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LAI THANH TUNG

PRODUCTION D'ENZYMES CELLULOLYTIQUES ET HEMICELLULOLYTIQUES À PARTIR DE MICROORGANISMES INDUSTRIELS (*BACILLUS SP.*, *TRICHODERMA RESEEI)* ET DE BOUES PAPETIÈRES

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Cette thèse a été dirigée par :

Simon Barnabé, directeur de recherche, PhD	Université du Québec à Trois-Rivières
François Brouillette, codirecteur de recherche, PhD	Université du Québec à Trois-Rivières

Jury d'évaluation de la thèse :

Simon Barnabé, directeur de recherche, PhD	Université du Québec à Trois-Rivières
François Brouillette, codirecteur de recherche, PhD	Université du Québec à Trois-Rivières
Daniel Montplaisir, examinateur interne, PhD	Université du Québec à Trois-Rivières
Marc Sirois, examinateur interne, PhD	Université du Québec à Trois-Rivières
Mathieu Drouin, examinateur externe, PhD	Centre Eau Terre Environnement, INRS

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Summary

Canadian pulp and paper industry is making an effort to transform their conventional pulp & paper mills into integrated forest biorefinery to overcome difficult situation such as the decline of the paper market. In addition, there is an inherently existing - problem of high cost waste management to meet strict environmental regulations that requires the pulp & paper industry to modify conventional technology of waste disposal in terms of recycling biosolids. Bioconversion of processing waste streams into value added products through fermentation of industrial microorganisms could be an economic and friendly environmental approach of waste management that would help the industry to diversify their products. Waste streams of pulp and paper mill such as sludges can be used as fermentation cheap substrate to produce bioproducts for on-site or local uses like cellulolytic enzymes. Moreover, such bioprocesses can be integrated in pulp & paper mill while some equipment's can be upgraded for fermentation. The paper sludges generated from wastewater treatment contain a lot of C, N and P sources and other nutrients. Thus, the paper sludges can be abundant and cheap substrates for production of cellulolytic enzymes at low cost from fungi and bacteria. However, the paper sludges have a complex and variable composition and a high heterogeneity that could be less favorable to microbial cultures in comparison with conventional culture media. Hence, it was required to investigate the feasibility of using of paper sludges for enzyme production and elaborate paper sludge – based culture media.

The aim of the present project is to study the production of cellulolytic and hemicellulolytic enzymes using paper sludge (primary, secondary, mix) as fermentation substrate. The microorganisms studied are bacterial strains of *Bacillus*, namely *B. licheniformis* (*Bl*), *B. pumilus* (*Bp*), and *B. subtilis* (*Bs*) and a fungi strain of *Trichoderma reesei*. Fermentations of these microorganisms in the paper sludges were carried out to evaluate their growth and enzyme production. To make them a better culture media for microbial production, the sludges were modified. Modifications were

consisting of an adjustment of solids concentration, the mixture of primary and secondary sludges at different ratio, an addition of inorganic or organic nitrogen source and a supplementation of lignocellulosic biomass (to induce enzyme production). Finally, enzymatic and hydrolysis tests were performed with the enzymes produced in the paper sludges using different lignocellulosic biomass to evaluate their efficacy to convert cellulose into fermentable sugars.

More precisely, the primary, secondary and mix paper sludges were used i as fermentation media to produce *Bl* ATCC 21424, *Bs IEBC SUB001*, *Bp ATCC 7061* and *T.reesei* RUT C30 ATCC 26921. The primary, secondary and mix sludges supported the growth of *T.reesei*, *Bl* and *Bs* while *Bp* grew well in the secondary and mix sludges. *T.reesei* produced cellulases (carbomethycellulase-CMCase, β-glucosidase) and hemicellulases (xylanse, β-xylosidase) in the secondary and mix sludges. *Bl* and *Bs* were able to produce only cellulases. *Bp* produced xylanases from the secondary sludge mixed with hydrolyzed primary sludge, corn stover and beechwood-extracted xylan. This last was selected for experiment at higher scale in a bioreactor. Fermentations of *Bp* in secondary sludge and bioreactor, supplemented with corn stover or beechwood-extracted xylan, were carried out. Xylanase activity of 37.8 IU/mL obtained in bioreactor fermentation of the secondary sludge with supplement of corn stover was higher than that of 35.5 IU/mL obtained in the secondary sludge with supplement of beechwood-extracted xylan.

This work demonstrated that secondary and mix sludges are good culture media of *Bl*, *Bs*, *Bp* and *T.reesei* for the production of their cellulolytic enzymes. The secondary and mix sludges stimulate the production of *T.reesei* multiple enzymes, which acts in synergistic function on conversion of lignocellulosic biomass in fermentable sugars. The secondary sludge, with supplement of corn stover, gives a higher xylanase activity with *Bp* than the secondary sludge supplemented with commercial xylan. The addition of nitrogen source to influence C/N ratio in the paper sludge is an efficient technique to increase the yield of the microorganisms and their enzyme production.

Résumé

L'industrie des pâtes et papiers envisage de transformer leurs usines classiques en bioraffinerie forestière pour surmonter leur situation difficile qui est entre autre attribuable à la baisse du marché du papier journal. De plus, en raison de coût élevé de la gestion des déchets pour répondre aux normes environnementales, l'industrie a besoin de changer ses modes d'élimination de traitement des déchets en vue de le valoriser. La bioconversion de ses déchets en produits à valeur ajoutée par fermentation de microorganismes industriels serait une approche économique et respectueuse de l'environnement qui pourrait également aider à l'industrie à diversifier ses produits. En effet, les déchets papetiers putrescibles comme les boues d'épuration pourraient être utilisés comme substrat de fermentation pour produire à coût moindre des produits biosourcés utilisables sur place ou localement. De plus, ces bioprocédés pourraient être intégrés dans les papetières en modifiant, de façon mineure, certains de ces équipements pour faire de la fermentation. Les boues papetières générées par le traitement des eaux usées du papetier contiennent beaucoup de nutriments pour supporter la croissance de microorganismes industriels. Elles peuvent dont être un substrat de fermentation abondant et bon marché pour la production à coût moindre d'enzymes de saccharification à partir des moisissures ou de bactéries. Cependant, les boues papetières ont une composition complexe et variable qui est peu favorable aux cultures microbiennes en comparaison avec des milieux de culture conventionnels. Il était donc nécessaire de vérifier la faisabilité d'utiliser des boues papetières pour la production d'enzymes cellulolytiques et déterminer des techniques pour en faire d'excellents milieux de culture.

Le présent projet avait pour but d'étudier la production microbienne d'enzymes cellulolytiques et hemicellulolytiques en utilisant les boues papetières (primaires, secondaires et mixtes) comme matière première. Les microorganismes étudiés étaient des souches bactériennes de *Bacillus sp.* dont *B. licheniformis* (*Bl*), *B. pumilus* (*Bp*), et *B. subtilis* (*Bs*) et une souche fongique de *Trichoderma reesei*. Des fermentations de ces souches dans les boues papetières ont été effectuées pour évaluer leur croissance et leur

production d'enzymes. Les boues papetières ont été modifiées pour en faire de meilleurs milieux de culture. Ces modifications consistaient en un ajustement de la concentration en solides, un mélange des boues primaires et secondaires à différents ratios, un ajout d'azote organique et inorganique et un supplément de biomasse lignocellulosique (pour induire la production d'enzymes). Finalement, des tests enzymatiques des enzymes produites à partir des boues papetières ont été réalisés pour évaluer leur efficacité à convertir la cellulose en sucres fermentescibles.

Les boues papetières primaires, secondaires et mixtes ont été utilisées dans ce travail comme milieu de fermentation pour produire les souches suivantes : *Bl* ATCC 21424, *Bs IEBC SUB001*, *Bp ATCC 7061* et *T.reesei* RUT C30 ATCC 26921. Les boues primaires, secondaires et mixtes ont supporté à la croissance de *T. reesei*, *Bl* et *Bs* tandis que *Bp* a bien crû dans les boues secondaires et mixtes. *T.reesei* a produit des cellulases (carbomethycellulase-CMCase, β-glucosidase) et des hemicellulases (xylanse, β-xylosidase) dans les boues secondaires et mixtes. *Bl* et *Bs* ont cependant seulement produit des cellulases. *Bp* a produit des xylanases dans un milieu constitué de boues secondaires mélangées avec des boues primaires hydrolysées, des résidus de culture de maïs broyés et du xylane extrait du bois d'hêtre. Ce dernier a été sélectionné pour réaliser des expériences à plus grande échelle en bioréacteur. L'activité des xylanases de *Bp* en bioréacteur dans les boues, supplémentés de résidus de maïs, soit 37,8 UI, est supérieure à celle dans les boues supplémentées du xylane, soit 35,5 UI/mL.

Ce travail a permis de conclure que les boues papetières secondaires et mixtes sont de bons milieux de culture de *Bl*, *Bs*, *Bp* et *T. reesei* et qu'ils supportent très bien la production de leurs enzymes saccharolytiques. Les boues secondaires et mixtes stimulent la production des enzymes de *Trichoderma reesei*. Les boues secondaires supplémentées de résidus de maïs permettent d'obtenir l'activité des xylanases de *Bp* plus élevée que dans les boues secondaires supplémentées de xylane commercial. L'ajout externe d'azote dans les boues est également une technique efficace pour accroître le rendement des souches et leur production d'enzymes.

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List of Abbreviations

ADMT	Air dried metric tons
Bl	*Bacillus licheniformis*
Bp	*Bacillus pumilus*
Bs	*Bacillus subtilis*
CFU/ml	Colony formed unit/millilitres
CMC	Carboxymethyl Cellulose
DNS	Dinitro Salicylic
DP	Degree of Polymerization
Fa	Fraction of β-glucosidic bond Accessible to cellulase
FRN	Fraction of Reducing Ends
PPS	Pulp and Paper Sludge
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
VAPs	Value Added Products
WWS	Wastewater Sludge

Chapter 1. Introduction

The global forest, paper and packaging industries have been under pressure during the last years. Financial situation of the companies felt dramatically because of economic downturn, weak markets, intense competition, environmental regulations and increase in production expense. Canadian companies were also hit with net losses rising 355% to approximately $4 billion from 2010 to 2012 (2010: $3.2 billion) [**1, 2**]. In this context, the forest and paper companies must develop new and creative strategies to reduce costs, improve margins and adapt with new regulations. A modern strategy is the application of biotechnology to increase yield of production, to save raw material and energy along with reducing the pollution generation.

In pulp and paper making processes, large volumes of water is consumed at different stages of production resulting in large amount of wastewater. The water consumption varies with the type of paper being produced and can be as high as 60 m^3 per tonne of paper produced, even with the most modern and efficient operational techniques [**3**]. The wastewater needs to be treated to reduce any possible impacts on the aquatic environment. Therefore, most Canadian pulp and paper mills are equipped with treatment systems and they have been successful in reducing the toxicity of their effluents [**4**]. However, the wastewater sludge generated during the treatment process is another problem. It is estimated that the cost of wastewater sludge management accounts for approximately 60% of the total wastewater treatment plant operating expenses [**5**]. Although there are new technologies that could reduce effectively wastewater sludge, the cost of sludge management is still high and consequently affects the price of final products. In addition, traditional disposal methods such as landfill and incineration are limited due to the few available space of land-filling, the potential of groundwater pollution and the greenhouse gases emissions. Therefore, the forest companies are urged to modify their conventional practice of waste management with the recycle and reuse of these biosolids.

Many researches have demonstrated that wastewater sludge (WWS) can be transformed into valuable bioproducts by industrial microorganisms through fermentation technique. Of these, value added products (VAPs) produced through bioconversion of WWS with industrial microorganisms include biopesticides [**6**] and biocontrol agents [**7**], industrial enzymes [**8**], bacterial bioplastics and other biopolymers [**9,10**]. These researches have shown that utilization of WWS as alternative of fermentation substrate to commercial sources could achieve significantly high yield of production. The industrial microorganisms like *Bacillus sp. and Trichoderma sp.* were chosen as VAPs producers because of their high adaptation, product diversification, and genetic manipulability **[11, 12].**

In regard to recycle organic component, pulp and paper industry wastewater sludge have been successfully used as substrate to produce various VAPs, namely biofuel (hydrogen, ethanol, methane), valued organic chemical (lactic acid) and enzyme (cellulase, hemicellulase, ligninase). Hence, bioconversion of paper sludge for production of VAPs would be an innovative approach of waste management in which the forest, paper and packing companies could reduce significantly the expense of sludge management along with diversification of their products that guarantee the competitiveness in market.

In fermentation process, microorganism is a key factor that determines whole design and scale of production. Therefore, selection of a suitable microorganism for VAPs production in paper sludge is a vital step. The global aim of this research is to investigate the utilization of paper sludge as culture medium in submerged fermentation of different industrial microorganisms for production of cellulase and/or xylanase. The study focused on the use of well-known strains included *Bacillus licheniformis, Bacillus pumilus*, *Bacillus subtilis and Trichoderma reesei.* The growth and enzyme production of these microorganisms in paper sludge were evaluated. In addition, the adjustment (enhancement) of medium using paper sludge in terms of solids concentration, sludge mixing, and ratio of C/N was applied in order to improve performance of fermentation.. Then, the application of enzymatic products in valorization of lignocellulosic residues

was explored. The results of the research might contribute to elucidate the feasibility of integrating fermentation processes into the pulp and paper industry in order to transform processing waste streams to valuable products in a perspective of biorefinery.

1.1 Overview of Pulp and paper making process

In general, the main steps in pulp and paper manufacturing processes include raw material preparation (wood debarking and chipping), pulping, bleaching, stock preparation and paper making.

There are two major ways to produce paper pulp from wood, namely mechanical processes or chemical processes. The combination of these technologies is also used widely such as semi-chemical process, mechanical sulfite process, etc. After pulping, the pulp is continuously treated by multi-process to make paper sheet. The typical processes for pulp and paper making are described in Table AI.1.

1.2 The pulp and paper sludge

Most pulp and paper products are manufactured from raw materials containing cellulose fibers such as wood and recycled paper. Residual solids are generated from pulping/bleaching, washing and de-inking unit operations. The amount of residual solids depends on the mill practices. The pulp and paper residual solids can be classified into several categories, which are described in **Table 1** [**13**].

Sludges generated at pulp and paper mills contain a mixture of inorganic compounds, organic compounds and wood fibers. Paper sludge can be divided into 3 main categories:

- The high solid content waste generated by the primary screening process of wastewater from pulp/paper machine, called *primary sludge*.
- The activated sludge from the biological treatment systems, called *secondary sludge*.
- The combination of sludges called *mix sludge (combined sludge)*.

Table 1 Classification of residual solids from pulp and paper mills

Types of residue	Description
Sludges	Sludges produced by mechanical treatment process, which removes the suspended matter in wastewaters or sludges generated by biological treatment of wastewaters.
Ashes from combustion of wastes	Volatile ashes (particularly recovered in treatment equipment of combusted gases) and ashes of grid (particularly recovered in ashtray) produced by the combustion of bark, wood residue or residues of some processes.
Bark and other wood residues	Residues include bark, sawdust, refusal of classification of chips, twigs, etc.
Rubbish of old papers and cartons	Residues of pulp, paper or paperboard produced during production processes.
Alkaline residues	Residues include lime mud, lees of green liquor and discharge of extinguisher from Kraft pulp mills
Miscellaneous	Wastes are collected during pulping of waste paper and cardboard or any other residue resulting from the production process and are not a hazardous material.

According to a survey performed by Elliott and Mahmood, the rate of paper sludge generation varies from 20 to 135 kg/admt of production. The majority of paper sludge generated from 43 Canadian mills is primary sludge. Newsprint mills with deinking facilities generate highest amount of residue that include three types of paper sludge of primary, deinking and secondary sludges. Mills using aerated stabilization basin generate fewer paper sludge. In general, all types of paper sludge were combined prior to their final disposal (**Figure 1.1**) [**14**].

Primary sludge contains wood fibers as the principal organic component. They contain also papermaking fillers (inorganic materials such as kaolin, $CaCO_3$, TiO_2, etc.), pitch (wood resin), lignin by-products, inert solids rejected during the chemical recovery process and other impurities. Almost compounds are carbonaceous that explains the high C:N ratio (above 145:1) in primary sludge. C:N ratio of deinking sludge is similar to sludge derived from virgin paper production. However, their other contents such as residual inks, clay fillers, coatings and other chemicals such as detergents or surfactants are different [**15**].

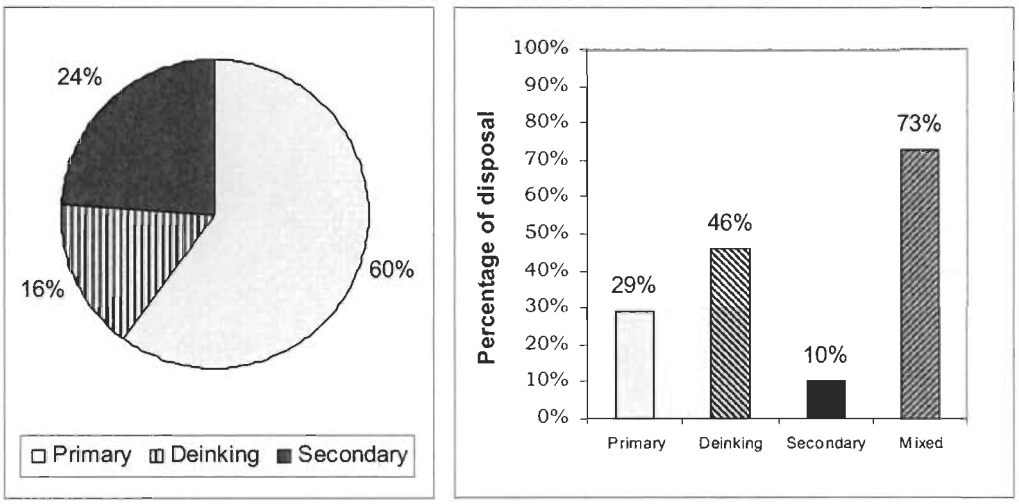

Figure 1.1 Percentage of paper sludge generated and disposed

The high C:N ratio is a limitation of microbial growth because of nitrogen depletion during bioprocess. Researches on primary sludge composting suggested that nitrogen supplementation will improve the yield of microbial growth and the stabilisation of organic matter of paper sludge [**16**]. Secondary sludge contains mainly biomass and by-products of bioconversion in biological treatment system. The low C:N ratio of secondary sludges is a result of the biological treatment process. However, significant parts of nutrients are trapped in microbial biomass or suspended matter that is an

obstacle to the bioconversion. Therefore, pretreatment of paper sludges is required to increase availability of nutrient sources [**17**].

The principal characteristics of primary and secondary sludges are described in **Table AI.2** and **Table AI.3** respectively. The data shows that paper sludge contains carbohydrate, nitrogen and phosphorus, which are the main nutrient sources for microbial growth. Most of these nutrients are present in solid content of paper sludge, and thus the nutrient concentration is directly proportional to the solids concentration. Hence, increasing solids concentration of paper sludge in medium could enhance nutrient availability and subsequently growth of microorganisms. In addition, there are macro-elements and oligo-element in paper sludge that are expected to induce enzyme production and other microbial derivatives. Also, important metals like sodium, potassium, magnesium and iron that play vital role in physiological activities of microorganisms are found in sludge.

1.3 Management of paper sludge

The pulp and paper industry is a huge water consumer that accounted 33.4% of total water consumed by Canadian manufacturing industries. Large volume of water is used for various tasks in pulp and paper mills and that generates large amount of wastewater. It has been estimated that the pulp and paper industry is responsible for 50% of all waste dumped into Canada's water. Particularly, in the Province of Quebec, approximately 507 million m^3 of wastewater is discharged from 52 pulp and paper mills to environment in 2007. Approximately 3 million m^3 of waste generated by pulp and paper mills are mainly disposed by landfilling (36.8 %), combustion (39.8 %), composting or agricultural valorization (19 %).

The Pulp and paper industry wastewater is treated to remove soluble inorganic compounds, which generates wastewater sludge. In general, wastewater is treated at least by a primary treatment process, which consists of neutralization, screening, and sedimentation to remove suspended solids. The secondary and tertiary treatments are

also employed widely to reduce the organics content of wastewater and destroy toxic organics and color (**Figure 1.2**) [**18**].

