme *Pyrsonympha* sp. dans le hindgut de *Reticulitermes santonensis*, sont fermement fixés aux parois de l'intestin et donc soumis à un flux d'oxygène, mais il s'agit là de cas particuliers [31].

Dans le cadre de cette étude, des isolements ont été tentés en atmosphères aérobies dans un premier temps car ces cultures sont plus faciles à mettre en œuvre que les cultures anaérobies. Trois milieux ont été testés, contenant soit du lait, soit de la cellulose, soit du riz [32-34]. Le milieu contenant du riz (et donc de l'amidon) a été le seul à permettre le développement de protistes, alors que celui contenant de la cellulose microcristalline n'a favorisé aucune croissance. Par la suite, des milieux modifiés contenant du riz additionné de son de blé, de cellulose ou de sciure de bois de sapin ont été testés en conditions aérobies et anaérobies. Les conditions anaérobies ont été obtenues par dégazage des fioles étanches par du dioxyde de carbone ou de l'azote. Le dioxyde de carbone n'a permis le développement d'aucun protiste, probablement à cause d'une acidification du milieu. En effet, le pH de l'intestin du termite *R. flavipes* est globalement proche de la neutralité [35, 36]. Une acidification a donc pu avoir un effet inhibiteur de croissance sur les protistes. En conditions aérobies, les protistes ont pu être observés dans tous les milieux de culture. Cependant, il n'a pas été possible d'entretenir ces cultures assez longtemps pour faire des tentatives d'isolement. Au final, seul le protiste identifié comme *Poterioochromonas* sp., classé dans les chrysophytes, a pu être cultivé et étudié dans un milieu contenant de l'amidon soluble. Les protistes anaérobies théoriquement présents dans le lumen du hindgut n'ont pas pu être cultivés.

Le problème principal qui se pose quant à l'isolement des protistes est la présence de bactéries qui empêchent l'obtention de cultures pures. La littérature propose différentes techniques de sélection : séparation sur base de la taille (filtration) ou de la densité,

enrichissement sélectif, cytométrie en flux, stratégie antibiotique, isolement à la micropipette ou dilution du milieu jusqu'à obtenir le protiste purifié [37]. Dans le cas présent, la stratégie antibiotique a permis de contrôler la contamination bactérienne sous une concentration maximale de 100 CFU/ml par utilisation de ciprofloxacine, dont la concentration a été choisie selon les résultats d'un antibiogramme appliqué aux deux souches bactériennes contaminant les cultures. Une séparation par filtration a aussi été tentée, mais sans succès. En revanche, une tentative de séparation par centrifugation en gradient de densité utilisant du Percoll® a permis une séparation partielle de la communauté bactérienne de celle des protistes identifiés comme *Poterioochromonas* sp. La purification n'a pas été complète. Les autres techniques n'ont pas été testées car la stratégie antibiotique a fourni des résultats satisfaisants.

Sur un plan pratique, les protistes sont probablement les microorganismes les moins exploités en termes de production industrielle. Le domaine le plus développé à ce jour est celui des micro-algues, dont la croissance dépend de la photosynthèse. Le succès des micro-algues dans le monde industriel est dû à plusieurs caractéristiques économiquement attractives : elles utilisent la lumière et le CO_2 pour croître, produisent des composés spécifiques (protéines, lipides, sucres, polymères et pigments) à hautes concentrations en conditions de stress, possèdent un cycle de division cellulaire relativement rapide et peuvent se développer dans des conditions de température, de salinité et de luminosité très variables. Le dispositif de production le plus courant est un bassin de profondeur comprise entre 0,2 et 0,5m, favorisant la transmission de lumière. Le milieu est homogénéisé constamment, et les conditions de stérilité ne sont pas nécessaires. Les micro-algues sont cultivées industriellement pour produire des caroténoïdes (β-carotène, astaxanthine), des biocarburants, et la biomasse elle-même trouve des applications dans l'aquaculture et le domaine des nutraceutiques [38]. La culture est relativement aisée et ne demande pas d'investissement particulièrement lourd. Cette caractéristique est liée à la croissance photosynthétique qui ne requiert pas un milieu de culture riche en nutriments, ce qui limite les problèmes de contamination bactérienne. Dans le cas des protistes de l'intestin du termite, la culture s'est avérée très difficile à mettre en œuvre. La meilleure solution applicable aux protistes de termites est donc vraisemblablement l'isolement des séquences génétiques codant pour les cellulases et hémicellulases. L'article de Tartar et al. [39] a notamment identifié des séquences de cellulases et d'hémicellulases de protistes à partir d'une banque génomique d'ADN issu du tube digestif de *R. flavipes*. La transformation de souches bactériennes par ces séquences est certainement la voie la plus prometteuse, évitant de résoudre les difficultés

inhérentes aux cultures de protistes. Il faut cependant noter que nos travaux ont permis de cultiver *Poterioochromonas* sp., que l'on peut ajouter à la liste des rares cas de protistes de termites cultivés à ce jour.