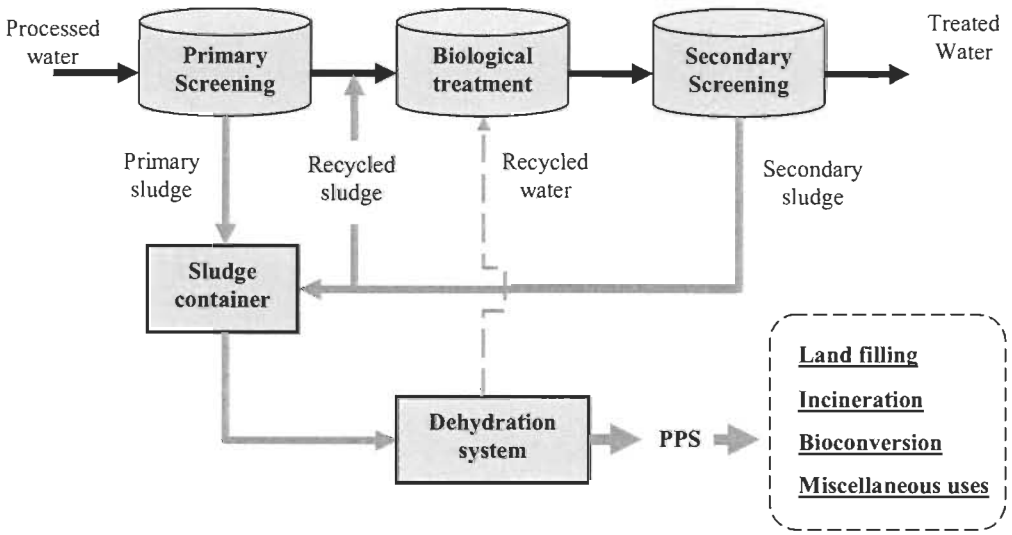

Figure 1.2 Schematic of pulp and paper wastewater treatment and management

In several decades, landfill was the main disposal method that accounted approximately About 50% of overall industry sludge production. The other disposal methods are incineration, land application and beneficial uses. When landfill space becomes rare and the public opposes against the incineration because of noxious gases and several other environmental hazards, alternative technology have been developed [**18,19**]. Recent methods include sonication and thermochemical pre-treatment to enhance biological treatment process [**20, 21, 22**], conversion of sludge to energy and fuel [**23, 24, 25, 26, 27, 28, 29, 30**], amendment to soil [**31, 32, 33, 34, 35, 36**] and composting [**16, 37, 38, 39, 40, 41**], or miscellaneous uses like cement, composite and other construct material [**42, 43, 44,45, 46, 47, 48**]. Of these, applications of sludge as amendment to soil and

composite have shown interesting results. According to field tests, paper sludge application to soils could increase soil nitrogen and modify soil properties for the benefice of crop growth and nutrition. In addition, mixed paper sludge that have high labile C content and low C/N ratios (from 12 to 25) can stimulate microbial growth, enzymatic activity, and nutrient availability as well as promote soil aggregation [**37**]. However, the limited area of proper soils is a main disadvantage of this method. More efficient approach is needed to achieve the maximum value of paper sludge.

Bioconversion of paper sludge is an attractive alternative for sludge management. It can generate products of relatively high value such as enzymes or organic chemicals. It is not hindered by the high water content typical of sludge when a medium of fermentation contains preferably 2 – 3% w/v of total solid [**49, 50**]. In addition, few or no waste material of non-biological origin results from the process. Recently, paper sludge as well as other type of plant-derived wastes are exploited by microbial bioconversion of lignocellulosic component into VAPs like organic chemical, bioenergy and various bioproducts. In Table 2, highlights of the researches on bioconversion of paper sludge and other lignocellulosic residues are described. The researches have shown that paper sludge is a promising alternative material for production of VAPs. Especially, comparison to lignocellulosic biomass, significant fraction of sludge does not require extensive pre-treatment to achieve near-theoretical yields of microbial products via bioconversion [**51**].

Among the biologically-derived products, ethanol has recently received a lot of attention as a product of lignocellulosic biomass conversion. According to Lynd et al., for sludge containing 50% carbohydrate (dry weight basis) and 67% moisture content (wet filter cake basis), the potential value of ethanol production is $91/dry ton sludge [**51**]. These authors also suggested that PPS represents the lowest cost opportunity to realize a commercial facility with positive cash flow-in fuel in comparison with other feedstock.

Production of ethanol using lignocellulosic biomasses includes three main stages. The first stage is pre-treatment of biomass to liberate polysaccharide (cellulose and hemicellulose) components from their complex structure. The second stage is hydrolysis of these polysaccharide components to fermentable sugars. The last step is fermentation of sugars to ethanol. These stages are described in the next sections to elucidate the relation between PPS and biorefinery.

Table 2 Recent studies on using of paper sludge and relative material as substrate for production of VAPs

Product	Substrate	Microorganisms	Process	Reference
Ethanol	PPS	*Zymomonas mobilis*	SSF	**[52, 53]**
	PPS	*Pichia stipiti*	SSF and SHF	**[54, 55]**
	PPS	*Saccharomyces cerevisiae*	SSF and SHF	**[56, 57,58]**
	PPS	*Clostridium thermocellum*	Fermentation	**[59]**
Hydrogen and Biogas	Paper sludge hydrolysate, switch grass	Extreme thermophile *Caldicellulosiruptor saccharolyticus*	Fermentation	**[60, 61]**
	PPS, Whet straw hydrolysate	Microbial consortia from anaerobic digesters	Anaerobic batch fermentation	**[62,63, 64]**
	PPS, sweet sorghum	*Ruminococcus albus.*	Bioconversion	**[65, 66]**
	Alkaline hydrolysed Miscanthus	*Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*	Batch cultures	**[67]**

	Secondary PPS	Microbial granules from anaerobic digester.	Anaerobic digestion	**[68]**
Chemicals				
Carboxylate salts	PPS	Mixed culture of acid-forming microorganisms	Countercurrent fermentation	**[69]**
Lactate, acetate	PPS	*Clostridium thermocellum* 27405	Solid substrate cultivations	**[70]**
Lactic acid	PPS, wet straw	*Thermophilic Bacillus* coagulan	Semi-continuous SSF	**[71, 72, 73]**
Lactic acid	PPS, corn stover	*Lactobacillus rhamnosus* ATCC 7469	SSF	**[74, 75]**
Lactic acid	Pretreated corn stover	*Bacillus* sp. NL01	Fermentation	**[76]**
Enzymes				
Laccase, Manganese, Peroxidase	PPS	White-rot fungi *Trametes versicolor* K 120a2 and *Cerrena unicolor* T 71.	Solid-state cultivation	**[77]**
Xylanases	Agro-industrial residues	*Aspergillus niveus, A. niger*, and *A. ochraceus*	Solid-state fermentation	**[78]**
Cellulase	Kraft PPS	*Trichoderma Reesei*	Submerged fermentation	**[79]**
Cellulase	Untreated waste paper sludge	*Acremonium cellulolyticus*	Submerged fermentation	**[80]**
Cellulase	PPS	*Clostridium thermocellum*	Submerged fermentation	**[59]**

1.4 Production of cellulosic ethanol

Lignocellulosic biomass

Lignocellulosic biomass is an excellent resource for production of biofuels because it is abundant, economic and production of such resources is environmentally friendly. The main sources include agricultural residues, waste from industrial and agricultural processes or dedicated energy crops including perennial grasses such as switch grass and other forage feedstock such as *Miscanthus*, *Bermuda* grass, *Elephant* grass [**81**]. The major component of lignocellulosic materials is cellulose (a homologous polymer comprised of long chains of D-glucose), followed by lignin (complex aromatic polymers) and hemicellulose (heterologous polymer of 5- and 6-carbon sugars). The composition and proportion of these components vary between plants [**82**].

Lignocellulosic biomass can be converted to simple sugars and fermented to liquid fuels through chemical and biological processes. The major bottleneck in conversion technology is the recalcitrance of lignocellulosic biomass to efficient conversion into simple sugars. Plant cell walls are highly recalcitrant to degradation in both microbial and mechanical processes. The main sources of recalcitrance are a crystalline structure of cellulose fibrils and a complex network of lignin component [**83, 84, 85**].

In order to hydrolyze efficiently hemicellulose and cellulose to fermentable sugars, a pre-treatment is necessary. The chemical, physical and morphological characteristics of lignocellulose are important to the digestibility of the substrate. Pre-treatment changes these characteristics and in particular makes the material more accessible for the saccharolytic enzymes. Thus, pre-treatment is a crucial steps in ethanol production as it influences on all other steps in the conversion process. Pre-treatment methods include physical (e.g. milling, grinding), chemical (e.g. alkali, dilute acid), thermal (e.g. steam treatment) and biological (e.g. wood degrading fungi). These methods can also be combined. Pre-treatment such as steam pre-treatment, acid pre-treatment and various

treatments under alkaline conditions are among the most widely reported and promising technologies [**86**].

Bioconversion process

Two important stages of bioconversion process are saccharification and ethanolic fermentation. The goal of saccharification is to hydrolyze the polysaccharides (e.g. cellulose and hemicellulose) in water - insoluble solid fraction that remains after pre-treatment. In cellulosic ethanol production, cellulolytic enzymes hydrolyze cellulose to glucose while hemicellulolytic enzymes also called "accessory enzymes" assist hydrolysis by increasing access to cellulose for the main enzymes [**87**]. The hydrolysate is fermented subsequently by microorganisms such as yeast, *Saccharomyces cerevisiae,* to produce ethanol. The hydrolysis and fermentation may be carried on separately or simultaneously depending on different factors including pre-treatment methods, characteristics of enzyme products and fermentation strain [**88**].

The separate hydrolysis and fermentation process (SHF) for cellulosic ethanol production includes four main steps: (i) pre-treatment, (ii) saccharification, (iii) fermentation and (iv) separation and purification of ethanol to meet fuel specifications. In SHF process, both yeast and enzymes can work at their optimal temperature, but an accumulation of end products can reduce the efficiency of enzymatic hydrolysis.

To overcome the product inhibition of cellulases, a strategy of simultaneous saccharification and fermentation (SSF) has been developed. The hydrolysis of cellulose and ethanol fermentation are conducted in the same reaction vessel [**89**]. Substrate, enzymes and yeast are present initially or may be gradually added during the process. In SSF process, end product inhibition can be avoided but the hydrolysis and the fermentation are not at optimal condition. Therefore, the simultaneous saccharification and consolidated fermentation (SSCF) is developed. Two or more bacteria can be co-cultivated to improve hydrolysis of cellulose, enhance product utilization and thus increase desirable fermentation products [**90**]. Finally, the approach of consolidated

bioprocessing (CBP) gains in popularity. It employs single microorganism that directly convert cellulose to ethanol. The microorganism not only ferment sugars to ethanol, but also generates the biocatalysts, saccharolytic enzymes that are needed to break down cellulose into fermentable sugars. Progress in developing novel strain is being made through two strategies. The first one is engineering naturally occurring cellulolytic microorganisms to improve product-related properties. The other is engineering non-cellulolytic organisms that exhibit high product properties to express a heterologous cellulase system enabling cellulose utilization [**91**].

In conclusion, there are many challenges to the production of cellulosic ethanol such as : cost of enzymes; conversion yield of sugars to ethanol; scale-up and integration processes; valuation of the lignin co-product; etc. Of course, enzyme is a key factor that determines the final goal of ethanol production. The structure, mechanism, biosynthesis and production of the cellulolytic enzymes is already understood for decades [**92, 93, 94, 95, 96, 97, 98, 99, 100, 101**]. To insure cost – effective and efficient production of bioethanol, desired properties of enzymes are : high adsorption capabilities; high catalytic efficiencies; high thermal stability; low end-product inhibition; and sufficient flexibility different feed-stock [**81**].

Even if many works to produce such desirable enzymes and significant results are conducted, cost of production and utilization of the enzymes is still high for ethanol producers. Therefore, enzyme producers are trying to advance to increase efficiency and affordability of enzymes For example, Dupont is developing new generation of products that has been expecting to reduce the cost of enzyme lower than 0.30$ per gallon of ethanol (information provided by Genencor, now DuPont Industrial Biosciences). Their enzyme cocktails shown to meet all requirements for the ethanol production process which act in synergistically way and efficient for hydrolysis of wide range of lignocellulose biomass. The strategies of reducing enzyme cost in the direction of decreasing enzyme production cost and increasing enzyme efficiency have been going on along with development of cost-effective bioethanol production.

Enzymes

Cellulolytic enzymes

The cellulose degrading enzymes have been studied intensively and the reaction mechanisms of key cellulases were revealed on molecular level. Generally, a non-complex cellulase system includes at least three classes of enzymes, which act in synergy to hydrolyse cellulose [**75**]:

- Endo-glucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4) attack randomly the endogenous part of cellulose chain.

- Exo-glucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91) attack the ends of the polymer, releasing cellobiose.

- β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21) ultimately cleaves cellobiose into two glucose molecules.

The cellulosome or complex cellulolytic enzymes systems are found in some anaerobic microorganisms such as *Clostridia* that are not a focus of this the present project but reviewed elsewhere [**102,103**]. The schematic of hydrolysis cellulose by cellulases is described in **Figure 1.3**. At the first period, insoluble cellulose is hydrolysed by the synergistic activities of several endoglucanases and exoglucanases. The products are cellobiose, cellodextrin (DP 3 – 6) and specially glucose. These soluble products are finally converted to glucose by β-glucosidases or consumed directly by organisms.

Various microorganisms have been evaluated for their ability to degrade cellulosic substrates into sugar monomers. However, relatively few microorganisms have been screened for their cellulase production potential. The most interesting microorganisms are the bacteria or fungi that produces extracellular cellulose complex with high levels of

endoglucanase, exoglucanase and β-glucosidase. Currently, industrial cellulases are almost all produced from aerobic cellulolytic fungi, such as *Trichoderma reesei* or *Humicola insolens*. This is due to : 1) the ability of engineered strains of these organisms to produce large amounts of crude cellulase (over 100 g per liter); 2) the relatively high specific activity of their crude cellulase on crystalline cellulose; 3) the ability to genetically modify these strains to tailor the set of enzymes they produce [**104**]. Recently, the bacterial cellulolytic enzymes are getting more attention. The main reason is that bacteria possess multi-enzyme complexes (e.g. *Paenibacillus curdlanolyticus* [**105**], *Bacillus licheniformis* [**106**]). These enzymes will increase efficiency of enzymatic products that using in cellulosic ethanol production.

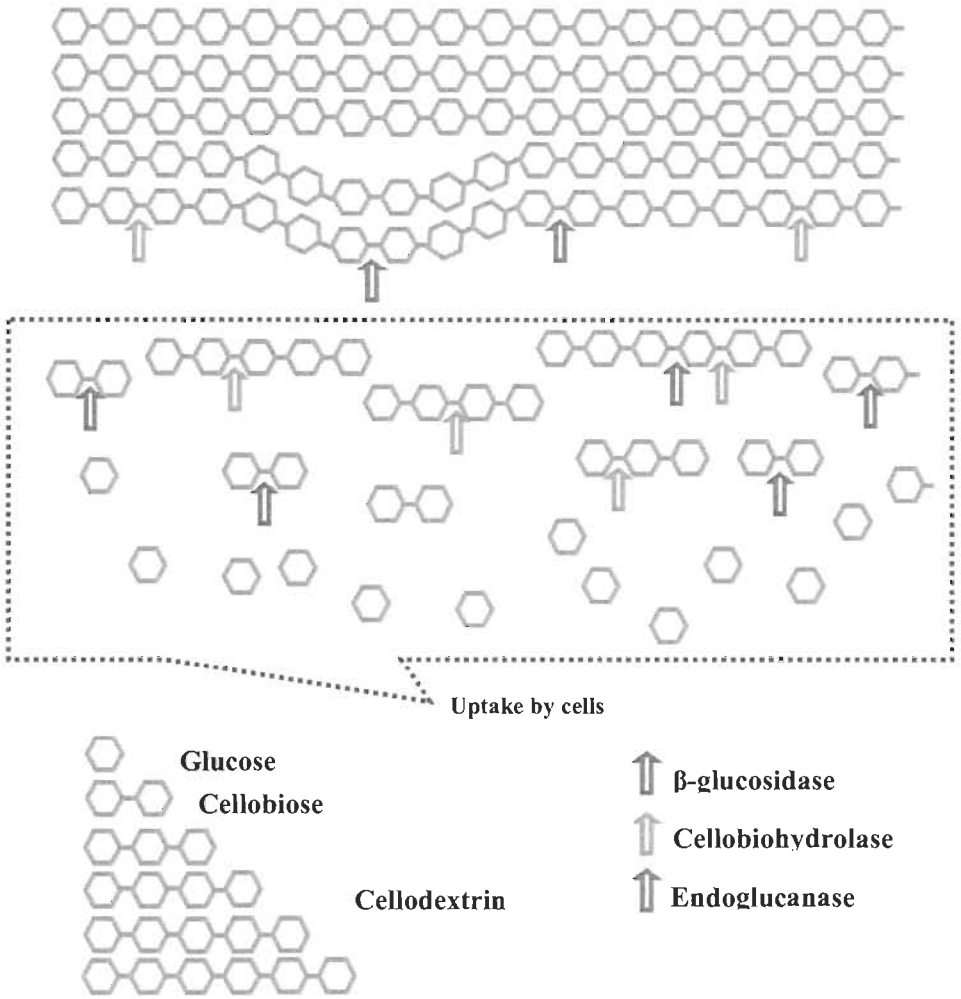

Figure 1.3 Schematic of cellulose hydrolysis by non-complexed cellulase system.

Hemicellulolytic enzymes

Next to cellulose, hemicellulose is the second most abundant renewable biomass which varies in composition from plant to plant and within different parts of the same plant. It is made up of mainly pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-

glucose, D-galactose) and sugar acids. Hydrolysis of hemicelluloses requires various types of enzymes. Briefly, xylan degradation requires endo-1-4,-β-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), α-glucuronidase (α-D-glucosiduronate glucuronohydrolase EC 3.2.1.139), α-L-arabinofuranosidase (α-L-arabinofuranoside arabinofuranohydrolase EC 3.2.1.55), as well as acetylxylan esterases (EC 3.1.1.72). In glucomannan degradation, β-mannanase (EC 3.2.1.78) and β-mannosidase (EC 3.2.1.25) are required to cleave the polymer backbone (**Figure 1.4**).

Among the enzyme responsible for hydroysis of hemicelluloses, xylanases are the most studied enzymes due to their biotechnology application. Over the years, applications of xylanases in the pulp and paper industry have increased steadily. Several applications have approached commercial use. The most effective application of xylanases is prebleaching of Kraft pulp to minimize use of harsh chemicals in Kraft pulp [**107**]. Also, xylanases are important in hydrolysis of hemicellulose component for second generation bioethanol production [**108**]. Most commercial xylanases are produced by submerged fermentation using fungi such as *Trichoderma sp.* and *Aspergilus sp.* Moreover, *Bacillus* sp. were chosen to produce bacterial alkaline xylanases in industrial scale because of their rapid growth rate, high adaptation comparison to fungi and stability of enzyme in high pH and temperature [**109**].

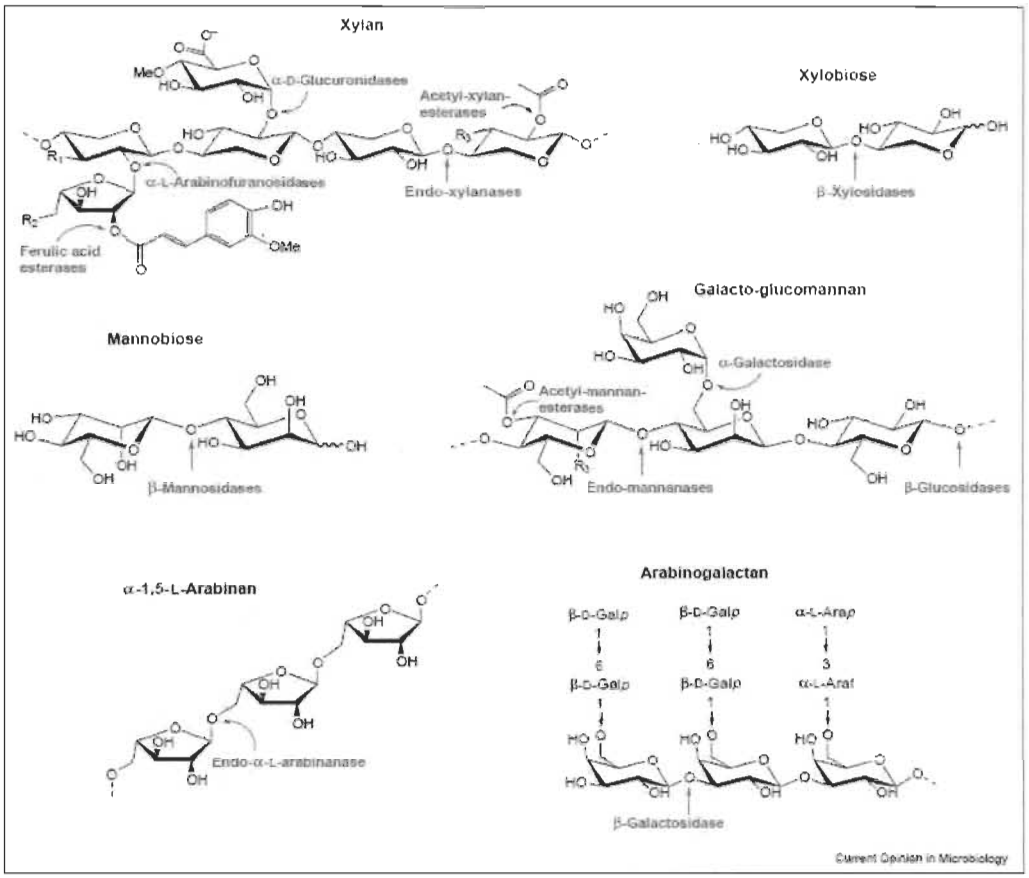

Figure 1.4 Structural components found in hemicellulose and the hemicellulases responsible for their degradation [110]

1.5 Enzyme producers

Trichoderma ressei

Filamentous fungi are the major source of commercial cellulases. To date, enzyme manufacturers have depended majority on fungal species mainly *Aspergillus sp*; *Trichoderma sp* to produce cellulase at industrial scale [**111, 112, 113, 114**]. These industrial strains, already improved in enzyme productionn via random mutagenesis, are extensively engineered using genetic and genomic tools to maximize protein productivity with high cellulase expression to deal with a diversity of lignocellulose

materials. Owing to a great capacity of producing a wide range of enzymes responsible for cellulose degradation, *T.reesei* has been employed as a key of cellulase manufacture for cellulose bioconversion. The cellulase system synthesized by *T.reesei* was reported to have at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two ß-glucosidases (BGLI and BGLII), 10 different hemicellulases and pectin degrading enzymes. Moreover, the catalytic enzymes of *T.reesei* are linked with carbohydrate-binding proteins (CIP1 and CIP2) and specific cellulose-binding domains, namely swollenin that increase hydrolysis activity on crystalline cellulose [**97, 115, 116**]. The strain has been successfully undergone classical mutagenesis and selection procedures to obtain advances in significant cellulase expression and catabolite derepression exhibition. In regard to increase the expression of target protein via genetic engineering, *T.reesei* is chosen as a host to be introduced heterologous proteins that would act synergistically or assistantly with its native cellulases and hemicellulases to achieve superior catalytic efficiency [**117, 118**]. Recent years, intensive research efforts and considerable government funding have been applied toward developing better industrial strains for producing bioethanol and a range of key biochemical building blocks, such as 1,4-dicarboxy acids (succinate, malate, fumarate), 3-hydroxypropionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, glycerol, sorbitol, xylitol/arabinitol and hydroxybutyrolactone, that are currently derived from non-renewable petroleum-based resources **[119, 120, 121, 122, 123, 124]**.