VIII.5. Étude des moisissures *Sarocladium kiliense* et *Trichoderma virens*

Les moisissures *S. kiliense* CTGxxyl et *T. virens* CTGxAviL ont montré des activités enzymatiques relativement diversifiées : α-amylase, β-glucosidase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase et FP-cellulase. Les levures et les moisissures possèdent de nombreuses applications dues à leur immense capacité à produire des molécules très diversifiées. Les moisissures sont en réalité les microorganismes les plus utilisés en termes de production d'enzymes, qu'il s'agisse ou non de souches recombinantes. Les moisissures regroupent 60% des microorganismes utilisés comme hôtes dans le domaine des producteurs d'enzymes, contre 27% pour les bactéries (recombinantes ou non) et 5% pour les levures (recombinantes ou non). Au total, 60% des enzymes industrielles proviennent des moisissures dont les principaux représentants appartiennent aux genres *Aspergillus* (27%), *Trichoderma* (6%), *Penicillium* (6%), *Rhizopus* (5%) et *Humicola* (2%). Les enzymes des moisissures trouvent des applications dans des domaines très diversifiés : l'industrie textile, le travail du cuir, l'industrie papetière, l'alimentation animale, le bioéthanol, la synthèse organique, les édulcorants, la boulangerie, les produits laitiers, l'hydrolyse des protéines, la brasserie, la distillerie, la fabrication des vins, des jus de fruits et la modification des lipides [40]. Les levures et moisissures possèdent aussi des aptitudes à la fermentation et à la production d'antibiotiques ; les levures fournissent également des extraits riches en vitamines et en minéraux [41].

Dans le cadre d'une exploitation industrielle, les moisissures ne peuvent en aucun cas avoir d'effet pathogène sur l'Homme, les animaux ou les plantes. En ce qui concerne les effets potentiellement négatifs sur l'être humain, l'Institut Scientifique de Santé Publique ne considère pas les genres *Sarocladium* (identique à *Acremonium*) et *Trichoderma* comme pathogènes, à l'inverse d'*Aspergillus fumigatus*. Du point de vue phytopathogène, le genre *Trichoderma* n'a pas d'effet négatif sur les plantes ; il a même montré des effets anti-pathogènes : la moisissure peut pénétrer les cellules des microorganismes phytopathogènes, entrer en compétition avec eux et sécrèter des antibiotiques empêchant leur développement [42]. Le cas de *Sarocladium* (*Acremonium*) *kiliense* est plus problématique. La moisissure *Acremonium cucurbitacearum* est reconnue comme étant phytopathogène, et la phytopathogénicité de *Sarocladium zeae* a déjà été démontrée. Le caractère phytopathogène

potentiel de *Sarocladium strictum* et *Sarocladium kiliense* a aussi déjà été proposé [43]. Par ailleurs, il a aussi été montré que la souche *S. kiliense* Samif11 a une forte activité antibactérienne et antifongique [44]. Les moisissures appartenant au genre *Sarocladium* ont donc une connotation très variable selon l'espèce. *S. oryzae* produit des antibiotiques tout comme *S. kiliense*, *S. strictum* est pathogène du sorgho et du fraisier et *S. zeae* est un endophyte mutualiste du maïs [45]. La phytopathogénicité de *S. kiliense* est donc encore à démontrer. Bien que cette moisissure ne soit pas reconnue comme pathogène de l'Homme au niveau légal belge, des études ont déjà rapporté des cas de dysfonction rénale, de péritonite, de cirrhose ou encore d'hypertension dues à *Acremonium kiliense* [46]. Par conséquent, l'usage de cette moisissure à l'échelle industrielle pourrait constituer un danger. Sur un plan légal, il est aussi probable que le statut de *S. kiliense* change suite à des études futures montrant le caractère pathogène de ce microorganisme. Une solution possible serait alors d'isoler les gènes codant pour les xylanases et les cellulases et de transformer une souche bactérienne receveuse, comme proposé au point VIII.3.

Les applications possibles des xylanases ont été présentées au point VIII.3., et l'intérêt pour les souches productrices de xylanases alcalines avait été souligné en raison de l'augmentation de la solubilité du xylane en pH alcalin [21]. La xylanase produite par *S. kiliense* CTGxxyl a montré un pH optimal de 8-10, l'enzyme tolère mieux les pH alcalins à plus long terme que les pH acides (voir **Chapitre VI**). Cette enzyme a montré une température optimale de 60^{o}C, bien que plus de 80% de cette activité soit conservée à plus basse température. Les températures supérieures ont un effet plus inhibiteur, une température de 70^{o}C faisant chuter l'activité à moins de 60% de la valeur maximale. En revanche, l'enzyme peut conserver son activité enzymatique originale presque inchangée après un chauffage de 30 minutes à une température allant jusqu'à 80^{o}C. Si l'on se réfère à quelques xylanases produites à l'échelle industrielle (voir **Tableau 1**, **Chapitre I**, p.5), il apparaît que l'enzyme produite par *S. kiliense* CTGxxyl possède les caractéristiques requises au niveau des conditions de travail en termes de pH et de température. Son avantage est d'hydrolyser plus favorablement à pH alcalin, condition dans laquelle le xylane est plus soluble, ce qui améliore le rendement de l'hydrolyse. Le cas de la xylanase produite par *T. virens* CTGxAviL est différent. L'enzyme montre un pH optimal acide (5-6), et tolère aussi des conditions de pH assez étendues (plus de 80% d'activité conservée après 4 heures à un pH de 4 à 10,8). L'enzyme se rapproche donc plus des xylanases industrielles courantes que celle de *S. kiliense* CTGxxyl. La tenue de l'enzyme à la température est peu satisfaisante et décroît fortement

après 30 minutes à 50°C. La température optimale de l'enzyme a été observée à 40-50°C, ce qui est en accord avec la plupart des xylanases industrielles.

Les cellulases trouvent aussi de nombreuses applications industrielles. La revue bibliographique de Kuhad et al. [47] peut être consultée pour plus d'informations. Les cellulases sont généralement identifiées chez les mycètes, les bactéries et les actinomycètes. L'industrie papetière exploite les cellulases pour produire la pulpe à partir du bois, procédé nettement plus économique que la méthode mécanique consommant beaucoup d'énergie. L'industrie textile est aussi demandeuse de cellulases. Les cellulases acides améliorent la tenue des fibres, et permettent d'obtenir des surfaces textiles plus nettes. La fabrication du bioéthanol de seconde génération est aussi basée en grande partie sur l'usage de cellulases qui hydrolysent le complexe lignocellulosique. La brasserie et la fabrication du vin font également appel aux cellulases. Les enzymes permettent dans ce cas d'améliorer la fermentation des sucres en éthanol. L'industrie alimentaire exploite les cellulases pour faciliter l'extraction des jus de fruits. L'alimentation animale requiert des cellulases pour augmenter la digestibilité des aliments. Certaines cellulases sont aussi utilisées comme moyens de lutte contre les phytopathogènes en agriculture. Il a aussi été démontré qu'elles peuvent avoir un effet bénéfique sur la qualité du sol. A ces applications peuvent être ajoutées l'industrie extractive de l'huile d'olive, des caroténoïdes, l'industrie des détergents et le traitement des déchets [47]. Le **Tableau 2** présenté dans le **Chapitre I** (p.6) montre quelques exemples de cellulases produites à l'échelle industrielle ainsi que leurs caractéristiques et applications mentionnées par les fournisseurs.

Une unité d'activité cellulase est définie comme la quantité d'enzyme capable de fournir 1 µmol de glucose en une minute. Toutefois, comme dans le cas des xylanases, il n'existe pas de standardisation des méthodes de mesure, et les valeurs fournies doivent être considérées avec précaution. Les cellulases présentées dans le **Tableau 2** (**Chapitre I**, p.6) montrent toutes des pH optimaux acides. Toutefois, les cellulases alcalines sont aujourd'hui recherchées, surtout au niveau de l'industrie textile. Durant la fabrication du jean, le polissage et le lavage ont tendance à décolorer le tissu. Cette tendance est fortement réduite à pH neutre et surtout alcalin, d'où l'intérêt pour des enzymes alcalines. De telles enzymes sont aussi beaucoup moins agressives que les cellulases acides sur les fibres de coton [48]. Les cellulases produites par *S. kiliense* CTGxxyl et *T. virens* CTGxAviL possèdent toutes les deux un pH optimal alcalin, respectivement de 10 et 9. Ces cellulases tolèrent également des pH alcalins plutôt qu'acides. La cellulase de *S. kiliense* CTGxxyl conserve plus de 70% de son

activité après avoir passé 4 heures à pH 10.8, alors que la valeur chute à moins de 5% quand le pH est fixé à 4. La cellulase de *T. virens* CTGxAviL, en revanche, ne supporte pas un pH de 10.8 à long terme, avec une activité résiduelle inférieure à 5% de la valeur maximale. Les cellulases des deux moisissures devraient donc être employées dans des procédés industriels ou les pH modérément alcalins sont requis. Les enzymes ont aussi montré une température optimale de 70^{o}C, mais cette température provoque une dénaturation rapide de l'enzyme même après 30 minutes. La cellulase de *S. kiliense* CTGxxyl ne conserve que moins de 40% de son activité originale, et celle de *T. virens* CTGxAviL n'en conserve que moins de 20%. Toutefois, les gammes de températures correspondent à celles des enzymes industrielles décrites dans le **Tableau 2** (**Chapitre I**, p.6). Les cellulases produites par les deux moisissures présentent donc des caractéristiques avantageuses du point de vue industriel, avec la particularité d'être actives à des pH non conventionnels.

VIII.6. Étude des communautés microbiennes sur base de diètes artificielles

Les résultats obtenus et présentés au **Chapitre VII** ont clairement montré une modification de la microflore du termite *R. flavipes* sous l'effet de diètes artificielles. Une étude avait été menée sur le termite *Coptotermes formosanus* et avait abouti aux mêmes conclusions [49]. Dans cette étude, le termite avait été soumis à différentes diètes (bois, cellulose, cellobiose ou glucose) et la microflore eucaryote et bactérienne avait été étudiée. Il est apparu que les populations des protistes *Pseudotrichonympha grassi*, *Holomastigotoides hartmanni* et *Spirotrichonympha leidyi* ont subi une forte diminution suite aux diètes à sources de carbone de bas poids moléculaires, suggérant ainsi l'importance des protistes dans la dégradation de la cellulose. Les communautés bactériennes se sont aussi révélées très différentes d'une diète à l'autre [49]. Les travaux menés par Bauwens et al. [50] ont aussi abouti à des conclusions bien claires. Cette étude a soumis des termites de l'espèce *Reticulitermes flavipes* à deux diètes composées de cellulose cristalline seule ou additionnée de lignine. La lignine a eu un effet positif sur certains protistes (*Trichonympha agilis*) et négatif sur d'autres (*Pyrsonympha major* et *Pyrsonympha vertens*). Il a aussi été possible de montrer une modification significative des activités enzymatiques globales dans les différents compartiments digestifs : les glandes salivaires et le foregut, le midgut et le hindgut (parois et lumen). L'effet sur la microflore bactérienne a aussi été mis en évidence [50]. Dans le cadre du présent travail, l'effet de la lignine sur *Trichonympha agilis* a été confirmé. L'hémicellulose s'est aussi avérée sélective des protistes de petite taille. Ces différentes observations suggèrent qu'il pourrait être possible de cibler certaines communautés de

protistes sur base des diètes artificielles. L'isolement des protistes dégradant la cellulose et le xylane pourrait être facilité par l'administration de diètes spécifiques aux termites avant la dissection, et préalablement à l'enrichissement en cultures spécifiques.