Bacillus – the important producers of industrial enzymes

According to the researches mentioned in the previous section, bacteria play an important role in valorization of lignocellulosic biomass. Studies are continuously published on the employment of bacterial sources, especially thermophilic [**125**] or psychrophilic [**126**], alkaliphilic [**127**] or acidiophilic, and halophilic strains [**128**]. These extremophilic species often produce systems of enzymes that are stable under extreme condition that is an advantage for industrial application.

In the field of applied bacteriology, *Bacillus sp.*, Gram positive endospore-forming bacteria, are important sources of various applications. Certain reasons are their high growth rates leading to short fermentation cycle times, their capacity to secrete large quantities (20–25 g/L) of protein into extracellular medium and the GRAS (Generally Regarded As Safe) status characterized by the Food and Drug Administration for species, such as *B. subtilis* and *B. licheniformis* [**11**]. In addition, complete genomes of important industrial strains like *B. subtilis* [**129**], *B. licheniformis* **[130, 131]** and others were published **[132, 133, 134]**. Therefore, advanced knowledge on biochemistry, physiology, and genetics of *Bacillus* species facilitates further development and greater exploitation of these organisms in industrial processes.

In decades, many *Bacillus sp.* are isolated and developed to be host of vast industrial products such as enzymes, biosurfactant, insecticides, antibiotics, vitamins, food additives, etc. Among them, the enzymes, namely proteases, amylases, xylanases represent about 50% of the total enzyme market [**4**]. The enzymes are used broadly in food processing, leather treating, household detergent, textile, and paper industry. The important applications of *Bacillus* enzymes are summarized in **Table 3** below.

Table 3 Main application of important enzymes from *Bacillus sp.*

Bacillus enzymes	Application	Source	References
Amylases and relatives			
Alkaline amylase			
Thermostable amylase	Biofuel, detergent, food processing, textile, pulp and paper making, person care.	*B. amyloliquefaciens* *B. licheniformis* *B. megaterium* *B. subtilis*	**[135, 136]**

Bacillus enzymes	Application	Source	References
CGTase	Cyclodextrin production	*B. circulans*	**[137]**
Glucose isomerase	Food processing, biofuel	*B. acidopullulitiicus* *B. coagulans*	**[138]**
Pullulanase	Food processing, biofuel	*B. acidopullulitiicus* *B. licheniformisd* *B. naganoensis* *B. subtilis*	**[139]**
Cellulolytic enzymes			
Cellulases β-glucanase	Animal feed, biofuel, detergent, Food processing, textile, pulp and paper making, deinking.	*B. subtilis* *B. amyloliquefaciens*	**[140, 141]**
Mannanase	Detergent	*B. circulans* *B. subtilis*	**[142]**
Xylanases	Biofuel, animal feeding, pulp and paper manufacture.	*B. circulans* *B. halodurans* *B. pumilus*	**[143]**
Proteases			
Proteases	Food processing, detergents, Pulp and paper making (Biofilm removal)	*B. amyloliquefaciens* *B. licheniformis* *B. stearothermophilus* *B. subtilis*	**[136, 144]**
Keratinases	Feather degradation, leather industry.	*B. licheniformis* *B. subtilis*	**[145]**

Additionally, there are several other enzymes of industrial application produced by different species of *Bacillus* such as levansucrases from *B. megaterium and Lactobacillus panis* [**146,147**]; esterases from *B. subtilis,* [**148**] and *B. pumilus* [**149**]; chitinases from *B. licheniformis* [**150**]; tannase from *B. licheniformis, Lactobacillus* [**151, 152**].

Production and application of cellulolytic and hemicellulolytic enzymes based *Bacillus* have been studied in several years [**81, 97, 140**]. Submerged fermentation was convenient technology of production of cellulolytic enzyme production. Recently, solid-state fermentation process is rapidly gaining interest as a cost effective technology of enzyme production. However, this technology is still required further development for large scale process. Cellulase-producing *Bacillus* have been isolated from different sources such as composting heaps, decaying plantmaterial from forestry or agricultural wastes, soil and organic matter, and extreme environments like hot-springs. Substrates for cellulase production are mainly cellulosic biomass, including straws, spent hulls of cereals and pulses, rice or wheat bran, bagasse, and paper industry waste. In regard to reduce production cost, utilization of inexpensive substrates from agro forestry residues for production cellulase and xylanase of *Bacillus* have been intensively studied. Recent exploitation of lignocellulosic biomass to produce cellulolytic and xylanolytic enzymes by *Bacillus* is summarized in **Table 4.**

Table 4 Recent studies on production of cellulolytic enzymes by *Bacillus* sp. used lignocellulosic residue (from 2005 up to now).

Enzyme	Bacillus	Substrate	Nitrogen source	Cultivated methods	Ref.
Cellulase					
Cellulases	*B. amyoliquefaciens*	Rice hull (2% w/v)	Yeast extracts (0.25% w/v)	Submerged ferm,	**153**

Endoglucanase	*B. pumilus EB3*	Pretreated Oil palm empty fruit bunch (1% w/v)	Yeast extracts (0.25% w/v)	Submerged ferm,	**154**
Endoglucanase	*B. subtilis*	Rice ban (2% w/v)	Yeast extracts (0.25% w/v)	Submerged ferm,	**155**
Hemicellulases					
Xylanase	*B. coagulans*	Pretreated Wheat straw (1% w/v)	Yeast extracts and pepton (0.5% each w/v)	Submerged ferm,	**156**
Xylanase	*B. pumilus*	Wheat bran (2% w/v)	Yeast extracts and pepton (0.5% each w/v)	Submerged ferm,	**157, 158**
Cellulase-free xylanase	*Thermoalkaliphilic Bacillus sp. JB-99*	Agricultural residues (50% w/v)	4% w/v of organic nitrogen	Solid state ferm.	**159**
Xylanase and mannase	*B. circulans*	Industrial fibrous soya residue (20% w/v)		Solid state ferm.	**160**
Xylanase	*B. coagulans*	Industrial fibrous soya residue (37.6% w/w)		Solid state ferm.	**161**
Xylanase	*B. megaterium*	Agro industrial residues (40-100%)	Soybean meal 1-5 % w/v	Solid state ferm.	**162**
Cellulase-free endoxylanase	*B. pumilus*	Agro industrial residues (ND)	Soybean meal 1-5 % w/v	Solid state ferm.	**163, 164**
Xylanase	*B. stearothermophilus*	Wheat bran (2.5% w/v)	2% of organic nitrogen	Solid state ferm.	**165**
Xylanase	*B. subtilis*	Agricultural residues (50% w/v)	4% w/v of organic nitrogen	Solid state ferm.	**166**

Many studies shown that enzyme production of *Bacillus* could be induced by polysaccharide content of lignocellulosic biomass. The activities of obtained enzymes are maintained at broad ranges of temperature and pH. These characteristics of *Bacillus* enzymes are advantageous over fungal enzymes in the simultaneous saccharification and fermentation process to produce ethanol where high temperature is preferred for ethano-producing microorganisms. In addition, *Bacillus* is capable of producing different complex enzymes or multifunctional enzymes supporting the bioconversion of cellulose. If these enzymes can be produced on industrial scale using lignocellulosic residues, they may have a great potential to reduce the cost of enzymatic hydrolysis for production of biofuels.

Hence, various lignocellulose residues were used successfully as carbon source and cellulase inducers for cultivation of cellulolytic bacteria and fungi with high specific activities of saccharolytic enzymes achieved. However, utilisation of high cellulose-containing residues such as crop and wood residues for enzyme production that are also biomass of cellulose ethanol production could give a competition of biomass supply resulting in reduction in both enzyme and ethanol yields. Waste streams from domestic operation and agriculture and forest industry such as wastewaters and sludges, daily manures that contain potential carbohydrates for induction of cellulosic enzyme and nutrient elements essential for microbial culture are alternative carbon sources for enzyme fermentation.

1.6 Integrating microbial fermentation for production of enzyme into the pulp and paper industry

Considering the reduction of the paper market, the pulp and paper industry is making an effort to change their business plan and look for the diversification of their products. Integration of lignocellulose - based biorefinery is a promising approach for the forest industry where large amount of cellulose residues are generated regularly. Bioconversion of paper sludge by fermentation technology would bring biorefinery activities to revitalize the industry. Scenario of application and integration of fermentation using

industrial microorganisms *Bacillus sp.* and *Trichoderma reesei* for enzyme production into pulp and paper mills is proposed in **Figure 1.5.** These enzymes produced from paper sludge could then be used directly to hydrolyse cellulose residues to obtain fermentable sugars for other microbial production. The enzymes can be also sold to local plants of cellulosic sugars and bioethanol production.

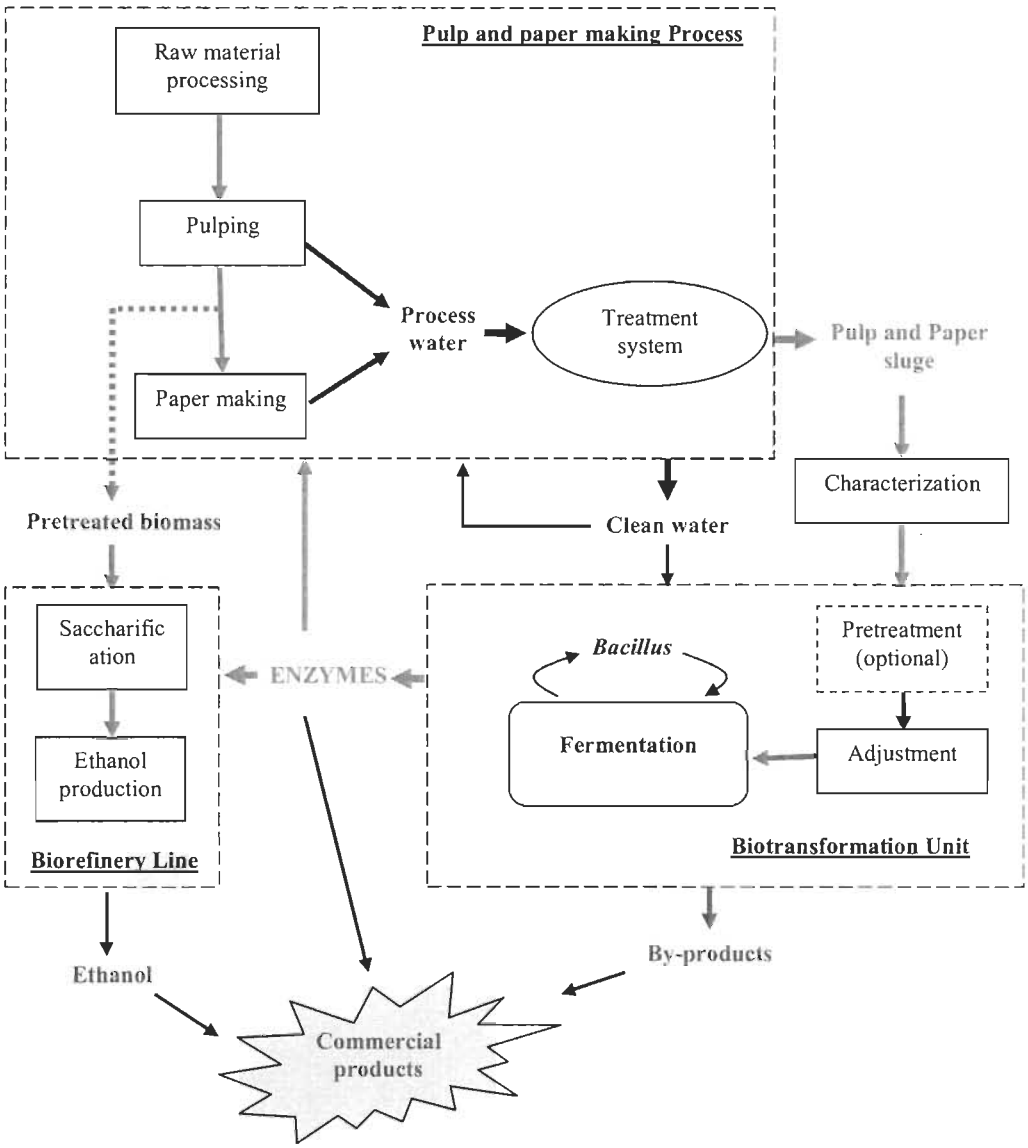

Figure 1.5 Enzyme production through fermentation integrated in a pulp and paper mill.

Besides maximizing value addition of lignocellulosic waste streams, existing equipments of pulp and paper mills can be upgraded and used for biorefining operation. Adapting existing equipments is a good way to reduce capital costs for industrial enzyme

production. As described in **Figure 1.6**, fermentation system can be setup using in-site facilities of pulp and paper mills, including vessels for inoculum preparation, aerobic biological reactor modified for fermenter, pumping system available for feeding nutriments, press system suitable for bioproducts recovery, and excess heat (steam) in plant recycled to control temperature of fermentation (and sterilize the fermentor and its accessories before fermentation).

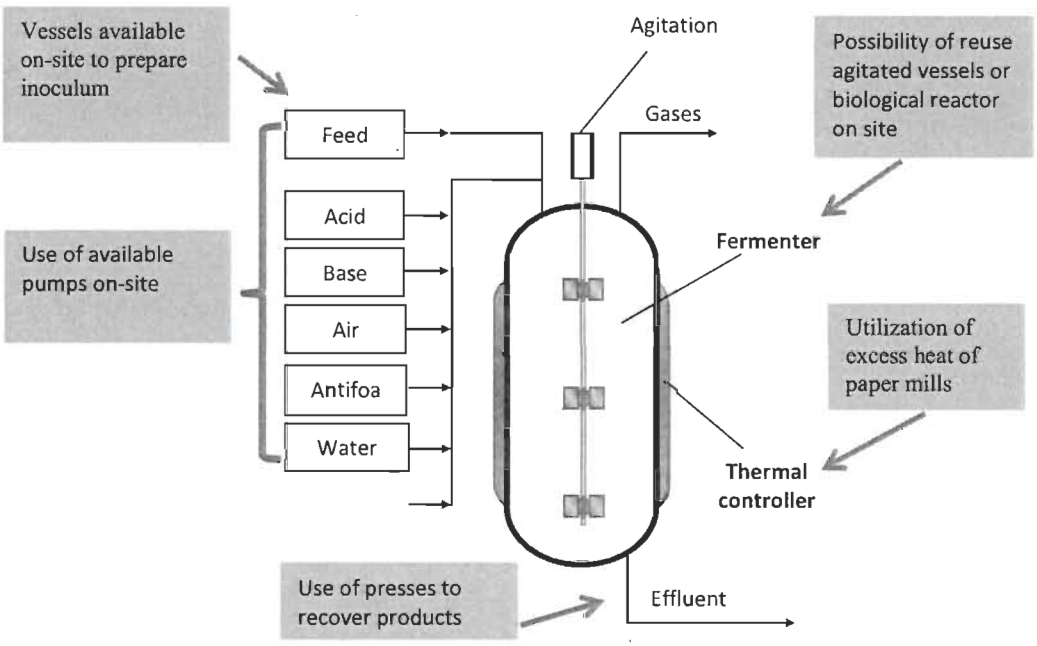

Figure 1.6 Equipments of pulp and paper mill that may be exploited to adapted for fermentation processes.

In summary, the main advantages of integrating bioconversion processes into pulp and paper mills are:

- Reduction of waste management cost for a mill when its paper sludge is used as raw material for fermentation;

- Reduction of enzymes or other microbial derivative cost production by using paper sludge as cheap, zero cost, even negative cost raw material;
- On – site enzyme manufacture (or other microbial derivatives) using paper sludge for further use of enzymes/microbial derivatives by the same mill producing the sludge or for local use of enzymes/microbial derivatives, bring down cost of enzymes/microbial derivatives;
- Possibility of revitalizing pulp and paper equipment's by using them for fermentation or downstream processing of fermented broths;
- Diversification of products for the mill.

Chapter 2. Hypothesis and objectives

2.1. Goal and Hypotheses

The goal of this PhD work is to study the use of pulp and paper industry wastewater sludge (paper sludge or PPS) as raw material for production of saccharolytic enzymes of *Bacillus sp.* and *Trichoderma reesei.*

Based on the literature review, it is shown that various industrial microorganisms can grow and produce cellulases and hemicellulases in fermentation using lignocellulosic residues. Some researches demonstrated that paper sludge was a good substrate to growth industrial microorganisms such as *Trichoderma sp.* or *Aspergillus sp.* Very few studies demonstrated that paper sludge can support cellulolytic enzymes production. As paper sludge contain cellulose residues and other macro or micro-nutrients, we can presume that paper sludge can be used as culture medium for growth and enzyme production of industrial strains of bacteria and fungi. Thus, the first hypothesis is:

- *Paper sludge including primary, secondary (biological) and mixed sludges can be used as culture media for growth and cellulases and hemicellulases production of Bacillus sp and Trichoderma sp.*

Despite of high water content, paper sludge is a highly heterogeneous, complex material. In addition, its composition varies according to many factors such as pulp and paper production processes, wastewater treatment operation, season, etc.. Thus, it is necessary to modify paper sludge and obtain a more homogenous culture medium. As most of nutrients in wastewater sludge are found in the solid particles or organic matter, the increase of solid concentration can result in a higher amount of nutrients. However, high solid content could lead to high viscosity and thus reducing mass and oxygen transfer in the medium during fermentation, which can potentially affect the growth of the microoorganisms. Then, the second hypothesis is:

- *Adjusting total solid of PPS for fermentation improves the growth and enzyme production of microorganisms.*

C:N ratio of a culture medium is a fundamental parameter for microbial growth. According to characterization of PPS, its C:N ratio varies following type of sludge and much higher compared to C:N ratio in synthetic culture medium, meaning nitrogen deficit in paper sludge when used as alternative medium. Unbalance between available carbon and nitrogen sources will cause a decline of microbial growth and enzyme production. According to recent researches on using lignocellulosic biomass as substrate in fermentation, a nitrogen supplement will increase the growth and enzyme production. In addition, previous researches suggested that the type of nitrogen source also influence the growth and enzyme production. Then, the third hypothesis is:

- *Adjusting C:N ratio of culture medium by adding nitrogen sources increases the growth and enzyme production of microorganism in fermentation using PPS as substrate.*

In complex culture media with organic matter, microorganisms secrete various extracellular enzymes to scavenge nutrient sources. Thus, type and functionality of the enzymes produced in complex media depend on substrates and could be different from the enzyme produced in conventional semi-synthetic media. Thus, it is presumed that... :

- *The cellulolytic and hemicellulolytic enzymes produced in complex culture media such as paper sludge, with a supplement of lignocellulosic residues, would be more efficient to convert cellulose and hemicelluloses into sugars in comparison with the same type of enzymes produced in conventional semi-synthetic media.*

2.2. Objectives

Based on the aforementioned hypotheses, the specific objectives of this project are:

1) Study of the growth and production of cellulase and hemicellulases of *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis and Trichoderma reesei* using PPS as culture media.

2) Study of the effect of modification of PPS on the improvement of the growth and enzyme production of *B. licheniformis, B. pumilus*, and *Trichoderma reesei* :
   - Effect of adjusting solids concentration of PPS;
   - Effect of supplementing the PPS with organic and inorganic nitrogen sources;
   - Effect of supplementing the PPS with a lignocellulosic substrate.

3) Study on the production of the more performant *Bacillus sp.* strain (in terms of growth and enzyme production) in bioreactor using PPS as culture media supplemented with a lignocellulosic substrate.
4) Study on characterization of enzymes produced in paper sludge.
   - Optimal pH and temperature of enzyme activity;
   - Ability of degrading and converting lignocellulosic residues into simple sugars.

This study is conducted in a context of intergating biorefining activities into the pulp and paper industry for which lignocellulosic residues could be used as feedstock to produce enzymes and biofuels for on – site or local utilization.

Originality of this research is the on-site manufacture of cellulolytic and hemicellulolytic enzymes in pulp and paper mills using their wastewater sludges as culture media, which is a new economical way of sludge disposal that contribute to diversification of pulp & paper industry products and to the raising of cellulosic biorefineries in region.

Chapter 3. Materials and methods

3.1. Material

Microbial strains

Strains used in this study are: *B. pumilus (ATCC7061)*; *B. subtilis (IEBC SUB001)* from strain collection of CRIEB; *B. licheniformis* ATCC 21424 and *T.reesei* ATCC RUT C-30 26921. General information about strains is given in **Table AII.1**.

Pulp and paper sludge

Different type of PPS was collected at the Crabtree Mill of Kruger company in the Lanaudière region (QC, Canada). The mill is part of Kruger Products' Region East Manufacturing Division that manufactures a wide range of tissue products, such as the following popular brands: Cashmere®, SpongeTowels®, White Swan® and Purex®. In addition to its four paper machines, the Crabtree Mill has a deinking and recycling plant, a transforming facility. After sampling, raw (fresh) PPS are stored at 4°C for a maximum of two weeks. Solid concentration of each sampled sludge is measured according to APHA et al. (2005) [**167**].

Semi-synthetic culture medium

The semi-synthetic culture medium normally used for production of the cellulase by *Bacillus sp.* contains the following components (w/v): 0.5% substrate (CMC, Xylan or lignocellulosic residue), 0.5% yeast extract, 0.5% pepton, 0.1% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4.7H_2O$ and 0.06% $(NH_4)_2SO_4$. For *Trichoderma ressei*, the culture medium contains the following components: 10 g/L carboxymethylcellulose (CMC); 0.3 g/L urea; 1.4 g/L $(NH_4)_2SO_4$; 2.0 g/L KH_2PO_4; 0.3 g/L $CaCl_2$; 0.3 g/L $MgSO_4$; 0.25 g/L yeast extract; 0.75 g/L proteose peptone, and trace elements (5 mg/L $FeSO_4.7H_2O$; 20 mg/L $CoCl_2$; 1.6 mg/L $MnSO_4$ and 1.4 mg/L $ZnSO_4$).

Chemicals

All chemicals are reagent grade purchased from companies Sigma or Fisher. Commercial cellulase preparations (Accelerase™ 1500) were obtained from Danisco US – Genencor (now DuPont Industrial Biosciences).

3.2. Method

Experimental plan

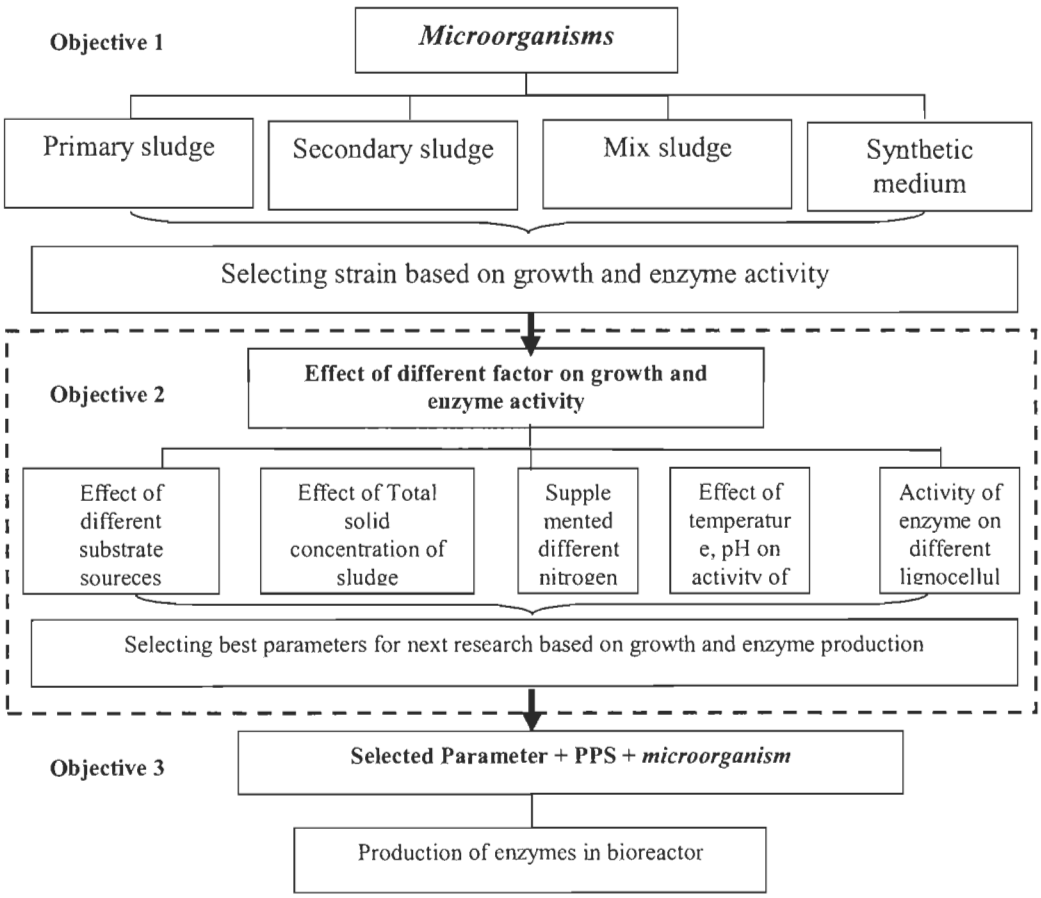

Figure 3.1 Experimental Plan

Many sets of experiments are planned to achieve the research objectives. The sets of experiments are illustrated in the figure below.

Starter culture and preculture preparation

For each strain, starter culture is prepared by transferring a single bacterial colony from stock culture into 500 ml Erlenmeyer flasks containing 100 ml of a tryptic soya broth. The starter culture is incubated for 12 to 16 h at the optimal temperature for the strain (normally 30°C) and an agitation of 200 rpm in a shake flask incubator. The starter culture is used to start the fermentation experiments. A volume of 2% (v/v) of the starter culture is inoculated into 100 ml of sterile PPS or control culture medium contained in 500 ml Erlenmeyer flasks. All culture media are previously adjusted at a pH 7 (with NaOH and H_2SO_4) and then autoclaved at 121°C during 30 minutes. For PPS, they are adjusted to a solid concentration of 1.5% (w/v) before pH adjustment and subsequent sterilization.

Fermentation experiments

All fermentations are conducted at the optimal temperature and pH of the strain and an agitation of 200 rpm in a shake flask incubator. For each experiment, samples will be withdrawn interval from the cultures to measures cell growth, spore production and the enzymatic activities of cellulases, beta-glucosidases and xylanases.

Cells and spore count

Growth of *Bacillus* is determined by the viable count plate technique [**168**]. For spore count, the suspension is previously heated at 70°C during 15 min before applying the viable count plate technique.

Measurement of the cellulolytic activity

There are two approaches to measuring the enzymatic activity: (1) measuring the individual enzymatic activities (endoglucanases, exoglucanases and the β-glucosidases); and (2) measuring the total cellulase activity. In general, hydrolase enzyme activities are expressed in the form of the initial hydrolysis rate for the individual enzyme component within a short time, or the end-point hydrolysis for the total enzyme mixture to achieve a

fixed hydrolysis degree within a given time. Because of interaction between components in cellulase enzyme system, different behaviours are exhibited when these components are present in combination or individually (purified). Therefore, there is always a gap between initial cellulase activity assays and final hydrolysis measurement [**169**]. For meaningful result, individual cellulase component assays must also be based on a reliable estimation of the amount of individual enzyme component present in the assay. This information, furthermore, allows the calculation of specific activity of enzyme.

In this project, vegetative cells (and spores) were removed from the culture broth. The pellet was washed two times with the buffer. Final supernatants were used for different enzymatic assays as described in the next sections. All enzymatic assays where performed as triplicate along with blank and negative control. The protocol of enzymatic assay will be based on standard procedure of Genencor **[170, 171]**.

Endo-glucanase activity

Endoglucanase activity is standardized on the basis of activity on CMC. One CMC unit of activity liberates 1 µmol of reducing sugars (expressed as glucose equivalent) in one minute under specific assay condition. The rate of reducing sugars released, as measured by the reaction with 3, 5 dinitrosailicylic acid (DNS), is proportional to the enzyme activity. The absorbance of the enzyme samples and control sample was measured at 540 nm against distilled water as zero absorbance **[170]**.

β-glucosidase activity

β-glucosidase activity is standardized on the basis of activity on 4-nitrophenyl-beta-D-glucopyranoside (pNPG). One pNPG unit of activity liberates 1 µmol of nitrophenol from para-nitrophenyl-B-D-glucopyranoside in one minute under specific assay condition. β-glucosidase hydrolyzes 4-nitrophenyl-beta-D-glucopyranoside to 4-nitrophenol and glucose. The reaction is stopped by adding a sodium carbonate. Absorbance of 4-nitrophenol released is measured at 400 nm [**171**].

Xylanases assays

The xylanase activity of sample will be expressed in Acid Beechwood Xylanase Unit (ABXU). One ABXU unit is defined as the amount of enzyme required to generate 1µmol of xylose reducing sugar equivalents per minute under the condition of experiment [**172**].

Effect of PPS solid concentration on growth and enzyme production

For experiments in objective 2, different solid concentrations (10, 15, 20, 25, g/L) will be tested. To adjust the solid concentration, sludge has normally settled during its storage. The supernatant is removed. Solid concentration is measured on the remaining sludge. Following the solid concentration, supernatant can be added to dilute the sludge at the desired solid concentration. If a higher concentration is desired, the remaining sludge is concentrated by centrifugation and the supernatant can be added to dilute the sludge at the desired concentration.

Effect of nitrogen source and concentration of nitrogen

Organic and inorganic nitrogen sources was added to primary sludge at different concentration (0.1, 0.15, 0.25, 0.5, 0.75, 1%, w/v) to the PPS having high C:N ratio.

Effect of pH on activity of enzymes

The activity of the enzymes at different pH is determined by incubating supernatant of the fermented broth with the substrate (mentioned in previous section) in the presence of appropriate buffers (See). The reaction mixtures in given pH buffers are incubated for 60 min at 30 °C and the enzyme activity was measured as described previously. The stability of the enzymatic activity at different pH (3 – 10) is determined after incubating the enzyme in the buffers described above for 24 h at 30 °C. After 24h, the residual enzymatic activity will be measured.

Thermal stability of enzymes

Thermal stability of the enzymes for hydrolysis of specific substrate at optimal pH is determined by incubating the supernatant of fermented broth with 1% (w/v) substrate for 60 min at different temperatures 20, 30, 40, 50, 60, 70, 80, 90 °C. The reaction is stopped and residual activity is then measured at intervals of 0, 5, 10, 20 and 30 min.

Saccharification of lignocellulosic biomass

The enzymatic hydrolysis experiments are carried out following the procedure of Genencor for their Accelerase products at the specific optimum temperature and pH for each fermented broth or its supernatant. The buffer will be supplemented mixture of antibiotic to prevent microbial contamination. The concentration of pretreated corn stover is 20g/L. The hydrolysis is conducted into 500 ml shake flasks and the work volume is kept at 100 ml. The shake flask is hermetically closed with a lid, and the hydrolysis is carried out in incubator-agitator at 150 rpm and 96h. The concentration of total reducing sugars is measured at each 24h with the DNS method. The concentration of glucose is measured at each 24h with the o-toludine method [**173**]. Comparison is made with the commercial enzyme cocktails Accellerase 1500.

Chapter 4. Investigating use of pulp and paper sludges as raw material for Bacillus sp. fermentation to produce cellulolytic enzymes

4.1. Preface

This article has been submitted. It compiles and discusses the experiments conducted on *Bacillus* sp. fermentation using pulp and paper sludges. As first author, Thanh-Tung Lai made all the experiments and wrote the article. Co-authors Thi-Thanh-Ha Pham and Kokou Adjallé assisted in preparing the experiments and doing the analysis. They have also contributed to the writing of this article. Co-authors François Brouillette and Simon Barnabé contributed to the writing of this article and to the data interpretation. They have also made the final correction.

4.2. Article no.1

Use of pulp and paper sludge as a raw material for Bacillus spp. fermentation to produce cellulolytic enzymes for biorefining activities

Thanh-Tung Lai, Thi-Thanh-Ha Pham, Kokou Adjallé, François Brouillette, Simon Barnabé*

Lignocellulosic Materials Research Centre, Université du Québec à Trois-Rivières, 3351, boul. des Forges, Trois-Rivières, Canada, G9A 5H7

(*Corresponding Author: E-mail: simon.barnabe@uqtr.ca; Phone: 1(819) 376-5011 # 4531; Fax: 1(819) 376-5148)

Significance and impact of the Study

The pulp and paper industry is looking for secure and economical ways to dispose of their sludge. At the same time, it is trying to be a part of the emerging bio-economy. The

use of pulp and paper wastewater sludge as a raw material for the fermentation of *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis* and the subsequent production of cellulolytic enzymes for further biorefining activities is proposed.

Abstract

Fermentation experiments were conducted with different types of sludge and strains of *Bacillus* spp. All of sludge samples supported the growth of *B. licheniformis* and *B. subtilis*, whereas *B. pumilus* was growing well in secondary and mixed sludge. The highest cell counts were obtained in mix sludge for *B. licheniformis* and *B. subtilis*, and in secondary sludge for *B. pumilus* (1.2×10^9, 9.6×10^8 and 3.6×10^8 CFU/ml, respectively). The results of enzymatic assays shown *B. licheniformis* and *B. subtilis* produced extracellular cellulase responsible for the depolymerization of cellulose only. However, the cellulases produced in paper sludge by *B. licheniformis* had a higher thermal stability than the ones produced in a conventional semi-synthetic medium. More experiments were conducted with *B. licheniformis*. The supplementation of inorganic and organic nitrogen sources in primary sludge, to decrease the C:N ratio, stimulated *B. licheniformis* growth. The highest cell counts of *B. licheniformis* were obtained in primary sludge having a supplement of yeast extract, followed by peptone, soy flour and ammonium chloride. Pulp and paper primary sludge must then be supplemented with nitrogen source to make it a proper culture medium for *Bacillus* spp.

Keywords

Bacillus, Enzymes, Fermentation, Sludge, Wastewater treatment

Introduction

The high management cost of wastewater sludge and the environmental impacts of land filling in the context of the pulp and paper industry is an undergoing problem. The industry is always looking for economical and environment friendly ways to recycle and reuse its residual biomass. Among the new promising beneficial ways to use this

biomass, many research efforts have been dedicated to the use of wastewater sludge to obtain value-added products (VAPS) through microbial fermentation [**1, 2**]. In fact, wastewater sludge is a cheap raw material for fermentation and it can reduce the production cost of microbial products. It may even be a negative cost material when considering the savings made in waste management cost for a pulp and paper plant. Some works have been published on the microbial fermentation of pulp and paper sludge (PPS) to various products such as biofuels, value-added organic chemicals, enzymes etc. [**3, 4**] All these microbial metabolites and products are being part of the emerging bio-economy. There is here an opportunity for the pulp and paper industry to play a role in this bio-economy by fermenting their sludge. They can contribute to biorefining activities or supply bio-based products to their host community [**5**]. They can also integrate biorefining activities in their mills. They may be a possibility of using pulp and paper equipment, with minor adjustments, to produce industrial microorganisms and process their products. The pulp and paper plant can also benefit from with bio-based products obtained from its own sludge. It can finally lead to a simultaneous product diversification, chemical replacement and waste management cost reduction for this industry.

Bacillus spp. are desirable industrial microorganisms that can grow well on sludge. They have high growth rate leading to short fermentation cycle times. They can also release large quantities of metabolites into the culture medium including enzymes [**6**]. In fact, natural or modified strains of *Bacillus* spp. produce enzymes that represent about 50 % of the world enzyme market [**7**]. *Bacillus* spp. are known to be low or medium producers of extracellular cellulases and hemicellulases having enzymatic activity over a wide range of temperature and pH. It was also reported that their enzyme production can be enhanced by the polysaccharides found in the lignocellulosic biomass. Some researchers used agro-forest and industrial residues as cheap fermentation substrates and enzyme synthesis inducers [**8, 9**]. With their high polysaccharides content, PPS could constitute adequate culture media to improve the production of *Bacillus* spp. cellulases and

hemicellulases over other semi-synthetic or complex culture media. However, the C:N ratio, which is an important parameter for microbial fermentation, is generally high in PPS. An additional supply of nitrogen could be needed to make them an adequate culture medium for *Bacillus* spp.

The objective of this study is to investigate the use of PPS as a substrate and an inducer for the production of *Bacillus* spp. cellulases and hemicellulases. Submerged fermentations of primary and secondary PPS were conducted with *Bacillus licheniformis*, *B. pumilus* and *B. subtilis*. Cell growth and enzyme activity were evaluated. Also, the addition of nitrogen to PPS to adjust the C:N ratio was investigated. This research will help to assess the possibility of using PPS to produce cellulases and hemicellulases from fast growing *Bacillus* spp.

Materials and Methods

Microorganisms

B. licheniformis, *B. pumilus* and *B. subtilis* were obtained from the Bacillus spp. collection of the Agriculture and Agri-Food Canada Research and Development Centre (Saint-Jean-sur-Richelieu QC, Canada). The strains were subcultured and streaked on tryptic soya agar (TSA) plates, incubated at 35°C for 24 hours and then preserved at 4°C for future use.

Pulp and paper sludge

Sludge samples were collected at the Kruger Crabtree Mill (Crabtree QC, Canada). The chemical composition of the sludge samples is shown in table 1. The adjustment of the sludge total solids concentration (TS) was carried out by centrifugation at 9000 g min^{-1} for 5 min at 4°C (Multifuge X3 FR centrifuge) or dilution with distilled water. TS was measured according to standard methods [**10**]. The sludge samples were stored for a maximum of one week at 4°C to minimize microbial degradation. The pH of the sludge samples was adjusted to 7.5 with NaOH 4 mol l^{-1}. Then they were sterilized at 121°C for

30 min before being used as culture media. A mixture of primary and secondary sludge (referred as mixed sludge) was also prepared by mixing primary and secondary sludge at a weight ratio of 1:1.

Shake-flask fermentation

A loopful of each *Bacillus* sp. from TSA plates was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of sterilized tryptic soya broth (TSB) medium. The flasks were incubated and agitated at 300 rpm and 35°C for 12h. A 2% (v/v) inoculum from these flasks was then used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of carboxymethylcellulose (CMC) medium or 100 ml of sterilized sludge media. The content of the CMC medium was: CMC (5 g l^{-1}), peptone (5 g l^{-1}), yeast extract (5 g l^{-1}), K_2HPO_4 (1 g l^{-1}); $MgSO_4$ (0.2 g l^{-1}). These flasks were incubated for 12h and used as a second inoculum. The Erlenmeyer flasks (500-ml) containing 100 ml primary, secondary and mixed sludge were inoculated with 2.0% (v/v) of the second inoculum. The flasks were then incubated at 300 rpm and 35°C for 48 h. Culture samples were withdrawn from the flasks at 3-hour intervals to determine the total cells and viable spores.

Determination of the total cell count

To determine the total cell count, the samples were serially diluted with sterilized saline water (0.9% NaCl). A 0.1 ml of the diluted samples (10^{-6}-10^{-8}) was spread on TSA plates and incubated at 35°C for 24 h. Cell counts are reported as colony forming unit per milliliter (CFU ml^{-1}). The standard deviation of total cell count measurements was 5%.

Qualitative estimation of the cellulolytic activity

Fermented broths were centrifuged at 10 000 g at 4°C for 10 min to harvest crude enzymes in the supernatant. Plates were prepared with 1.5% added agar and 2 % (w/v) commercial cellulose (CMC or Avicel). A 100 µL aliquot of the crude enzyme was dropped in the middle of the Petri dishes in which a 1cm-diameter spot of agar was

removed. The plates were kept at 4°C for 3 h, then incubated at 30°C for 24 h and subsequently flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 3 to 5 minutes. The binding of the iodine reagent with CMC unhydrolysed polysaccharides creates a blueish black coloration of the agar medium. The enzyme activity was estimated through the presence of a clear zone where the substrate was degraded into monosaccharides [**11**]. The crude enzymes, treated by boiling at 100°C for 15 min were used as negative control.

Enzymatic activity

All enzyme activities were determined using the same experimental conditions: 50°C and pH 7.0, adjusted with a 0.05 mol l^{-1} acetate buffer. Only the reaction time varied. Cellulase activities were determined in terms of carbomxyethylcellulase (CMCase) and hemicellulases were determined in terms of xylanase. The CMCase activity was determined by measuring the release of reducing sugars from the enzymatic hydrolysis of 2 % CMC for 30 min according to the dinitrosalicylic acid (DNS) standard method [**12**]. The reducing sugars released from the enzymatic hydrolysis of 1 % birchwood xylan for 15 min were measured to calculate the xylanase activity according to previously described methods [**13**]. One unit of activity (IU) of CMCase or xylanase is defined as the amount of enzyme that releases 1 μmol of glucose or xylose, respectively as reducing sugar equivalent per minute.

Enzymatic hydrolysis

Extracellular enzymes were separated from the fermented broth by centrifugation (10000 *g*, 10 min, 4°C) and used directly as enzyme cocktails for hydrolysis. Saccharification experiments were performed in 125-ml shake flasks with a working volume of 45 ml [**14**]. Commercial celluloses, namely Avicel and CMC, were used as substrates for the enzymatic hydrolysis assays. The enzymatic digestibility tests were carried out at 50°C and pH 7.0. Enzyme loading was 0.1 mL supernatant per g substrate. The

monosaccharide content of hydrolysed samples was determined by anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) with a Dionex ICS-5000 system. C5 and C6 sugars were separated using CarboPAC® PA100 columns at 30°C with 0.05 mol l^{-1} NaOH used as eluent at a flowrate of 1 ml l^{-1}. Hydrolysate sugar concentrations were quantified using glucose and cellobiose standards.