L'analyse des communautés microbiennes par les microplaques BIOLOG a fourni de très nombreuses informations. L'avantage de ce système est qu'il fournit des données sur les activités enzymatiques développées par les différents membres des consortia microbiens. En effet, une source de carbone présente dans un puits va stimuler la croissance de la ou des souches microbiennes capables de la dégrader. De cette manière, il est possible d'avoir une vue très générale des potentialités de la microflore intestinale du termite. Le puits de la microplaque peut donc être considéré comme un micromilieu d'enrichissement. On pourrait envisager d'y prélever des échantillons pour caractériser les microorganismes sélectionnés, tout en gardant en mémoire que le développement des protistes n'y est pas possible en raison des conditions de culture très sévères. La production de β-glucosidase a notamment pu être mise en évidence au vu de la dégradation du β-méthyl-D-glucoside et du cellobiose. Ces substrats sont bien évidemment indicateurs de la dégradation de la cellulose. Toutefois, la dégradation d'autres molécules est liée à de nombreux types d'applications. La γ-lactone d'acide D-galactonique est une lactone qui requiert la présence de lactonases pour être hydrolysée. Ces enzymes dégradant les lactones trouvent notamment des applications dans le secteur des biotechnologies [51]. La dégradation de l'acide hydroxybenzoïque, présent sous forme d'acides 2- et 4-hydroxybenzoïques dans les microplaques, est un autre domaine de recherche. La dégradation de tels composés aromatiques est recherchée dans le secteur de la dépollution des effluents [52]. Un autre exemple est celui des cyclodextrinases qui catalysent l'hydrolyse des cyclodextrines. Ces enzymes trouvent notamment des applications dans les secteurs alimentaire, cosmétique et pharmaceutique en tant qu'agents de stabilisation, de solubilisation ou de libération contrôlée de molécules spécifiques [53]. Ces exemples caractérisent la portée de l'utilisation des microplaques BIOLOG, et la possibilité d'y enrichir des souches particulières apparaît très intéressante et innovante.

Ce travail a pu mettre en évidence la forte différence entre les résultats d'identification génomique et protéomique. La présence de séquences spécifiques de microorganismes est un indicateur de leur présence, mais les données apportées par la protéomique apportent des informations complémentaires. L'expression des protéines des microorganismes dépend de nombreux facteurs, et ceux-ci varient selon le type d'organisme considéré. Pour rappel, la synthèse protéique repose tout d'abord sur la transcription de l'ADN en ARN messager, qui

va lui-même servir de base à la traduction en séquence d'acides aminés dont l'enchaînement définit la protéine. A titre d'exemple, chez les bacilles (notamment *Bacillus subtilis* dont une souche xylanolytique a été présentée au **Chapitre IV**), les ARN-polymérases qui procèdent à la transcription sont composées de facteurs σ et des ARN-polymérases proprement dites. La transcription est initiée quand il y a interaction entre les séquences -35 et -10 des promoteurs des gènes et les facteurs σ. Ces facteurs σ sont très diversifiés (σ^A, σ^B, σ^D, σ^E, etc.), et chacun interagit avec une séquence bien spécifique. Certaines conditions peuvent être nécessaires pour activer les promoteurs. Par exemple, les facteurs σ^B sont liés aux conditions de stress, les facteurs σ^W sont liés aux chocs alcalins, les facteurs σ^M sont liés au stress osmotique, etc. L'activité des facteurs σ peut aussi dépendre de l'expression et de la stabilité de facteurs anti-σ, voire de facteurs anti-anti-σ. Certaines kinases interviennent également dans l'activation des gènes par l'intermédiaire de transferts de phosphates dépendant des conditions environnementales. Différents systèmes à deux composantes ont aussi été identifiés chez les bacilles, tels que les systèmes DegSU, CssRS, YccGF, etc. A ces différents systèmes viennent s'ajouter les régulateurs de transcription spécifiques qui s'appliquent à des gènes ciblés. De plus, les protéines extracellulaires, telles que les enzymes excrétées, nécessitent en plus de traverser la paroi cellulaire à l'aide de transporteurs membranaires [54]. Tous ces systèmes régulateurs rendent compte de la complexité des facteurs qui influencent l'expression des protéines. Le cas des moisissures est aussi très complexe. Chez les moisissures *Aspergillus* et *Trichoderma*, la production des cellulases, hémicellulases et amylases est soumise à la répression catabolique des sucres solubles, sous l'effet du gène CreI/CreA. La nature des agents inducteurs et répresseurs est variable selon la moisissure. Par exemple, les cellulases sont produites par *T. reesei* en présence de cellulose, de cellobiose, de lactose ou de sophorose. Chez *Aspergillus*, les amylases sont produites en présence d'amidon, de maltose, d'isomaltose et d'autres disaccharides [55]. Cette constatation permet une fois encore de démontrer la complexité de l'expression des protéines chez des organismes différents. Il est aussi nécessaire de considérer les interactions entre les différents microorganismes. En effet, l'action des protéines extracellulaires produites par une souche spécifique peut avoir une influence directe sur d'autres souches. La protéomique est donc riche en informations mais aussi très complexe à traiter.