Results and discussions

Growth of Bacillus spp. in pulp and paper sludge

The growth profiles of the culture of *B. licheniformis*, *B. subtilis* and *B. pumilus* using PPS as culture media for a 72 h fermentation time are illustrated in Fig 1. *B. licheniformis*, *B. subtilis* and *B. pumilus* were able to grow in secondary and mixed sludge. In addition, the mixed sludge (MS) was the best culture medium, followed by secondary and primary sludge (SS and PS). PS has high carbohydrate content, mostly due to the presence of cellulosic residues, which could serve as a carbon source for the bacilli. Meanwhile, SS, generated by a biological treatment system, contains macro and micro-elements such as nitrogen and phosphorus, that can be used for the bacilli. Thus, MS gives an adequate culture medium in terms of C:N:P ratio for Bacillus spp.. It can also be observed that the growth profiles of *B. licheniformis* and *B. subtilis* in PPS were similar. The highest cell concentrations in the SS and MS were obtained after 48h of fermentation (1.2 x 10^9 and 8 x 10^8 CFU ml^{-1} respectively for *B. licheniformis*, and 9.6 x 10^8 and 5.6 x 10^8 CFU ml^{-1} respectively for *B. subtilis*). On the other hand, the PS supported *B. licheniformis* and *B. subtilis* with the highest cell production at 48h of fermentation (4.8 x 10^8 and 1.1 x 10^8 CFU ml^{-1} respectively for *B. liheniformis* and *B. subtilis*), but not the growth of *B. pumilus* . However, *B. pumilus* did not grow in the primary sludge. This type of sludge contains other components like lignin degradation products and ash (from inorganic additives used in papermaking) that might inhibit specifically the growth of *B. pumilus* [**15, 16**] and not *B. licheniformis* and *B. subtilis*.

Production of cellulolytic and hemicellulolytic enzymes

The cellulase assay on agar plates allowed the screening of the cellulases production by *Bacillus* spp. in PPS. Results are presented in Fig 2. Distinct and clear zones of cellulose hydrolysis were obtained on agar plates inoculated with the crude enzymes produced by *B. licheniformis* and *B. subtilis* in primary and mixed sludge. This indicates that *B. licheniformis* and *B. subtilis* produce cellulose degrading enzymes in PPS. Previous studies shown that *Bacillus* spp. produce the endo – β – 1,4 – glucanase only and does not produce the complete cellulase enzyme complex to convert cellulose into monosugars [**17, 18**]. Therefore, the activity of the enzymes of Bl and B. subtilis was measured by the DNS method along with the performance of saccharification using these enzymes to produce reducing sugars. There were no reducing sugars of glucose or cellobiose detected as shown in table 2. Then, in the present case, there is a possibility that the cellulase enzyme systems produced by *B. licheniformis* and *B. subtilis* in PPS consists of endoglucanases responsible for the hydrolysis of the internal glycosidic bond and the decrease of the cellulose chain length. Interestingly, in the case of *B. licheniformis*, the clearance zone was still observed on the agar plates when enzymes were supposed to be deactivated by heat. It suggests that the enzyme remained active after a heat treatment and had a higher thermal stability.

As observed in Fig 2c, there was no zone of clearance in agar plates with added crude enzymes of *B. pumilus*. Also, no difference was observed between these plates and the plates with the added deactivated enzymes of *B. pumilus*. It suggests that *B. pumilus* did not produce cellulase. In addition, it has been demonstrated that *B. pumilus* produced xylanases from PPS [**19**]. Then, *B. pumilus* produced cellulase free xylanases from PPS. It could be interesting for the pulp and paper industry to use *B. pumilus* cellulase free xylanases for biobleaching **[20]**.

The screening of cellulase and xylanase activity on agar plates of the three Bacillus in PPS is summarized in Table 2. Cellulase and avicelase activities were detected in *B.*

licheniformis and *B. subtilis*, whereas xylanase activity was detected in *B. pumilus*. For *B. licheniformis* and *B. subtilis*, the PS and MS supported cellulase production due to the presence of cellulose residues in PS that can induce enzyme synthesis. As discussed earlier, the SS supported the growth of *B. licheniformis* and *B. subtilis* but do not induce the cellulase production of these microorganisms. For *B. pumilus*, only the MS could support both the growth and xylanase production.

Effect of nitrogen supplementation on the growth and cellulase production of B. licheniformis

As the PS was assumed not to have enough nitrogen content for *Bacillus spp.* to grow as well as in SS, various supplemental nitrogen compounds were studied in the culture of *B. licheniformis*. Fig 3 presents the growth of *B. licheniformis* in the PS with addition of different organic or inorganic nitrogen sources such as yeast extract, peptone, soya flour, ammonium chloride, ammonium nitrate, ammonium phosphate and ammonium sulphate. The supplementation of each nitrogen source increased the growth of *B. licheniformis* while the exponential phase was achieved after 24h of fermentation instead at 36h when there was no supplement. Higher cell counts were obtained with organic nitrogen supplements. The highest cell counts were obtained in the medium with the yeast extract (1.4×10^9 CFU ml^{-1}), followed by peptone (1.4×10^9 CFU ml^{-1}) and soya flour (9.8×10^8 CFU ml^{-1}). The addition of inorganic nitrogen also enhanced the growth and the highest cell counts were obtained with ammonium chloride (6.0×10^8 CFU ml^{-1}). In addition, all the crude enzymes produced by *B. licheniformis* in the PS with added nitrogen sources exhibited CMCase and Avicelase activities. Thus, the use of PS supplemented with nitrogen sources for the fermentation of Bacillus spp. such as *B. licheniformis* is a promising way for sludge disposal and to simultaneously obtain cellulolytic enzymes at low cost.

Conclusions

PPS can support the growth of *B. licheniformis*, *B. subtilis* and *B. pumilus* while the mixed sludge (primary and secondary sludge) is the best PPS based culture medium. In this culture medium, *B. licheniformis* and *B. subtilis* were able to produce endoglucanase responsible for cellulose depolymerization in mixed sludge. Primary sludge served as cellulase inducer and the secondary sludge was a nutriment source. Cellulolytic enzymes of *B. licheniformis* produced in PPS have an improved thermal stability. Supplemental supplies of both inorganic and organic nitrogen compounds to the primary sludge made it a better culture medium for the growth and cellulase production of Bl. The fermentation of PPS with Bacillus spp. is certainly an interesting option for the pulp and paper industry to obtain value added products from their sludge. It could become part of the new bioeconomy by supplying biorefineries with low cost cellulolytic enzymes.

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Conflict of interest

I declare that we have no conflicts of interest in the authorship or publication of this contribution.

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Table 1 Chemical analysis of PPS samples

Properties	Primary sludge	Secondary sludge
pH	6.5 – 6.8	5.8 – 6.2
Total solids (g/L)	40.0 ± 0.5	12.0 ± 0.5
Suspended solids (g/L)	37.0 ± 0.5	8.0 ± 0.5
Dissolved solids (g/L)	0.80 ± 0.02	3.80 ± 0.02
Ashes (g/L)	0.28 ± 0.02	0.080 ± 0.005
Total organic matter (g/L)	3.70 ± 0.02	4.50 ± 0.02
Elemental analysis		
Nitrogen (% w/w)	0.33 ± 0.01	4.34 ± 0.02
Carbon (% w/w)	39.44 ± 0.01	42.50 ± 0.01
Hydrogen (% w/w)	5.47 ± 0.01	6.39 ± 0.01
Sulphur (% w/w)	LOQ	1.23 ± 0.01

Table 2 Cellulase and xylanase activities detected by colorimetric plate (CP) assay, DNS assay and ion chromatography (IC)

	B. licheniformis						*B. subtilis*						*B. pumilus*	
	CMCase			Avicelase			CMCase			Avicelase			Xylanase	
	CP	DNS	IC	CP	DNS	IC	CP	DNS	IC	CP	DNS	IC	CP	DNS
PS	++	nd	nd	++	nd	nd	++	nd	nd	++	nd	nd	nd	nd
SS	+-	nd	nd	+-	nd	nd	+-	nd	nd	+-	nd	nd	+-	0.2 IU
MS	++	nd	nd	++	nd	nd	++	nd	nd	++	nd	nd	++	2.5 IU

++ : Positive activity; +- : Very low activity; nd : none detected; na : not applicable

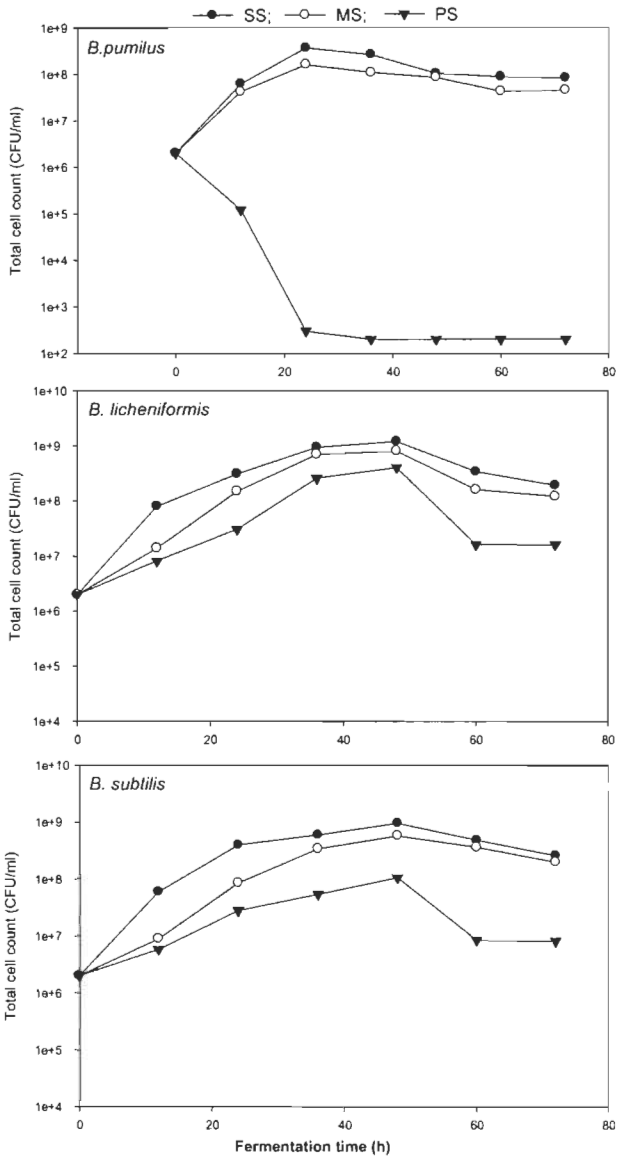

Fig. 1 Growth of *Bacillus sp.* on different sludge types

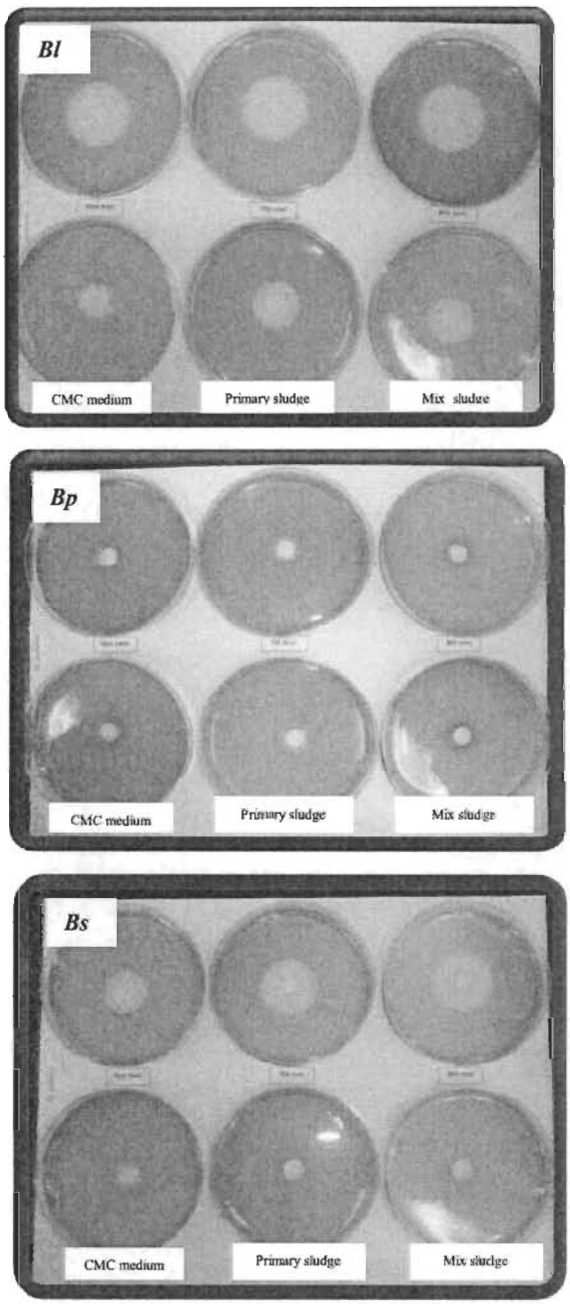

Fig. 2 Cellulase detection in CMC agar plates. Top: active enzyme; bottom: deactivated enzyme.

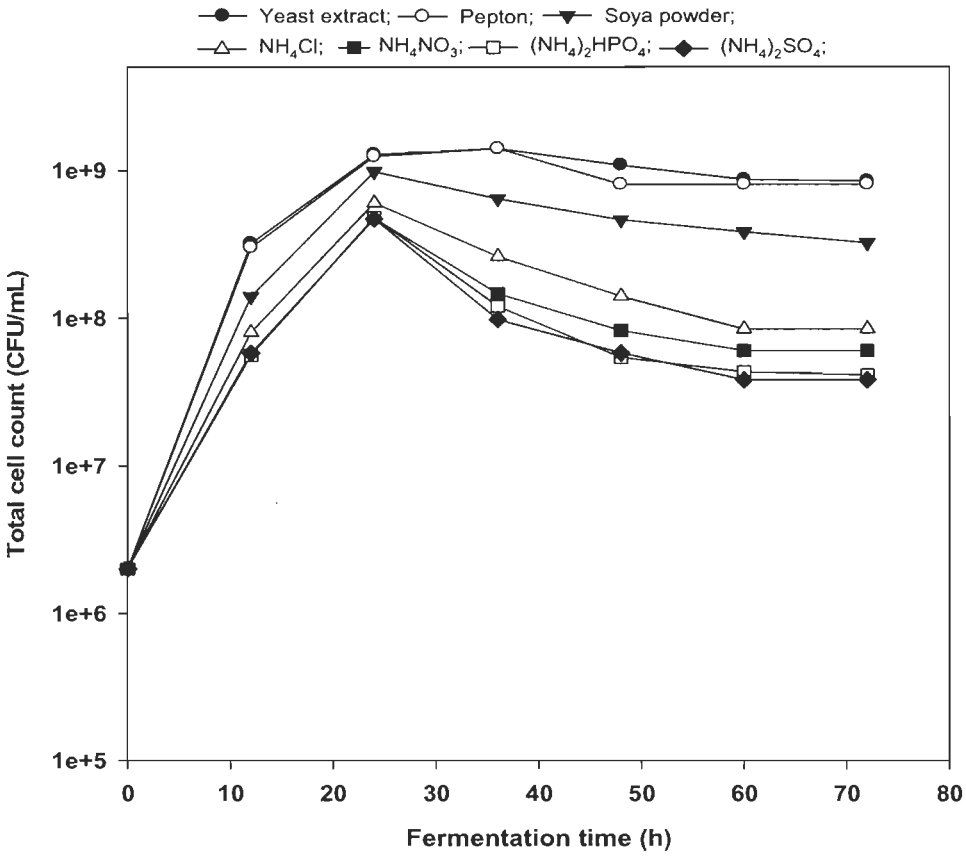

Fig. 3 Effect of nitrogen sources on *B. licheniformis* growth in PS

Chapter 5. Production of *Trichoderma reesei* RUT C-30 lignocellulolytic enzymes using paper sludge as fermentation substrate : an approach for on-site manufacturing of enzymes for biorefineries

5.1. Preface

This article is published. It compiles and discusses the experiments conducted on *Trichoderma reesei* RUT C-30 fermentation using pulp and paper sludges. As first author, Thanh-Tunng Lai made all the experiments and wrote the article. Co-authors Thi-Thanh-Ha Pham and Kokou Adjallé assisted in preparing the experiments and doing the analysis. They have also contributed to the writing of this article. Co-authors François Brouillette and Simon Barnabé contributed to the writing of this article and to the data interpretation. They have also made the final correction. Co-author Daniel Montplaisir contributes to the experimental plan and to the funding of the laboratory works.

5.2. Article no.2

Production of Trichoderma reesei RUT C-30 lignocellulolytic enzymes using paper sludge as fermentation substrate : an approach for on-site manufacturing of enzymes for biorefineries

Thanh Tung Lai, Thi Thanh Ha Pham, Kokou Adjallé, Daniel Montplaisir, François Brouillette, Simon Barnabé*

Lignocellulosic Materials Research Centre, Université du Québec à Trois-Rivières, 3351, boul. des Forges, Trois-Rivières, Canada, G9A 5H7

(*Corresponding Author: E-mail: simon.barnabe@uqtr.ca; Phone: 1(819) 376-5011 # 4531; Fax: 1(819) 376-5148)

Abstract

Different types of pulp and paper sludge were used as raw materials for the production of a cocktail of lignocellulolytic enzymes of Trichoderma reesei RUT C-30 (T.reesei RUT C-30). The fungus were grown in pellets to produce cellulases (carbomethylcellulase, β-glucosidase) and hemicellulases (xylanase, β-xylosidase) in three types of sludge: primary (PS), secondary (SS) and mixed sludge (MS). The highest carbomethylcellulase activities obtained after 7 days of fermentation were 7.3, 4.8, and 1.5 IU/ml in MS, SS, and PS, respectively. Sludge modification such as the mixing SS and PS at 1:1 (v/v) ratio, the addition of ammonium sulfate as an inorganic nitrogen source, and the increase of the solids content were shown to improve enzyme production. The crude enzyme mixture obtained from the sludge samples showed a synergistic effect to hydrolyze various biomasses into monosugars. The tested biomasses included highly purified CMC, xylan from birch wood and lignocellulosic materials (corn stover and primary pulp and paper sludge). The addition of a surfactant (polysorbate 20) to the enzyme cocktail enhanced the saccharification efficiency of the sludge, in particular the hydrolysis of the corn stover which contains lignin. The obtained results contribute to the assessment of the feasibility of on-site low cost enzyme production at paper mills generating the sludge, or for neighboring or local biorefineries.

Keywords

Cellulase, enzyme, on-site manufacturing, paper sludge, lignocellulosic biomass, *Trichoderma reesei*, xylanase.

Introduction

Lignocellulosic biomass is a renewable and abundant source of carbohydrates that can be converted into simple sugars to produce biofuels and biochemicals through microbial fermentation technologies. Agricultural and industrial residual biomasses are an interesting alternative to crop biomass as secondary generation feedstock for biorefinery that would not contribute to the global food crisis. However, high amounts of various saccharolytic enzymes are required to convert complex lignocellulosic materials into fermentable sugars. This particular situation contributes to the high cost of industrial scale biofermentation processes. Several solutions are being investigated to minimize enzyme consumption. Among them, engineering of cellulase enzymes with high hydrolytic efficiency and yield, biomass pretreatment, and alternate raw materials for enzyme production are particularly promising [**1, 2, 3**].

Various lignocellulosic residues have already been successfully used as carbon source and cellulase inducers in the cultivation of cellulolytic bacteria and fungy. Saccharolytic enzymes with high specific activities were obtained [**4, 5, 6**]. However, competition for the biomass supply could arise from the fact that the same material, which contains high amounts of cellulose, would be used for the production of enzymes and also as a feedstock for cellulosic ethanol manufacturing. This could result in both enzyme and ethanol yield reductions [**7**]. Waste streams from agricultural and forestry industries, such as wastewaters and wastewater sludge, contain carbohydrates potentially capable of inducing cellulosic enzymes production and essential nutrient for microbial culture [**8, 9**].

Several studies have reported the successful production of enzymes from semi-alternate media containing paper sludge as a carbon source [**8, 10, 11**]. Paper sludge contains high amounts of organic material and water that are suitable for the submerged culture fermentation of industrial microorganisms. In addition, residual cellulose fibers found in

paper sludge can induce the enzyme secretion of cellulolytic bacteria and fungi. In consequence, replacement of the whole synthetic medium with paper sludge would provide a low cost culture medium for enzyme production. Moreover, an on-site concept of enzyme production from paper sludge located near bioethanol plants can be applied to reduce biofuel production cost, since the produced enzymes are directly used for biomass-to-sugar conversion without any need for stabilization, formulation or transportation [**12**].

The present work focuses on the utilization and modification of paper sludge as a culture medium for *T.reesei* RUT C-30 saccharolytic enzyme production. Enzymatic activity and hydrolysis assays were conducted by using the fermented broth on different biomasses to evaluate the saccharification efficiency of the enzyme cocktail.