Les diètes artificielles ont permis de modifier significativement les microflores bactériennes mais ont aussi favorisé le développement de certaines moisissures. Les diètes artificielles ont eu un effet d'enrichissement spécifique sur les souches microbiennes

initialement présentes. Le **Chapitre VII** a montré que des souches spécifiques ont pu être isolées selon les diètes : la bactérie *Chryseobacterium* sp. XAvLW a été extraite des microflores issues des diètes artificielles cellulose cristalline+lignine, xylane et sciure de bois ; la moisissure *Trichoderma virens* CTGxAviL a été extraite des microflores issues des diètes cellulose cristalline (avec ou sans lignine) ; la moisissure *Sarocladium kiliense* CTGxxyl a été extraite de la microflore issue de la diète xylane ; la moisissure *Fusarium solani* AvL a été extraite de la microflore issue de la diète cellulose cristalline (avec ou sans lignine). Les propriétés des moisissures *Sarocladium kiliense* CTGxxyl et *Trichoderma virens* CTGxAviL ont été développées dans le **Chapitre VI**. Le choix a été fait d'écarter la moisissure *Fusarium solani* AvL en raison de sa phytopathogénicité, bien qu'elle ait montré les mêmes activités enzymatiques que les deux autres moisissures. La moisissure *Fusarium* est phytopathogène et produit diverses mycotoxines telles que les trichothécènes, la zéaralénone, la fumonisine, la moniliformine et l'acide fusarique. La moisissure peut se développer sur les céréales, les fourrages et la nourriture animale de manière générale. Des cas de mycotoxicose ont été observés en Europe, en Asie, en Nouvelle-Zélande et en Amérique du Sud. Les mycotoxines produites par la moisissure peuvent avoir des effets néfastes sur les porcs, les volailles ou encore les chevaux. La revue bibliographique de D'Mello et al. [56] peut être consultée pour plus d'informations. Au vu des effets négatifs de la moisissure sur les plantes et les animaux, une étude plus poussée de la souche *Fusarium solani* AvL devrait probablement faire appel à l'extraction des gènes codant pour les cellulases et les xylanases suivie de la transformation de souches receveuses inoffensives. La bactérie *Chryseobacterium* sp. XAvLW a elle aussi montré des activités enzymatiques intéressantes (α-amylase, β-glucosidase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, filter paper-cellulase). Certaines souches du même genre ont par ailleurs déjà montré d'autres activités enzymatiques. A titre d'exemple, *Chryseobacterium* sp. kr6 sécrète de la kératinase, enzyme exploitable dans le secteur de l'épilage des peaux ou de l'hydrolyse des plumes de volailles trouvant une application dans l'alimentation animale [57]. La recherche d'autres activités enzymatiques sur la souche *Chryseobacterium* sp. XAvLW pourrait donc être intéressante. Pour terminer, les autres moisissures isolées et ayant fourni des réponses positives aux tests enzymatiques qualitatifs ont aussi produit des xylanases et des cellulases. Ces souches demandent donc une investigation plus poussée et une détermination quantitative des activités enzymatiques. En effet, il avait été décidé de se focaliser sur les moisissures montrant les activités les plus diversifiées (*Sarocladium kiliense* CTGxxyl et *Trichoderma*

virens CTGxAviL), mais il est possible que les autres souches non identifiées produisent des enzymes en quantités appréciables.

VIII.7. Combinaison des approches microbiologique, génomique, protéomique et métabolomique appliquées au termite *R. flavipes* (ex. *santonensis*)

Ce travail a été effectué dans le cadre du projet ARC Termitofuel (Gembloux Agro-Bio Tech no. ARC 08-13/02) et mené selon des approches différentes et complémentaires. Les différentes manipulations réalisées durant le projet ont abouti à des résultats très diversifiés. Ces travaux ont été menés selon les approches suivantes : microbiologique, génomique, protéomique et métabolomique.

L'approche microbiologique a permis d'isoler des microorganismes directement à partir de leur milieu. Il a été possible d'extraire des souches de l'intestin du termite, de les cultiver et de les faire produire des enzymes dans des milieux de culture adaptés. Cette approche a mené à l'isolement de tous les microorganismes présentés dans ce travail. La difficulté de cette méthode est la reproduction des conditions régnant dans le tube digestif des termites dont la complexité a été mentionnée de nombreuses fois. Par conséquent, il apparaît inévitable de perdre une partie de la microflore d'origine lorsqu'on tente de réaliser un enrichissement suivi d'un isolement. Le **Chapitre III** a notamment montré la difficulté d'isoler des souches microbiennes anaérobies. Cette observation est paradoxale étant donné que la majeure partie de la population des protistes vit principalement dans la zone anaérobie de l'intestin des termites. L'approche microbiologique de la problématique a donc ses limites. Elle a toutefois permis d'isoler des souches intéressantes du point de vue enzymatique, telles que *Bacillus subtilis* ABGx et *Pseudomonas* sp. ABGxCellA.