Materials and Methods

Strain

The fungus *T.reesei* RUT C-30 (ACCT 56765) was used in this study. The fungal culture was recovered from freeze-dried spores (2 days at 25°C and 200 rpm) in a potato dextrose broth. A loop of *T.reesei* RUT C-30 cells inoculum was transferred to potato dextrose agar slants and incubated for 7 days at 25°C. The greenish conidia, appearing on the slants, were suspended in sterile saline water (0.9 % NaCl) and harvested to determine the spore concentration with a hemocytometer equipped with a microscope Axio scope.A1 (Carl Zeiss company) [**13**]. The harvested spores were stored in 10% glycerol at -80°C [**14**].

Culture media

A synthetic Mandel medium was prepared with the following composition: 10 g/L carboxymethylcellulose (CMC); 0.3 g/L urea; 1.4 g/L $(NH_4)_2SO_4$; 2.0 g/L KH_2PO_4; 0.3 g/L $CaCl_2$; 0.3 g/L $MgSO_4$; 0.25 g/L yeast extract; 0.75 g/L proteose peptone, and trace elements (5 mg/L $FeSO_4.{}_7H_2O$; 20 mg/L $CoCl_2$; 1.6 mg/L $MnSO_4$ and 1.4 mg/L $ZnSO_4$).

Primary (PS) and secondary (SS) paper sludge samples were collected from the wastewater treatment facility of the Kruger Crabtree mill, Quebec, Canada. The total solids concentration of the sludge samples was adjusted to 15 g/L. The SS (initial solids concentration of 8 g/L) was concentrated by gravity clarification for 1 day at 4°C followed by centrifugation at 3 000 x *g* for 5 min at 4°C (Multifuge X3 FR, Thermo Scientific). The supernatant was used to dilute the precipitate to targeted solids concentrations. The PS (initial solids concentration of 40 g/L) was diluted with demineralized water to 15 g/L. Mixed sludge (MS) was prepared by mixing the 15 g/L PS and SS to different ratios. The pH of both synthetic and sludge-based media was adjusted to 5 with 1 N HCl before sterilization. The chemical characterization of sludge was followed standard methods for examination of water and wastewaters [**15**] and Sluiter et al. [**16**]. Sludge properties are presented in **Table 1**.

Cellulase production

A starter culture of *T.reesei* RUT C-30 was prepared by transferring 4 ml of a 1.0×10^8 spore/ml stock culture in 200 ml of potato dextrose broth and inoculating in a shaking incubator at 25°C and 200 rpm for 2 days. A 10% v/v solution of the actively growing cells (starter culture) was used as inoculum for enzyme production by shake-flash fermentation.

Submerged culture fermentation was carried out in 1-L shake flask with 200 ml of working volume at 25°C and 200 rpm for 10 days. Samples were taken at 1-day intervals. The fermented broth supernatant containing extracellular enzymes was separated from the fungal biomass by centrifugation at 10 000 x *g* for 10 min at 4°C for further enzyme activity assays.

Enzyme activity

All enzyme activities were determined using the same experimental conditions: 50°C and pH 4.8, adjusted with a 0.05 M acetate buffer. Only the reaction time varied.

Cellulase activities were determined in terms of carbomethylcellulase (CMCase) and β-glucosidase while hemicellulases were determined in terms of xylanase and β-xylosidase. CMCase activity was determined by measuring the release of reducing sugars from the enzymatic hydrolysis of 2 % CMC for 30 min according to the dinitrosalicylic acid (DNS) standard method [**17**]. The reducing sugars released from the enzymatic hydrolysis of 1 % birchwood xylan for 15 min were measured to calculate xylanase activity according to previously described methods [**18, 19**]. One unit of activity (IU) of CMCase or xylanase is defined as the amount of enzyme that releases 1 μmol of glucose or xylose, respectively as reducing sugar equivalent per minute.

β-glucosidase and β-xylosidase activities were determined by the enzymatic hydrolysis of 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-β-D-xylanopyranoside for 10 min. The release of 4-nitrophenol was measured according to method C210-00 of Genecor (now Dupont Industrial Biosciences), which using a modification of the assay described by Hagerdal et al [**20**]. A 1 ml portion of a 1 mM substrate was heated to 50oC. Then, 0.2 ml of the diluted sample was added to the substrate, and the reaction was stopped by adding 0.5 ml of 1 M sodium carbonate. The solution was diluted by 10 ml distilled water and mixed well by vortex. The absorbance was read at 400 nm. One IU of β-glucosidase or β-xylosidase is defined as the amount of enzyme that releases 1 μmol of 4-nitrophenol per minute.

Enzymatic hydrolysis

Cultivations of *T.reesei* RUT C-30 in Mandel and MS media were carried out in 2-L shake flasks for 7 days under the conditions described above. Extracellular enzymes were separated from the fermented broth biomass by centrifugation (10 000 x *g*, 10 min, 4°C) and used directly as enzyme cocktails for hydrolysis. Saccharification experiments were performed in 125-ml shake flasks with a working volume of 45 ml according to the NREL protocol [**21**]. Commercial celluloses, namely Avicel pH101 and CMC, were used as substrates for the enzymatic hydrolysis assays. Lignocellulosic residues

including filtered PS and corn stover pretreated with 5 % NaOH at 105°C in a twin-screw extruder were also used. The total sugar content of substrate were 97%, 92%, 64% and 33,5% for Avicel, CMC, PCS and PS respectively. The substrate suspended in a 0.05 M sodium citrate buffer to 2% solid loading (w/v), were sterilized at 121°C for 20 min and cooled down to ambient temperature before enzyme addition. The enzymatic digestibility tests were carried out at pH 4.8, temperature varied from 50 to 70 oC and surfactant varied 0.5 to 2% tween 20. Enzyme loading is 266 CMCase IU per gram of substrate. The commercial enzyme - Accellerase 1500 of Genecor (now Dupont) was used as positive control. The monosaccharide content of hydrolysed samples was determined by anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) with a Dionex ICS-5000 system. C5 and C6 sugars were separated using CarboPAC® PA100 columns at 30°C with 50 mM NaOH used as eluent at a flow rate of 1 ml/L. The injected volume was 10 µL. Hydrolysate sugar concentrations were quantified using glucose, fructose, xylose, mannose, arabinose, galactose, and cellobiose standards.

Statistical analysis

The experiments of fermentations and enzymatic hydrolysis have been performed in duplicate in which the results were analysed using software Sigmaplot 13. Enzyme activity data are presented with the standard deviation of triplicates. The significance of variation in sugar concentrations at different enzymatic hydrolysis was determined by considering sample triplicates using paired t-test at $p < 0.01$.

Results and Discussion

Growth of T.reesei RUT C-30 in paper sludge

The growth of *T.reesei* RUT C-30 was observed through the formation of pellets in all three sludge media (PS, SS and MS). *T.reesei* RUT C-30 grew mainly in mycelia form in the synthetic medium, meanwhile pellets dominated in fungal morphology in the

sludge based media. Loose, fluffy and hollow spherical pellets were formed from the close attachment of fungal mycelia on sludge insoluble matters. This observation is consistent with many other studies reporting that the presence of insoluble substrates stimulates pellet formation at high agitation speed in submerged fermentation [**22**, **23**]. Interestingly, the prevalence of pellets may help to reduce the medium viscosity thus improving mass transfer during fermentation. The pellet formation may also promote a simple downstream recovery of enzymes.

Characterisation of saccharolytic enzymes produced from paper sludge

Paper sludge was able to support the growth of *T.reesei* RUT C-30 and its enzyme secretion. The profile of CMCase activity with time, shown in **Fig.1**, demonstrates that the highest CMCase activity for MS, SS, and PS enzymes obtained after 7 days of fermentation are 7.3, 4.8, and 1.5 IU/ml, respectively. Since *T.reesei* RUT C-30 needed time to adapt to the complex composition of the sludge samples, the production of enzyme in the sludge media occurred 3 days later than in the synthetic medium with the highest activity obtained on the fourth day (data not shown). Moreover, MS was the better medium for enzyme production compared to the PS and SS. In pulp and paper wastewater treatment systems, PS is generated from wastewater sedimentation. It contains mainly cellulosic fibers residues and waste that could supply cellulose and xylan for cellulase and xylanase induction. The same observation on enzyme activity was reported by Wang et al. working on cellulase production of *T.reesei* RUT C-30 on Kraft paper mill sludge [**24**]. SS, generated from activated sludge treatment, contains nutritive elements from endogenous activity of bacteria that Activities of cellulolytic and hemicellulolytic enzymes of Mandel, SS and MS were continuously measured and highest results are presented in **Table 2**. The mixture of enzymes responsible for the complete conversion of biomass into monosugars including CMCase, Xylanse, β-glucosidase, and β-xylosidase was found in both SS and MS enzymes. The proportion of β-glucosidase per CMCase was very low with 9%, 10% and 12% for SS, Mandel and MS, respectively. The same low level of β-glucosidase and β-xylosidase production in

paper sludge was also reported previously [**8**]. In fact, many studies proved that a low secretion of these enzymes is an intrinsic character of *T.reesei* RUT C-30 strains. In addition, the major part of these enzymes is tightly bound to the cell wall of the fungus during cultivation [**2, 25**]. The higher enzyme mixture yield was obtained in MS. CMCase and xylanase were 1.7 and 2.1 times higher than in SS. This confirmed the important role of PS in the induction of both cellulases and hemicellulases. For this reason, the utilization of MS as enzyme production medium could be the best choice for the production of a lignocellulolytic enzyme cocktail from paper sludge.

Improvement of sludge-based culture media for enzyme production

Paper sludge composition, which is known to be variable, can influence the performance of microbial fermentation. The primary/secondary mixing ratio, the C/N ratio, and solids concentration are important parameters that might need to be adjusted to stabilize the sludge and make it a proper culture medium to improve enzyme production [**26**].

Modification of mixing ratio

As mentioned above, fermentation using MS reached higher enzyme activity than using only PS or SS. Residual cellulose in PS could be required for *T.reesei* RUT C-30 enzyme secretion. However, the primary sludge contains high amount of ash which could be detrimental to fungal growth and consequently to cellulase production. Optimizing the PS to SS ratio is necessary to determine the best fermentation medium. Therefore, different PS to SS mixing ratios (1:3; 1:1; and 3:1 (v/v)) were prepared for fermentation. The 1:1 mixing ratio was the most suitable for enzyme production with the highest CMCase activity of 7.3 ± 0.2 IU/ml as presented in **Fig.2.** Compared to the optimal ratio of 1:1, adding a lower concentration of PS could not meet the demand of substrate inducer of the strain, but adding more substrate could lead to substrate inhibition and lower enzyme production. The presence of more inorganic compounds (ash) could explain this phenomenon. In addition, the high amount of residual cellulosic

fibers found in PS increases the viscosity of sludge medium, thus hindering mass transfer of the strain during fermentation [**27**].

Effect of nitrogen supplementation

The MS have a C/N ratio of 18, which is much higher than that of the Mandel synthetic medium (3.7 C/N) as shown in **Table 3**. It was indicated that MS contains abundant C, but insufficient N for fungal growth and enzyme production. Thus, in order to modify the C/N ratio of MS to meet nitrogen demand, inorganic and organic nitrogen sources (urea, yeast, and peptone) were supplemented in the 1:1 MS. The concentration of nitrogen sources as follows: 1.4 g/L $(NH_4)_2SO_4$ in MS adding inorganic nitrogen (MS+IN); 0.3 g/L urea 0.25 g/L yeast extract and 0.75 g/L proteose peptone in MS adding organic nitrogen (MS+ON). The MS adding total nitrogen (MS+TS) comprised of both inorganic and organic nitrogen at the same concentration as the ones in MS+IN and MS+ON. . As shown in **Fig.3**, supplementation of organic nitrogen compounds, alone or with ammonium sulfate, affected enzyme production. A significant decrease in enzyme activity was observed. Otherwise, the addition of the inorganic nitrogen enhanced enzyme production. Enzyme activity slightly increased from 7.5 to 8.9 IU/ml. Protein rich nitrogen compounds such as peptone and yeast extract could induce a co-secretion of extracellular protease with cellulases. The proteolysis of cellulase by protease was suggested as the main reason for cellulase reduction [**28**].

Effect of total solids concentration

Total solids concentration is one of the most critical parameters of fermentation bioprocesses, especially when residues and waste media are used. High solids concentrations suggest more nutrients for microbial use, but it can lead to a higher viscosity that affects mass transfer during fermentation. Thus the effect of MS solids concentration on enzyme production was evaluated in the 5 to 40 g/L range. As shown in **Fig.4**, enzyme activity shows an increasing profile with solids concentration. The highest enzyme activity (12.6 IU/ml) was obtained at 30g/L solids concentration. The enzyme

activity decreased slightly of 12.2 and 11.8 IU/ml at higher solids concentration of 35 and 40 g/L, respectively. It was reported that the viscosity of PS increased at higher solids concentration [**29**]. That may limit the mass transfer during fermentation and thus inhibiting the growth and enzyme production of the strain.

Effect of temperature and surfactant on enzymatic hydrolysis

Effect of temperature

To investigate the effect of temperature on CMCase activity of fermented broth, an enzymatic essay was carried on using 2 % of substrate concentration at temperature range from 50 °C to 90 °C in 30 min. The results presented in **Fig.5** show that CMCase activity in sludge medium did not change, while in Mandel medium, the CMCase activity decreased significantly (26 %) in temperature range of 50-70 °C . The enzymes produced in complex medium of sludge may have better properties than convenient medium that could be other advantage of using sludge as fermentation medium. Therefore, the enzymatic hydrolysis was carried out at different temperatures from 50 to 70 ° C to investigate the effect of temperature on performing of enzyme during conversion process. Unfortunately, the sugar yield decreased significantly for all substrates at high temperature of 60 and 70°C (**Fig.6**) thus the cellulases of *T.reesei* RUT C-30 produced from the sludge are not stable over 50°C.

Effect of surfactant concentration

Non-ionic surfactants such as polysorbate 20 and 80 are known to have multiple functions in enhancing hydrolysis efficiency: biomass disruptor, enzyme stabilizer, and enzyme effectors [**30**]. **Fig.7** shows the improvement of hydrolysis with the addition of 0.5 to 2 % polysorbate 20 for all substrates. CMC to sugar conversion yield was improved slightly (7 % of sugar released) with the addition of 0.5 % polysorbate 20, but further increases did not have any effect on the hydrolysis. A linear relationship between polysorbate 20 concentration (below 1%) and sugar yield was observed in the hydrolysis

of Avicel, PCS, and PS with an increase in sugar amount of 61 %, 95 %, and 21 %, respectively. The effect of the surfactant addition was more important on the enzyme hydrolysis of insoluble and complex structure substrates, particularly PCS, which was assumed to have high lignin content. The positive effect of the surfactant addition might be explained by a strong affinity of polysorbate 20 for this substrate, leading to a hydrophobic interaction with lignin on substrate surface that prevented unspecific adsorption of the enzymes on lignin [**31, 32**].

Hydrolysis of lignocellulosic biomass

Cultivations of *T.reesei* RUT C-30 in Mandel and 30g/l MS + IN media were carried out in 2 L shake flasks for 7 days. The supernatant of sludge fermented broth of *T.reesei* RUT C-30 was used directly for enzymatic hydrolysis. To evaluate the performance of the enzyme cocktail, different cellulosic biomass were used as substrates. They consisted in Avicel, CMC, lignocellulosic residues (pretreated corn stover (PCS) and primary sludge (PS)). Specific sugar concentrations obtained by hydrolysis were calculated based on total detected sugars. Perform of hydrolysis are presented in **Fig.8**. The main reducing sugars found in all substrate hydrolysates are glucose and xylose, as determined by ion chromatography. In addition, small amounts of arabinose and cellobiose were detected in PCS and Avicel. The presence of monosaccharides in the hydrolysates of all four substrates indicates that there is a good functional and synergistic integration of the sludge-produced enzyme mixture responsible for degrading these lignocellulosic substrates into fermentable sugars. However, the biomass to sugar conversion efficiency of the enzymes was affected by the complexity of the composition and structure of the biomass. For the hydrolyse using the enzyme from sludge, maximum conversion was obtained from CMC (43%), containing mainly soluble and amorphous cellulose, followed by PCS (36.5%). The low rate conversion of Avicel (23%), can be explained by the substrate containing mainly microcrystalline cellulose. Also, the paper sludge-based enzyme cocktail is capable of degrading PS, resulting in 16.2% sugar yield, In the case of the enzymes from Mandel medium, the conversion rate was obtained higher slightly

(52 % for CMC, 28.3 % for Avicel, 46 % for PCS and 20.3 % for PS). The significantly higher conversion was observed on the use of Accellerase 1500 (90 % for CMC, 65 % for Avicel, 75 % for PCs and 45 % for PS). It was noted that the proportion of β-glucosidase per CMCase (20 -25%) of this commercial enzyme cocktails is two-fold higher compared to the one of the enzymes from sludge and Mandel medium. Thus, a supplementation of β-glucosidase to enzyme cocktail from sludge is recommended to improve the conversion. The twin-screw extruder treatment was effective at removing lignin and extractives present in corn stover. These products inhibit enzyme accessibility, resulting in significant sugar amounts in the PCS hydrolysate, the remaining lignin (8%) could be minimized by help of supplemented surfactant 1% [**33, 34**]. PS contains mainly small cellulose fibers and other materials extracted during the deinking process such as ink pigments, clay and adhesives that could hinder the catalyst activity of the enzymes. Previous study suggested that, pretreatment process could improve the sugar content in PS as well improve the digestibility [**24**].

Conclusions

The activity of various lignocellulolytic enzymes, including CMCase, β-glucosidase, xylanase, and β-xyloxidase, was determined in the fermented broth of *T. reesei* obtained with pulp and paper sludge. It was necessary to modify the sludge to obtain a stable and efficient culture medium for *T. reesei* and enhance enzyme production. The optimal PS and SS mixing ratio for enzyme production was found to be 1:1 (v/v) in this particular case. In addition, enzyme production was enhanced with an inorganic nitrogen supplementation and an increase in sludge solids concentration. The enzyme mixture obtained with paper sludge was efficient to hydrolyse pretreated corn stover and the pulp and paper primary sludge into fermentable sugars. Moreover, the sugar yield was improved by adding polysorbate 20 during the hydrolysis of the lignocellulosic substrates. Overall, paper sludge can be used to produce lignocellulolytic enzymes with minimal chemical supplementation. When such paper sludge are available near to a biorefinery using enzymes to convert biomass components into valuable products such

as second generation sugars, on-site production could be considered to decrease the manufacturing cost of enzymes.

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Table 1 Chemical analysis of pulp and paper sludge sample

Properties	Primary sludge	Secondary sludge
pH	6.5 – 6.8	5.8 – 6.2
Total solid (g/L)	40.0 ± 0.5	12.0 ± 0.5
Suspended solid (g/L)	37.0 ± 0.5	8.0 ± 0.5
Dissolved solids (g/L)	0.80 ± 0.02	3.80 ± 0.02
Ashes (g/L)	0.28 ± 0.02	0.080 ± 0.005
Total organics mater (g/L)	3.70 ± 0.02	4.50 ± 0.02
Elemental analysis		
Nitrogen (% w/w)	0.33 ± 0.01	4.34 ± 0.02
Carbon (% w/w)	39.44 ± 0.01	42.50 ± 0.01
Hydrogen (% w/w)	5.47 ± 0.01	6.39 ± 0.01
Sulfur (% w/w)	LOQ	1.23 ± 0.01
Glucan (% w/w)	22.28 ± 0.01	2.10 ± 0.01
Xylan (% w/w)	9.32 ± 0.01	0.61 ± 0.01
Hemicellulose (% w/w)	11.29 ± 0.01	2.72 ± 0.01

Table 2 Specific enzyme activities of *T.reesei* RUT C-30

Sample	CMCase (IU/mL)	ß-glucosidase (IU/mL)	Xylanase (IU/mL)	ß-xylosidase (IU/mL)
SS	4.2 ± 0.1	0.38 ± 0.003	3.1 ± 0.06	0.02
MS (1:1)	7.3 ± 1.18	0.9 ± 0.01	6.6 ± 0.13	0.45 ± 0.004

Table 3 Estimated C/N ratio of synthetic and pulp and paper sludge media

	Synthetic	PS	SS	MS	N added MS
C , % (w/w)	66.7	39.4	42.5	12.5	12.5
N, % (w/w)	18.0	0.3	4.3	0.7	3.4
C/N	3.7	120	10	18	3.7

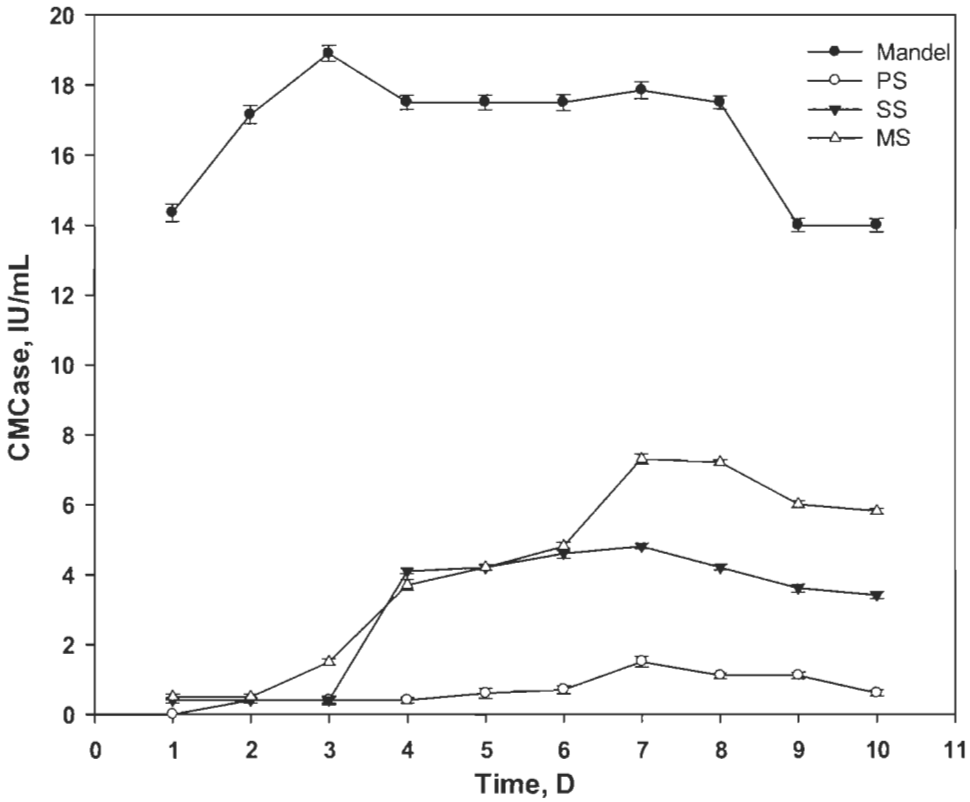

Fig.1 CMCase production during *T.reesei* RUT C-30 fermentation of pulp and paper sludge

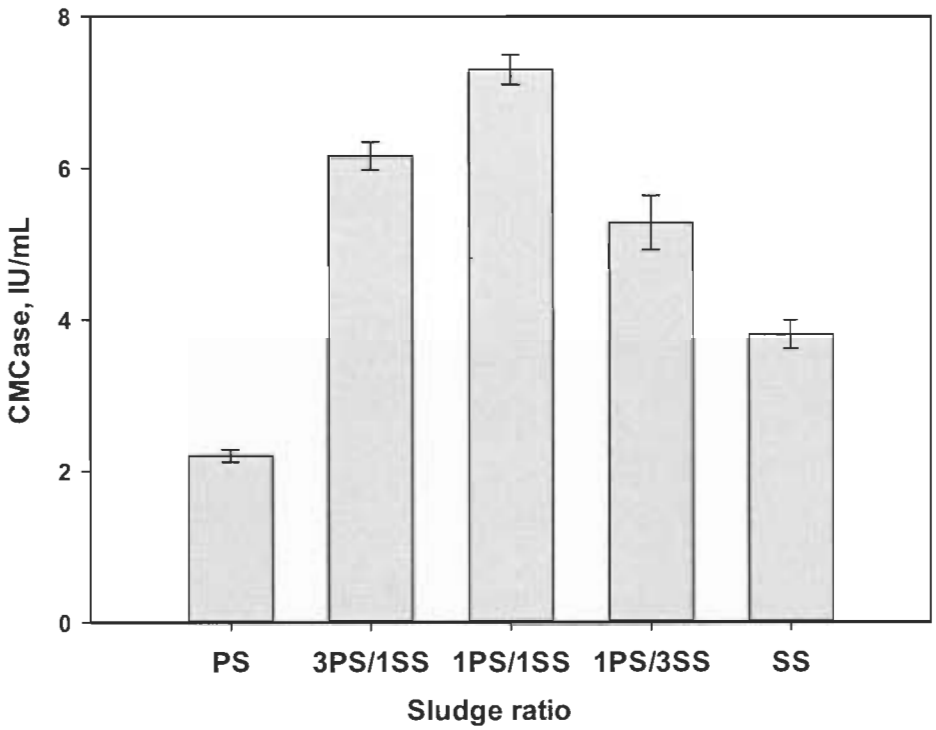

Fig.2 CMCase production at different PS and SS mixing ratios

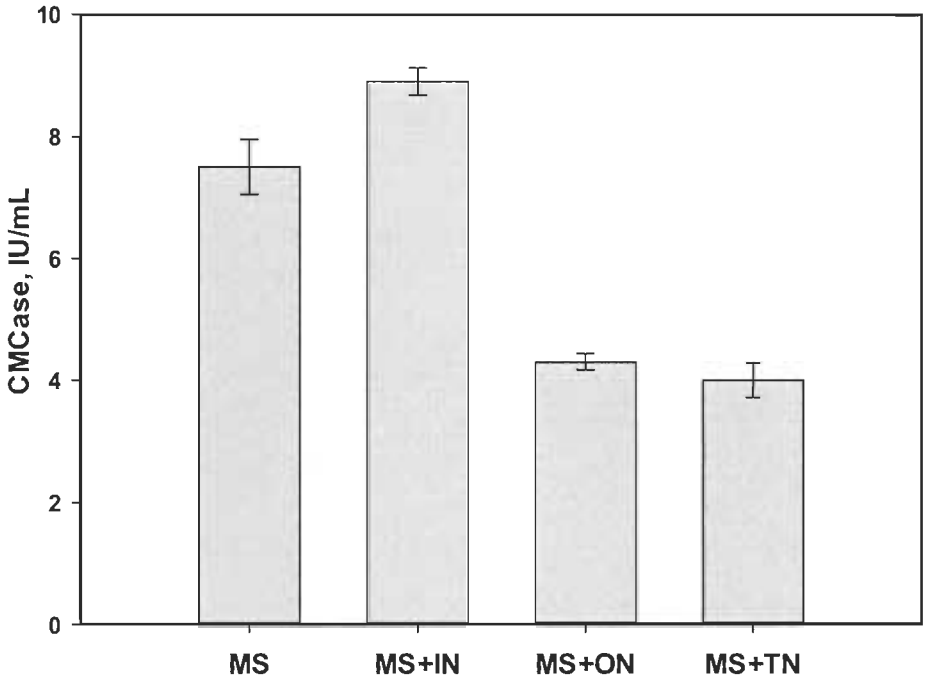

Fig.3 CMCase production with the addition of different nitrogen sources: IN: inorganic nitrogen; ON: organic nitrogen; TN: inorganic and organic nitrogen

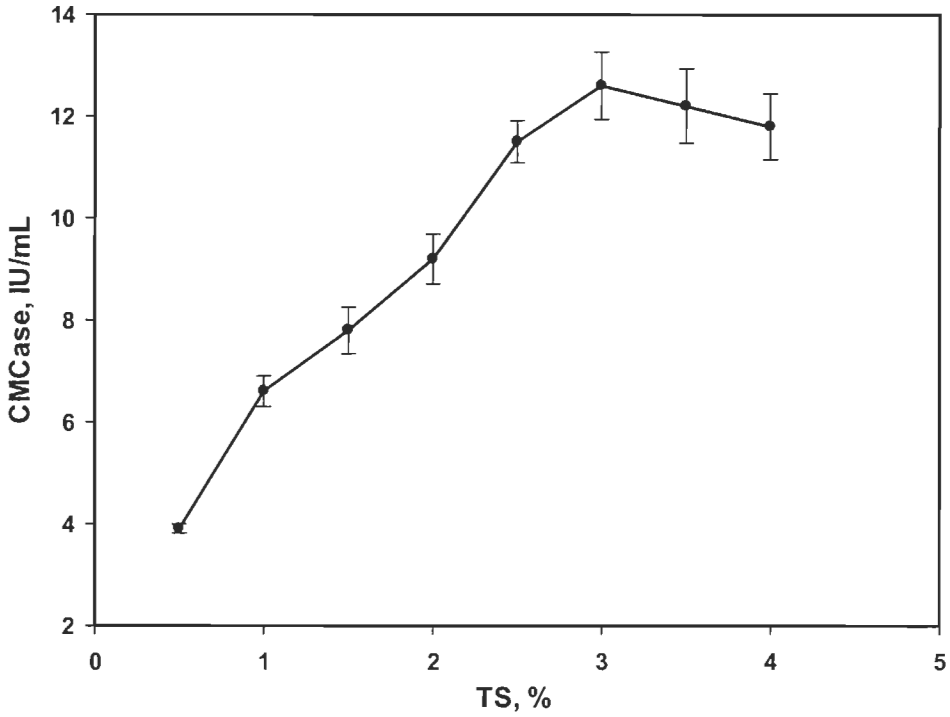

Fig.4 Effect of sludge solids concentration on CMCase production

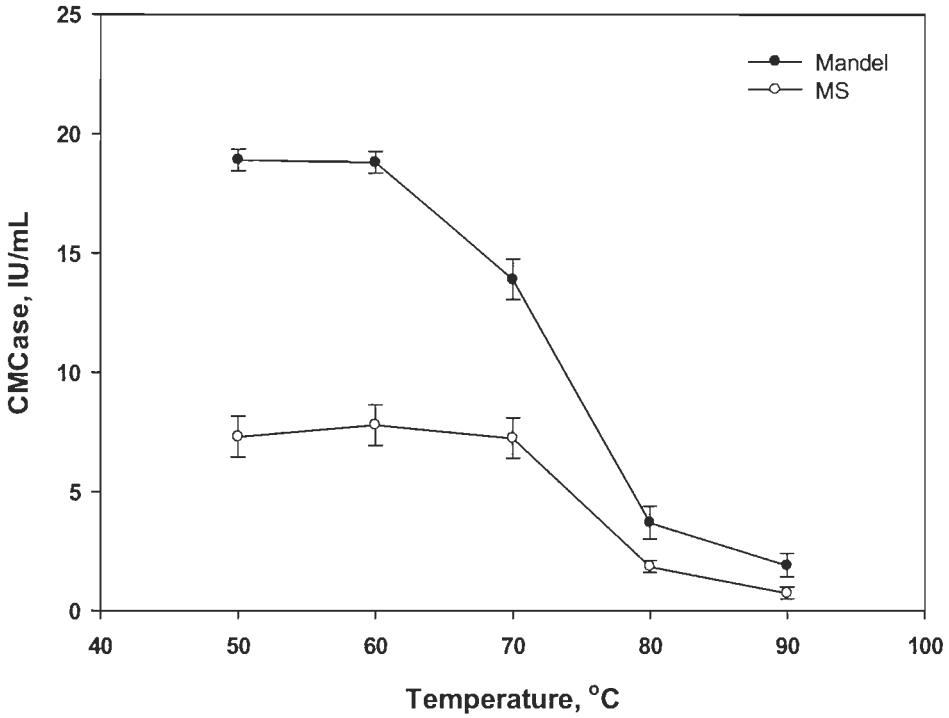

Fig.5 Effect of temperature on CMCase activity (2% substrate in 30 min)

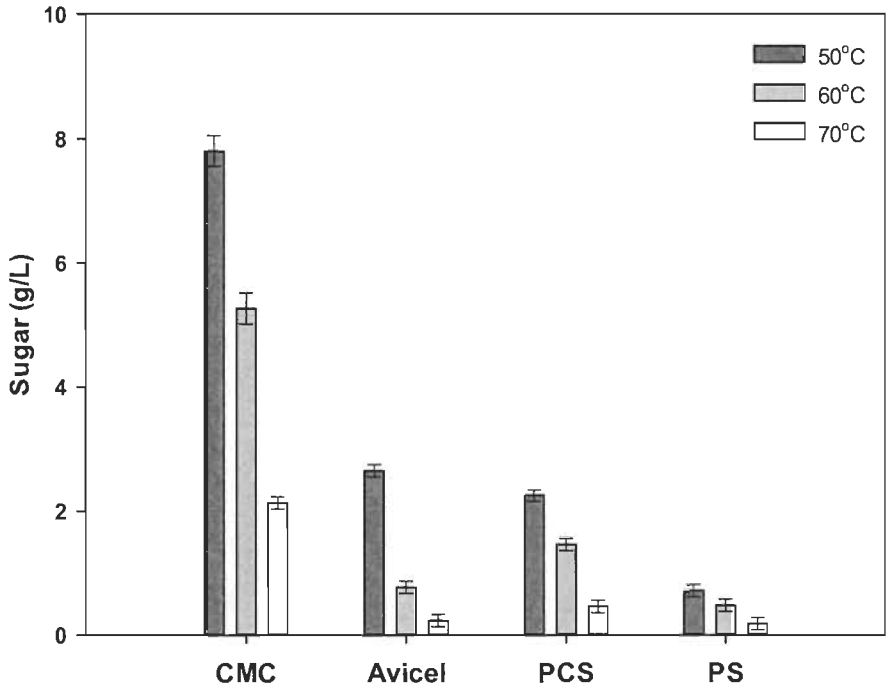

Fig.6 Effect of temperature on enzymatic hydrolysis using sludge fermented broth (2% substrate in 72h)

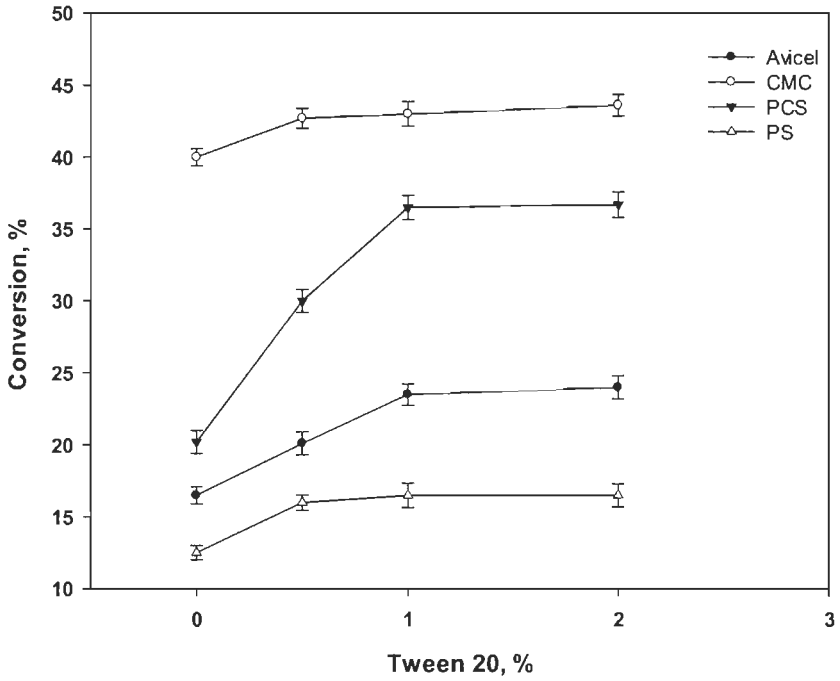

Fig.7 Effect of polysorbate 20 addition on the enzymatic hydrolysis of different substrates (CMC = carboxymethyl cellulose; PCS = pretreated corn stover; Avicel = bacterial microcrystalline cellulose; PS = primary sludge)

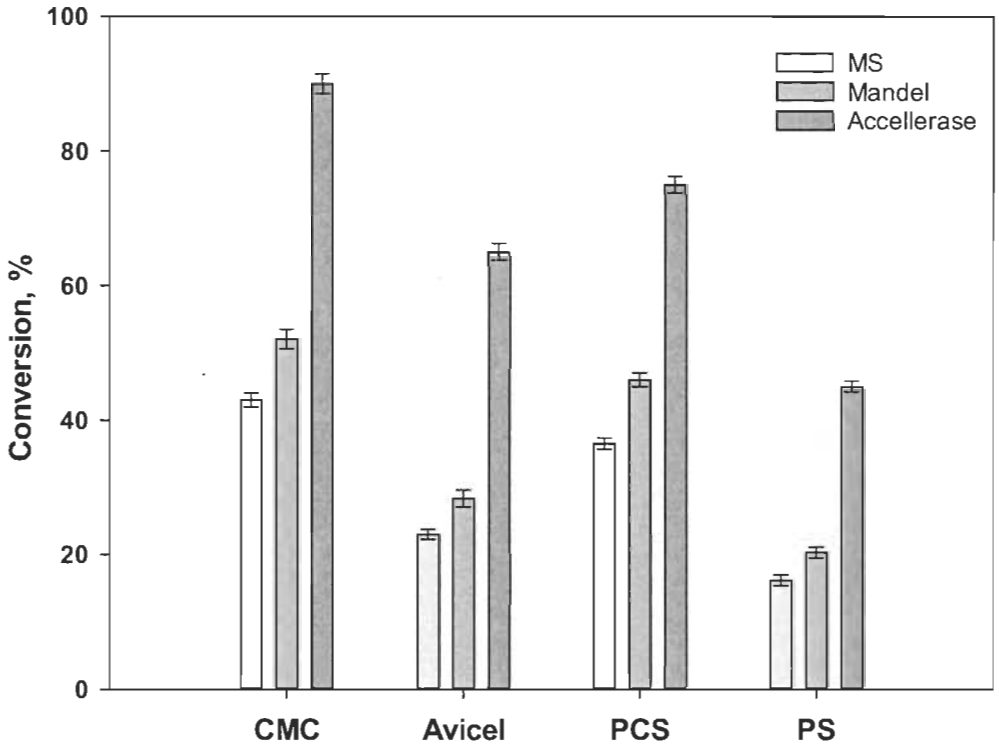

Fig.8 Enzymatic hydrolysis of different substrates at pH 4.8, 50°C, 1% Tween 20 and 72h (CMC = carboxymethyl cellulose; Avicel = bacterial microcrystalline cellulose; PCS = pretreated corn stover; PS = primary sludge)

Chapter 6. Strategies of using pulp and paper sludges as culture media for xylanase production of *Bacillus pumilus*

6.1. Preface

This article is published. It compiles and discusses the experiments conducted exclusivly on *Bacillus pumilus* fermentation using pulp and paper sludges. As first author, Thanh-Tung Lai conducted all the experiments and wrote the article. Co-authors Thi-Thanh-Ha Pham and Kokou Adjallé assisted in preparing the experiments and doing the analysis. They have has also contributed to the writing of this article. Co-authors François Brouillette and Simon Barnabé contributed to the writing of this article and to the data interpretation. They have also made the final correction.

6.2. Article no.3

Strategies for using pulp and paper sludges as culture media for xylanase production with Bacillus pumilus

Thanh Tung Lai, Thi Thanh Ha Pham, Kokou Adjallé, François Brouillette, Simon Barnabé*

Université du Québec à Trois-Rivières, 3351, boul. des Forges, Trois-Rivières, Canada, G9A 5H7

(*Communicating Author: E-mail: simon.barnabé@uqtr.ca; Phone: 1(819) 376 5011 # 4531; Fax: 1(819) 376 5148)

Abstract

Fermentation of *Bacillus pumilus* (*Bp*) using different pulp and paper sludges as culture media were performed in this work to produce at lower cost industrial enzymes such as xylanases. Secondary sludge was shown to be a suitable alternative culture medium for

Bp growth, while primary sludge may serve as xylanases inducer. Mixing primary (PS) and secondary (SS) sludges at 1PS : 2SS (w/w) ratio having 15 g/L total solids concentration resulted in the highest cell concentration of 2 x 10^8 CFU/mL and the highest xylanase activity of 3.8 IU/mL under shake flask fermentation. Other lignocellulosic biomasses were tested as potential xylanase inducers. Addition of corn stover to SS showed the highest xylanase activity (10.7 IU/mL). When using a 7 L bioreactor, total cell concentration and xylanase activity obtained in the secondary sludge medium supplemented with commercial xylan (2.5 x 10^9 CFU/mL and 35.5 IU/mL, respectively) and corn stover (3.4 x 10^9 CFU/mL and 37.8 IU/mL, respectively) were comparative to a semi-synthetic based medium (5.8 x 10^9 CFU/mL and 47 IU/mL, respectively). The xylanase activity of *Bp* produced in paper sludge is stable at pH 6-9 at 50°C that offered a potential application of the enzyme for biobleaching in pulp and paper industry.

Key words: *Bacillus pumilus*, xylanase, pulp and paper sludge, lignocellulosic biomass.

Introduction

The global forest, paper and packaging industry has been under pressure over the last years. The financial situation of the pulp and paper sector was dramatically affected by economic downturns, weak markets, intense competition, environmental regulations, and increasing production costs. The industry must develop new and creative strategies to reduce costs, improve margins and adapt to new regulations. Such strategies could be the application of biotechnology to increase production yields, reduce input cost, energy consumption and pollution generation. One example is the use of enzymes in the pulp and paper mills to mitigate pitch deposits [**1, 2**] or to convert waste streams into valuable coproducts like C5 sugars or building block molecules [**3, 4**]. In this study, the potential of the xylanase enzyme to generate interesting coproducts and be part of a value added strategy pulp and paper mills is investigated.

Xylanases are hydrolytic enzymes well known to catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan of hemicellulose. They find applications in various industrial processes: food (fruit and vegetable processing, brewing, wine production, baking), animal feed, starch, textile, bioremediation. One current application of xylanases in pulp and paper is the prebleaching of kraft pulp to minimize the use of harsh chemicals. Xylanases are also useful for the production of second generation biofuels. They are commonly used as accessory enzymes, to supplement multi-enzyme cocktails, in some biofuel production processes in bioethanol fermentation. They have a synergistic effect with cellulases in the hydrolysis of the lignocellulosic biomass to simple sugars [**5, 6, 7**].

Various microorganisms, including bacteria, fungi, yeasts, and actinomycetes can produce multiple xylanases [**5, 6, 8, 9**]. Although fungi are considered as host producers of xylolytic enzymes, bacteria like *Bacillus sp* have also received attention since these microorganisms can produce xylanases having specific properties. They are cellulase free, neutral or alkaline, and thermo- and halostable. These properties make them suitable for applications in the pulp and paper industry [**10, 11, 12, 13**]. Moreover,

Bacillus sp is fast growing and tolerant to extreme conditions which lead to a high enzyme productivity even under harsh conditions.