Le travail réalisé par Mattéotti et al. [58] a utilisé une méthode différente, consistant à construire en premier lieu une banque d'ADN génomique à partir d'isolats obtenus sur boîtes de Pétri. L'ADN génomique a ensuite été digéré enzymatiquement avant d'être inséré dans une souche d'*E. coli*, puis les bactéries transformées ont été soumises à des tests enzymatiques. De cette manière, une β-glucosidase a été mise en évidence, montrant un pH optimal de 6 et une température optimale de 40^{o}C. La même banque génomique a par la suite fourni 7 β-glucosidases différentes, proches de celles des genres *Klebsiella* et *Enterobacter* [59]. Par la suite, une xylanase a été isolée selon la même méthode, phylogénétiquement associée aux actinobactéries et montrant un pH optimal de 5 et une température optimale de 55^{o}C [60]. Selon l'approche microbiologique, une actinobactérie a aussi pu être isolée

(appartenant au genre *Streptomyces*), mais cette souche était différente car elle n'a montré aucune activité xylanase. La complémentarité des méthodes apparaît ici étant donné que les différents genres bactériens identifiés suite à la création de banques génomiques n'ont pas été retrouvés selon la méthode microbiologique employée dans le présent travail.

La biodiversité relative à la microflore du termite a été mise en évidence par la protéomique. Cette approche a été présentée dans le travail réalisé par Bauwens et al. [61]. Dans cette étude, les protéines ont été étudiées à partir d'échantillons provenant directement de l'intestin de termites disséqués, sans introduire de biais par une mise en culture intermédiaire. Deux approches ont été menées : l'analyse par chromatographie à deux dimensions couplée à un spectromètre de masse (2D-LC-MS/MS) et l'analyse par électrophorèse bidimensionnelle couplée à un analyseur MALDI-TOF (2DE-MALDI-TOF-MS). Ce travail a notamment mis en évidence la difficulté d'interprétation due à un manque d'informations dans les bases de données utilisées comme bases de comparaison. En effet, les bases de données ne sont pas assez documentées. Cette observation concerne surtout les protistes, identifiés majoritairement sur base de peptides issus de tubulines. Les deux techniques employées ont abouti à des résultats très différents. La 2D-LC-MS/MS a permis d'identifier des séquences peptidiques issues des protistes, mais la 2DE-MALDI-TOF-MS a été inefficace dans ce domaine. La microflore procaryote a plutôt été identifiée selon la 2DE-MALDI-TOF-MS, alors que les mycètes ont pu être identifiés par les deux techniques.

La spectrométrie de masse appliquée à l'étude des métabolites entreprise dans l'étude de Brasseur et al. [62] constitue une approche différente. Il ne s'agit pas à proprement parler de métabolomique car cette technique est définie comme l'étude du métabolome par l'analyse quantitative et qualitative des métabolites présents dans un système biologique [63]. Dans le cas présent, des extraits ont été testés sur des substrats commerciaux et les métabolites ont été analysés suite à la réaction, ce qui ne correspond pas exactement à la définition sensu stricto. Cette étude a consisté à tester des extraits d'intestin de termite sur des substrats lignocellulosiques : la carboxyméthylcellulose, la cellulose cristalline et le xylane. Cette étude a permis de fournir une vue d'ensemble des potentialités de la microflore du termite. Des fragments de 3 à 12 unités de glucose ont été observés après l'hydrolyse de la carboxyméthylcellulose ; des fragments de 3 à 12 unités de xylose ont été mis en évidence après l'hydrolyse du xylane. Ces fragments ont prouvé l'existence d'activités enzymatiques de type endo-1,4-β-D-glucanase et endo-1,4-β-D-xylanase présentes dans le contenu intestinal des termites. Toutefois, l'utilisation du contenu intestinal sur de la cellulose cristalline n'a pas

fourni d'oligosaccharides [62]. La spectrométrie de masse a aussi été utilisée dans l'étude de *Bacillus subtilis* ABGx, présenté au **Chapitre IV**, démontrant l'activité endo-1,4-β-D-xylanase développée par le bacille, sur base des xylo-oligosaccharides obtenus [64]. Pour terminer, la méthode a aussi pu démontrer les activités de type endo-1,4-β-D-xylanase développées par les moisissures présentées au **Chapitre VI**.

Un bon exemple montrant la complémentarité des méthodes est sans conteste celui du **Chapitre VII**, présentant une analyse pluridisciplinaire des microflores issues de termites nourris à base de diètes artificielles. L'effet de ces diètes a pu être mis en évidence par les techniques suivantes : la microscopie, les microplaques BIOLOG, l'analyse protéomique et génomique des cultures issues des diètes ainsi que l'isolement de souches microbiennes combiné à la détermination qualitative des activités enzymatiques développées. La microscopie a apporté des informations sur les protistes alors que les microplaques BIOLOG ont apporté des informations sur les activités métaboliques développées par les bactéries et les mycètes. L'analyse génomique des cultures a fourni une vue d'ensemble des microorganismes présents, et l'analyse protéomique a souligné certaines différences. Pour terminer, la culture suivie de l'isolement des souches et du criblage des activités enzymatiques a permis de caractériser des souches très intéressantes, telles que *Sarocladium kiliense* CTGxxyl et *Trichoderma virens* CTGxAviL, faisant l'objet du **Chapitre VI**.

VIII.8. Références

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CHAPITRE IX.

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Conclusions et perspectives

En se focalisant sur le termite *Reticulitermes flavipes* (ex. *santonensis*), ce travail a permis de caractériser de manière précise les potentialités de la microflore en termes de production enzymatique. Il a fourni différentes techniques d'isolement et de caractérisation d'activités enzymatiques exploitables dans des domaines relativement diversifiés. Il a notamment été confronté à la difficulté de l'isolement de souches microbiennes en raison des conditions physico-chimiques très spécifiques au tractus intestinal du termite *R. flavipes*.

De manière très générale, ce travail se compose de deux grandes parties :

- Une première partie focalisée sur la recherche de souches microbiennes productrices d'enzymes et extraites du tractus intestinal du termite à l'état originel,
- Une seconde partie focalisée sur la recherche de souches microbiennes productrices d'enzymes et extraites du tractus intestinal du termite soumis à des diètes artificielles, et dont l'effet modificateur a clairement été démontré.

La première partie du travail a d'abord montré que les souches microbiennes anaérobies vivant au sein du tractus intestinal du termite sont relativement difficiles à isoler, en comparaison aux souches aérobies. Les souches anaérobies sont d'ailleurs caractérisées par un métabolisme plus lent, ce qui les rend moins attractives en termes de production enzymatique. La mise en culture n'est donc pas un moyen adapté à l'étude de tels microorganismes, et il serait plus judicieux dans ce cas d'extraire l'ADN total du contenu intestinal et de cribler ce matériel génétique afin d'en isoler des séquences d'enzymes potentiellement intéressantes.

Le travail s'est par conséquent porté sur la recherche de souches productrices d'enzymes de type aérobie. Cette approche s'est révélée beaucoup plus fructueuse, et a mené à l'isolement d'un bacille producteur de xylanase et d'un protiste, type de microorganisme dont la difficulté d'isolement a été rapportée de nombreuses fois dans le cadre de l'intestin des termites.

Cet aspect de la recherche a mené à une première série d'expérimentations et d'observations intéressantes. Tout d'abord, les conditions d'isolement de souches microbiennes les plus efficaces à partir du tractus intestinal du termite ont pu être investiguées. Cette recherche a présenté un intérêt considérable car elle a conditionné l'ensemble des expérimentations subséquentes. Cette approche a mené à l'isolement de deux microorganismes en particulier qui ont pu être caractérisés.

La première souche, *Bacillus subtilis* ABGx, de type bactérien, a montré une bonne capacité de production d'endo-1,4-β-D-xylanase. L'étude a aussi évalué l'efficacité de différentes sources de carbone en tant qu'inducteurs d'activité hémicellulolytique. A ce titre, les résidus agricoles ont montré un effet inducteur d'activité enzymatique tout à fait satisfaisant. Cette observation est d'autant plus importante que ces sources de carbone sont considérées comme des déchets.

La seconde souche, *Poterioochromas* sp., est le seul protiste qu'il a été possible d'étudier dans le cadre de la présente étude. Les protistes résidant dans le système digestif des termites inférieurs sont caractérisés par une difficulté d'isolement jusqu'ici incontournable au vu du faible nombre de publications disponibles à ce sujet. Le présent travail a montré l'existence et l'isolement d'un protiste produisant une enzyme de type α-amylase. Bien qu'aucune activité de type cellulolytique ou hémicellulolytique n'ait pu être mise en évidence, il s'agit ici de la première observation de la présence de protistes mixotrophes au sein du tractus intestinal du termite, ce qui n'a encore jamais été rapporté dans la littérature. Sur un plan plus pratique, les manipulations réalisées fournissent également une technique d'isolement de protistes basée sur l'exploitation d'antibiogrammes. La méthode utilisée peut être appliquée à la recherche d'autres protistes. Il est encore important de signaler ici que de nombreux protistes vivant dans le tractus intestinal des termites entretiennent des relations symbiotiques avec des bactéries intra- ou extracellulaires. Par conséquent, l'emploi d'antibiotiques est susceptible de rompre des symbioses. Cet aspect ne doit pas être négligé en ce qui concerne la méthode décrite. Par ailleurs, la spectrométrie de masse a été utilisée en tant qu'outil de caractérisation du type d'activité enzymatique, sur base des fragments observés. Cette technique a été appliquée à la bactérie *Bacillus subtilis* ABGx et au chrysophyte *Poterioochromas* sp., et les spectres ont fourni de précieuses informations sur les activités enzymatiques développées par les microorganismes.

La seconde partie du travail a tenté de déterminer l'effet de perturbations alimentaires sur la composition de la microflore endosymbiotique du termite. Cet aspect a permis de mettre en évidence de profondes modifications de la microflore sur base de techniques relativement diversifiées : la microscopie, la métagénomique, la protéomique et l'analyse par microplaques BIOLOG®. Cet effet a été démontré sur les communautés des protistes, des mycètes et des bactéries.

A travers cette étude, il a été possible d'isoler des souches microbiennes intéressantes du point de vue enzymatique. Une souche bactérienne et plusieurs souches de moisissures ont pu être caractérisées. Qu'il s'agisse de la première ou de la seconde partie du travail, les moisissures semblent être les microorganismes les plus prometteurs en termes de production d'enzymes. En effet, la moisissure *Aspergillus fumigatus* ABGxAviA2, isolée du tractus intestinal des termites originaux, a montré des activités de type α-amylase, β-glucosidase, endo-1,4-β-D-glucanase et endo-1,4-β-D-xylanase. Dans la seconde partie du travail, les moisissures *Sarocladium kiliense* CTGxxyl et *Trichoderma virens* CTGxAviL ont montré des activités de type α-amylase, β-glucosidase, endo-1,3-β-D-glucanase, endo-1,4-β-D-glucanase, endo-1,4-β-D-xylanase et Filter Paper-cellulase.

De manière globale, la seconde partie du travail a également fourni de précieuses informations. Tout d'abord, les différentes techniques appliquées ont permis de démontrer l'effet modificateur de l'application de diètes artificielles à la microflore des termites. La profonde perturbation apportée par la modification de régime alimentaire est donc indéniable, qu'il s'agisse des bactéries, des mycètes ou des protistes. Cette partie du travail a clairement démontré la complémentarité des techniques qui ont été utilisées dans le cadre de ces manipulations. D'autre part, l'application de ces diètes « artificielles » a pu favoriser le développement de souches microbiennes « cachées ». Plus précisément, une bactérie et plusieurs moisissures ont été mises en évidence et montré des propriétés enzymatiques exploitables du point de vue de l'hydrolyse des matières lignocellulosiques. L'étude appliquée aux moisissures *Sarocladium kiliense* (*Acremonium kiliense*) CTGxxyl et *Trichoderma virens* CTGxAviL a démontré la complexité qui existe entre les sources de carbone utilisées comme inducteurs d'activité et les activités enzymatiques réellement développées. Cette observation est d'une grande importance dans le domaine de la production d'enzymes. Pour terminer, l'importance des bases de données utilisées dans le cadre de l'analyse protéomique d'échantillons complexes a pu être mise en évidence. Cette conclusion met en avant l'intérêt de vérifier le contenu des bases de données testées, qu'il s'agisse de séquences d'acides aminés ou d'ADN.

Sur un plan plus général, les manipulations effectuées dans le cadre de cette thèse soulèvent certaines perpectives. Tout d'abord, la recherche de souches microbiennes anaérobies n'a pas été concluante par les méthodes utilisées. Une piste d'amélioration serait l'utilisation d'azote moléculaire dans toutes les expériences menées, et un temps plus long serait nécessaire aux microorganismes pour se développer et être isolés. Une autre piste serait

le criblage de l'ADN total contenu dans le tube digestif des termites (soumis ou non à des diètes artificielles). Cette méthode permettrait de considérer à la fois les souches aérobies et anaérobies. Par ailleurs, certaines souches présentées dans ce travail ont montré des activités enzymatiques de type cellulase (β-glucosidase, endo-1,4-β-D-glucanase et filter paper-cellulase) et xylanase (β-xylosidase et endo-1,4-β-D-xylanase). Dans certains cas, d'autres activités ont aussi été détectées : α-amylase et endo-1,3-β-D-glucanase. Ces enzymes possèdent aussi de nombreuses applications industrielles, et leur caractérisation précise n'a pas été entreprise en raison de l'intérêt focalisé sur les cellulases et les xylanases. Ces souches microbiennes constituent donc un intérêt supplémentaire à l'hydrolyse de la cellulose et du xylane, et l'utilisation de milieux spécifiques pour favoriser ces activités enzymatiques constitue une piste de recherche supplémentaire. En ce qui concerne les protistes, de précieuses observations ont pu être retirées des expériences réalisées. Ces microorganismes sont considérés, chez le termite inférieur, comme majoritairement responsables de la dégradation de la cellulose, et leur mise en culture est particulièrement difficile à mettre en œuvre. L'isolement des enzymes sécrétées par de tels microorganismes semble nécessiter l'extraction de leur ADN suivi d'un criblage au niveau des séquences enzymatiques. Cette méthode permettrait de contourner toutes les difficultés posées par la mise en culture. Cette recherche d'enzymes à partir du matériel génétique serait aussi bien applicable aux protistes qu'aux bactéries et aux mycètes non mis en évidence par les techniques utilisées dans le présent travail. Un autre aspect relatif à la recherche et à la production d'enzymes est celui de la transformation de souches microbiennes. Cette approche exploiterait des souches réceptrices de séquences d'enzymes provenant de bactéries, mycètes ou protistes. Une autre perspective est la transformation des souches originales à l'aide d'agents mutagènes. Toutefois, l'utilisation de cette technique sur les protistes paraît difficilement concevable en raison de la fragilité des cellules. Une autre perspective concerne la source de microorganismes étudiée tout au long de cette thèse. L'intérêt de cette étude a été focalisé sur la fraction du hindgut, reconnue comme étant la plus riche portion du tractus intestinal en termes de biodiversité. Toutefois, la recherche de souches microbiennes productrices d'enzymes peut aussi être appliquée aux autres parties : le foregut et le midgut. Une telle recherche permettrait probablement de mettre en évidence d'autres bactéries ou mycètes. Cependant, la recherche de protistes, dont la présence est prépondérante au niveau du hindgut, ne serait certainement pas possible par cette voie.

Les différents résultats obtenus au cours des différentes étapes de ce travail ont permis de présenter le termite comme source indiscutable d'enzymes exploitables dans le domaine de la synthèse des biocarburants de seconde génération. A cette application peuvent aussi s'ajouter d'autres secteurs industriels, exploitant eux aussi les xylanases et les cellulases. Pour conclure, les organismes vivants qui exploitent des symbioses complexes sont une source indéniable de souches microbiennes sécrétant des enzymes exploitables dans des domaines aussi utiles que diversifiés.