Because of potential operating cost reductions related to the use of *Bacillus*-based xylanase, many researches focused on the utilisation of agricultural and industrial residues as complex culture media instead of expensive semi-synthetic culture media [**12, 14, 15, 16**]. These studies reported high levels of xylanase production with complex culture media that could be comparable to the production of xylanases using commercial xylan as the carbon source. In addition, wastewaters and wastewater sludge have been successfully used as raw materials for *Bacillus sp* fermentation at laboratory and pilot scales [**17, 18**]. Among *Bacillus* sp. strains, *B. pumilus* is recognized as a very good producer of xylanases, particularly cellulase-free xylanases for the bio-bleaching of pulps. Enzyme production of *B.pumilus* using alternative and cheap raw materials such as agricultural residues has been previously demonstrated in several studies [**10, 19**].

The pulp and paper industry generates high amounts of residual materials from various processes. These materials must be disposed at low cost and in an environmentally friendly way [**20**]. Pulp and paper sludges usually contain high levels of carbohydrates and water that makes them suitable for fermentation into valuable products [**21**]. In addition, they are continuously available and low cost. However, to make sludge a stable and effective complex culture media for microbial production, modification might be required either by mechanical and chemical pretreatment, nutrient supplementation or solids concentration adjustment [**16**].

Hence, this research features the use of pulp and paper sludge as an alternative culture medium to produce xylanase enzymes from *Bacillus pumilus* in submerged culture fermentation. Several treatments were investigated in order to improve *Bacillus pumilus* growth and xylanase production: sludge mixing, solids concentration adjustment and addition of pretreated lignocellulosic biomass as inducer. Finally, the optimal conditions for enzyme activity (pH and temperature), were determined.

Material and methods

Microorganism

Bacillus pumilus ATCC 7061 was obtained from the American Type Culture Collection (ATCC). The strain was stored at -80 ° C and was grown for inocula preparation following ATCC guidelines and Reddy et al. [**22**].

Fermentation media

A defined xylan medium (DXM) was used as a reference culture medium containing (g/L): peptone (5), yeast extract (5), K_2HPO_4 (1), $MgSO_4$ (0.2), and xylan from beechwood (5). The medium was adjusted to pH 7.5±0.1 and sterilized at 121 ° C for 30 minutes.

Different lignocellulosic biomasses used as alternate xylan sources were hydrolysed primary sludge (HPS), untreated (RCS) and pretreated corn stover (PCS), untreated (RF) and pretreated flax (PF), untreated (RR) and pretreated reed canary grass (PR). Corn stover residus, flax (*Linum usitatissimum*), and reed canary grass feedstocks were supplied by Agrosphère company (Québec, Canada), Schweitzer Mauduit (SWM) international (Manitoba, Canada), International Institute for sustainable development (IISD) (Manitoba, Canada), respectively. Theses biomass residues were pre-treated by reactive extruder fractionation at conditions of 5% NaOH, 200 rotations per minute (rpm), 180 ° C. This preprocessing allows essentially burst fibers to release their main components (cellulose, hemicelluloses, proteins) recruited by lignin. Commercial beechwood-extracted xylan from Sigma-Aldrich was also used. All biomass was dried and ground to powder before use. Hemicellulose and xylan content of the biomasses was determined by method of Suliter et al. [**23**].

Pulp and paper sludge samples were collected at the Kruger Crabtree tissue mill (Crabtree, Québec, Canada) wastewater treatment facility. Primary sludge was sampled

from primary clarifiers while secondary (biological) sludge was sampled from the activated (excess) sludge treatment system. Sludge properties are presented in Table 1. Sludge samples were pelletized by centrifugation at 3000 x *g* for 5 min at 4 ° C (Multifuge X3 FR of Thermo Scientific). The pellets were diluted to the desired solids concentration with deionized water. The total solids concentration (TS) of the final sludge samples was measured according to standard methods [**24**]. Sludge samples were stored for a maximum of one week 4 ° C to minimize microbial degradation. Before being used as microbial culture media, the sludge samples pH was adjusted to pH 7.5 and they were sterilized at 121 ° C for 30 minutes.

Fermentation procedure

To prepare an inoculum of growing cells (starter culture), a loopful of *B.pumilus* from a Tryptic Soya Agar (TSA) plate was transferred into a 500-mL Erlenmeyer flask containing 100 mL of sterilized tryptic soya broth (TSB). The mixture was incubated in a rotary shaker at 250 rpm at 35 ° C for 16 to 18 h until the cell concentration reached 10^8 CFU/mL.

Shake flask fermentation was carried out in 1-L Erlenmeyer flasks containing 200 mL of DXM and sludge media, namely primary, secondary and mixed sludge. The inoculum was transferred in the flasks (2% v/v) and incubated in a rotary shaker at 250 rpm and 35 ° C for 48h.

Fermentation was conducted in a 7.5 – L Labfors III stirred tank bioreactor (Infors HT,Bottmingen, Switzerland) equipped to control fermentation parameters. The Iris 5.2 software allowed automatic set point control and integration of all reaction parameters. The pH electrode was calibrated using pH 4 and 7 buffers. The oxygen probe was calibrated to zero using nitrogen degassed water and 100 % with air saturated water. The culture medium was added to the bioreactor vessel and sterilized at 121 ° C for 30 min in a vertical-loading laboratory autoclave. It was then cooled to 35 ° C before being

inoculated with 2 % (v/v) starter culture. The temperature was regulated at 35 ° C with a water circulation pump. The pH was adjusted at 7.5 using 4 N NaOH or 4 N H_2SO_4 through computer – controlled peristaltic pumps. Mixing speed (200 – 500 rpm) and aeration rate (1.5 – 2 L per minute) were varied in order to keep dissolved oxygen (DO) values above 30% saturation, which ensured the oxygen concentration was above the critical level. An anti-foam agent was added automatically (0.1%, v/v) to control foaming during fermentation. Culture samples were taken at specific intervals during fermentation to evaluate the growth and enzyme production of *B.pumilus*.

Estimation of cell and spore count

For each experiment, viable cell counts were determined by the plate count technique [**17, 22**]. Samples were sequentially diluted, plated on TSA and incubated at 35 ° C for 24h. The same method was used for viable spore counts, but samples were heated at 80 ° C in a heating bath for 10 min and then chilled on ice for 5 min before being placed on TSA plates and incubated. Counts were reported as colony forming units (CFU) per mL and expressed as total cell or spore counts per mL. The relative standard deviation of cell and spore measurements was respectively 6 and 7 %.

Volumetric oxygen transfer coefficient (k_La)

The volumetric oxygen transfer coefficient k_La was measured in the bioreactor using the dynamic gassing-out method [**17**]. k_La values were determined during fermentation at different sampling times.

Enzyme activity assays

Culture samples were centrifuged at 10 000 x *g* and 4° C for 10 min and the supernatants were used directly to measure xylanase activity. It was determined by measuring the release of reducing sugars from the enzymatic hydrolysis of 0.5 % (w/v) beechwoodextracted xylan (Sigma Chemicals Co.) at 35 ° C and pH 7 (50 mM

phosphate buffer) for 15 min. Reducing sugars measurement was conducted following the dinitrosalicylic acid (DNS) standard method according to IUPAC [**25**]. One unit of activity (IU) of xylanase is defined as the amount of enzyme that releases 1 µmol of xylose as reducing sugar equivalent per minute.

Determination of optimal pH and temperature

The optimal pH of xylanase activity was determined by measuring enzyme activity at 35 ° C in the 5.0 to 11.0 pH range using different buffers (50 mM): citrate phosphate (pH 5.0 and 6.0), phosphate (pH 6.5; 7.0; 7.5; 8.0), borate (pH 9.0; 9.5; 10.0; 11.0). The optimal temperature of xylanase activity was determined by measuring the enzyme activity at various temperatures ranging from 10 to 80 ° C at pH 7.0 (50 mM phosphate buffer).

Results and discussion

Growth and xylanase production in pulp and paper sludge

To investigate the use of pulp and paper sludge as culture media, shake – flask fermentation of *B.pumilus* in three types of sludge, primary, secondary and primary-secondary mixed sludge, at a solids concentration of 15 g/L and in DXM were carried out. The evolution of the total cell count and the xylanase production of *B.pumilus* during a 72 h fermentation is illustrated in **Fig 1**. *B.pumilus* was able to grow and utilize the nutrient contained in secondary and mix sludge but not in primary sludge. The trend of *B.pumilus* growth in secondary and mixed sludge was similar to the DXM medium (Fig 1a). The enzyme activity profiles (Fig 1b) show that xylanase production reached a maximum level after 24 h, which seems to coincide with the exponential growth phase of *B.pumilus*. This was observed in previous studies using agro-industrial residues as substrate for *B.pumilus* enzyme production [**10, 15, 16**]. The composition of the sludge samples may have influenced *Bacillus* growth and enzyme production. Besides high carbohydrate content (cellulose and hemicellulose), low nitrogen content measured in

primary sludge (Table 1) did not satisfy the nutrient need of microorganisms. Meanwhile, the secondary sludge generated from the activated sludge treatment contains primary and secondary metabolites of microbial endogenous activities such as amino acids or vitamins and components of dead cells that may stimulate *B.pumilus* growth and metabolite production. The comparative data on maximum cell concentration, maximum specific growth rate, and maximum xylanase activity in all media are summarized in table 2. Secondary sludge was the best sludge-based medium for *B.pumilus* growth with the highest cell concentration at 3.6×10^8 CFU/mL, followed by 1.1×10^8 CFU/mL in mixed sludge, which are less than the cell concentration in DXM (1.2×10^9 CFU/mL). In spite of the lower maximum specific growth rate in mixed sludge (0.25 h^{-1}) than in secondary sludge (0.29 h^{-1}), xylanase activity in mixed sludge (3.8 IU/mL) was about –8 times higher than in secondary sludge (0.5 IU/mL). It could be attributed to xylanase induction by xylan found in primary sludge, which is part of the mixed sludge. *Bacillus* required that xylan be added as inducer to the substrate for enzyme synthesis as proposed by [**12, 26**].

Optimizing the mixed sludge medium for Bp growth and xylanase production

Mixed sludge supported well the growth and xylanase production of *B.pumilus* because of its xylan content, originating from the primary sludge, and the nutrients from the organic matter and cell debris of secondary sludge. For this reason, it was proposed to improve the xylan and nutrient content of mixed sludge with different proportions of primary and secondary sludge at different solids concentrations. A set of experiments was conducted at different primary and secondary sludge ratios, which in turn resulted in different solids concentration as shown in table 3. Increasing primary sludge proportion did not support *B. pumilus* growth or xylanase production. As explained previously, primary sludge was effective at inducing xylanase production, but it was not adequate for microbial growth due to its lack of nitrogen. Meanwhile, increasing the secondary sludge proportion, to bring more nitrogen and other nutrients, improved the microbial growth as well as xylanase production. However, the growth of *B. pumilus* decreased gradually

followed increase of solids concentration (Table 3). According to previous study, high solids concentration may cause mass transfer limitations during fermentation. Therefore, solids concentration at 15 g/l was used for further studies.

Effect of xylan sources on xylanase production

As shown by previous results, adding primary sludge to secondary sludge improves the xylanase production of *B. pumilus*. In consequence, it was necessary to verify if the xylan content of the primary sludge would trigger the cell growth and the xylanase production of *B. pumilus*. A set of experiments was conducted in which lignocellulosic residues were supplemented with secondary sludge. Different lignocellulosic biomasses were used as alternate xylan sources including hydrolysed primary sludge, untreated and pretreated corn stover, untreated and pretreated flax, untreated and pretreated reed canary grass those xylan contents are 17.03%, 20.25%, 22.19%, 10.89%, 13.17%, 10.37%, 16.57%, respectively. The xylanase activities achieved during *B.pumilus* fermentation of secondary sludge supplemented with these different lignocellulosic residues are presented in **Fig 2**. Higher xylanase activities were obtained in all the sludge supplemented with these residues than that in secondary sludge, thus indicating the presence of xylan in these residues could induce – xylanase production in *B. pumilus*. The addition of residual corn stover gave the highest xylanase activity at 10.7 IU/mL, followed by pre-treated corn stover (8.0 IU/mL). These highest activities were observed from 24h to 36h of fermentation. It is also observed in Fig 2 that the supplementation of untreated corn stoves has more impact on xylanase activities than pretreated ones. In the present case, the pretreatment not only removes lignin in biomass but also reduces about 70% of the hemicelluloses contained in corn stove, leading to the reduction of intact xylan in the pretreated residues. It may explain why higher xylanase activities were observed when using untreated corn stover as sludge culture medium supplements. Even untreated corn stover gave xylanase activity (10.7 IU/mL) approximately to commercial xylan (9.8 IU/mL). Thus corn stover could be an alternative to commercial xylan to induce xylanase production in *B. pumilus* using complex and inexpensive culture

medium such as secondary sludge, which could offset the cost of enzyme production. Consequently, untreated corn stover powder and beechwood-extracted xylan were chosen as xylanase substrates for subsequent experiments in the bioreactor. With controlled pH, agitation and aeration, it was expected to obtain higher total cell count and xylanase activity.

Bioreactor production of xylanase

Batch fermentations of *B. pumilus* in DXM, xylan-added secondary sludge media supplemented with xylan (SSX) and corn stover (SSC) were conducted into a 7.5L bioreactor with a work volume of 5 L. Total solids concentration (TS) were adjusted to 15 g/L. The evolution of the volumetric mass transfer coefficient (k_La), oxygen uptake rate (OUR), and oxygen transfer rate (OTR) along with the total cell count, spore count, and xylanase activity during 60h fermentation are illustrated in **Fig 3**, **Fig 4** and **Fig 5** for DXM, SSX and SSC, respectively. The profiles were similar from one medium to another, except for the spore production that was triggered earlier in SSX and SSC media. The highest value of k_La occurred at 12 h on DXM and at 15h on SSX and SSC and then decreased continuously to the end of fermentation (Fig 3b, Fig 4b and Fig 5b, respectively). The decline of this parameter could be explained by a decrease in oxygen demand of *B. pumilus* when substrate exhaustion occurred [**17**]. The exponential phase of *B .pumilus* in DXM occurred during the first 9-10 h in DXM and 18-20 h in both SSX and SSC. The production of spores was observed sooner in SSX and SSC (at 5 h of fermentation) than DXM (at 9 h of fermentation). Some sludge components may trigger spore production earlier compared to a synthetic or semi-synthetic culture medium. Xylanase activities in the three culture media show a maximum enzyme level at 24h for SSX and DXM and at 30h for SSC when the total cell count cell concentration was maximal and decreased after 36h of fermentation when sporulation was at its maximum in all media.

Table 4 summarizes the maximum growth and enzyme production of *B. pumilus* fermentation in the bioreactor. Compared to results obtained in shake flask fermentation (Table 1), the growth and xylanase production of *B. pumilus* were foundto be much higher in the bioreactor in both synthetic (DXM) and sludge-based (SSX) media. It was attributed to a better mass transfer in the bioreactor due to the control of aeration and mixing. High growth and enzymatic activity were achieved in both SSX and SSC media. The highest values of k_La and specific growth rate calculated in both SSX and SSC are 56 h^{-1} and 0.49 h^{-1}, respectively. These values were higher in DXM with 68h^{-1} for k_La and 0.69 h^{-1} for the maximum specific growth rate. Lower values of k_La in sludge media compared to a synthetic soluble medium are attributed to the complexity of the media in terms of nutrient accessibility and rheological properties [**27, 28, 29**]. Moreover, the better growth and xylanase activity observed in SSC (3.4 x 10^9 CFU/mL of cell count and 37.8 IU/mL of xylanase activity) compared to that in SSX (2.5 x 10^9 CFU/mL of cell count and 35.5 IU/mL of xylanase activity) demonstrated that corn stover is a good alternative to the costly commercial xylan used to induce xylanase production.

Xylanase activity at different pH and temperature

The impact of the culture medium on xylanase properties has been studies. It is expected that complex culture media such as wastewater sludge can improve enzyme tolerance to pH and temperature variation. The fermented broths of DXM, SSX and SSC were used to determine the pH and temperature optimum for their xylanase activities. There are no xylanase activity was observed at pH 5 and pH 10 for all three media (DXM, SSX and SSC) and the enzyme activities were observed in a pH range from 6 to 9 as shown in **Fig 6a**. The results showed that, xylanase produced from sludge had highest activity at pH 6 - 7 and slightly reduce at higher pH, these result accorded to the optimal pH for *B.pumilus* is 7 to 7.5. The alkalothermophilic properties of xylanase produced by *B. pumilus* were reported in previous studies [**30, 31**]. The optimal temperature for xylanase activity was 50 ° C (**Fig 6b**) and the enzyme activity in range temperature from 30 to 55 ° C. These results suggest that sludge based media have no influence on the pH and

temperature tolerance of xylanase produced by *B. pumilus* as expected. In addition, *B. pumilus* xylanase produced from sludge was determined to be almost cellulase free in our other results (data not shown), which could be interesting for the use of these enzymes in the pulp and paper industry.

Conclusions

Pulp and paper sludge can be used as a complex and inexpensive culture media for the xylanase production of *Bacillus pumilus*. It is recommended to mix primary and secondary sludge at ratio of 1:2 at 15 g/L TS to get high cell counts and xylanase activity. Supplementation of pulp and paper sludge media with lignocellulosic residues such as raw and ground corn stover increases xylanase activity. Compared to commercial xylan, untreated powder corn stover could be a cost effective inducer for xylanase production. A fermentation time of 30 h is proposed to recover the enzyme from the fermented broth or even use the broth as accessory enzymes for bioethanol production. In fact, it would be interesting to verify if the fermented broth can be used as a supplement for multi-enzyme cocktails in the saccharification process for bioethanol production.

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Abbreviations

Bp	*Bacillus pumilus*
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen
DXM	Defined xylan medium
HPS	Hydrolyzed primary sludge
MS	Primary – secondary sludge mix
OTR	Oxygen transfer rate (mmol/h)
OUR	Oxygen uptake rate (mmonl/h)
PCS	Pretreated corn stover
PF	Pretreated flax
PR	Pretreated reed
PS	Primary sludge
RCS	Raw corn stover
RF	Raw flax
RR	Raw reed

SS	Secondary sludge
SSC	Secondary sludge added corn stover
SSX	Secondary sludge added xylan
TC	Total cell count (CFU/mL)
TS	Total solids concentration (g/L)

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Table 1 Chemical analysis of pulp and paper sludge sample

Properties	Primary sludge	Secondary sludge
pH	6.5 – 6.8	5.8 – 6.2
Total solid (g/L)	40.0 ± 0.5	12.0 ± 0.5
Suspended solid (g/L)	37.0 ± 0.5	8.0 ± 0.5
Dissolved solids (g/L)	0.80 ± 0.02	3.80 ± 0.02
Ashes (g/L)	0.28 ± 0.02	0.080 ± 0.005
Total organics mater (g/L)	3.70 ± 0.02	4.50 ± 0.02
Elemental analysis		
Nitrogen (% w/w)	0.33 ± 0.01	4.34 ± 0.02
Carbon (% w/w)	39.44 ± 0.01	42.50 ± 0.01
Hydrogen (% w/w)	5.47 ± 0.01	6.39 ± 0.01
Sulfur (% w/w)	LOQ	1.23 ± 0.01
Hemicellulose (% w/w)	11.29 ± 0.01	2.72 ± 0.01
Xylan (% w/w)	9.32 ± 0.01	0.61 ± 0.01

Table 2: *B. pumilus* fermentation in shake flasks.

Medium	Initial total cell count (CFU/mL)	Total cell count after 48h (CFU/mL)	Maximum specific growth rate (h^{-1})	Maximum xylanase activity (IU/mL)
DXM	2.0×10^6	1.2×10^9	0.35	19.80 ± 0.09
Primary sludge	2.0×10^6	2.0×10^2	-	-
Secondary sludge	2.0×10^6	3.6×10^8	0.29	0.50 ± 0.01
Mix sludge	2.0×10^6	1.1×10^8	0.25	3.80 ± 0.07

Table 3 Total cell count and xylanase activity at different mix ratios

TS, g/L	Mix ratio (w/w)	Total cell count, CFU/mL	Xylanase activity, IU/mL
15	2 PS : 1 SS	1.00×10^6	0.220 ± 0.03
15	1 SS : 1 PS	7.10×10^7	2.70 ± 0.04
15	2 SS : 1 PS	2.06×10^8	3.80 ± 0.03
20	3 SS : 1 PS	1.02×10^8	3.80 ± 0.06
25	4 SS : 1 PS	9.20×10^7	3.00 ± 0.04

Table 4 Performance of Bioreactor *B. pumilus* fermentation

Parameters	DXM	SSX	SSC
Initial total viable cell count (CFU/mL)	$2.0x\ 10^6$	2.0×10^6	2.0×10^6
Final total viable cell count (CFU/mL)	5.8×10^9	2.5×10^9	3.4×10^9
Final total viable spore count (CFU/mL)	3.4×10^9	9×10^8	9.4×10^8
Maximum specific growth rate (h^{-1})	0.69	0.49	0.49
Maximum xylanase activity (IU/mL)	47.0 ± 0.4	35.5 ± 0.3	37.8 ± 0.3
Maximum $k_L a$ (h^{-1})	68	56	56

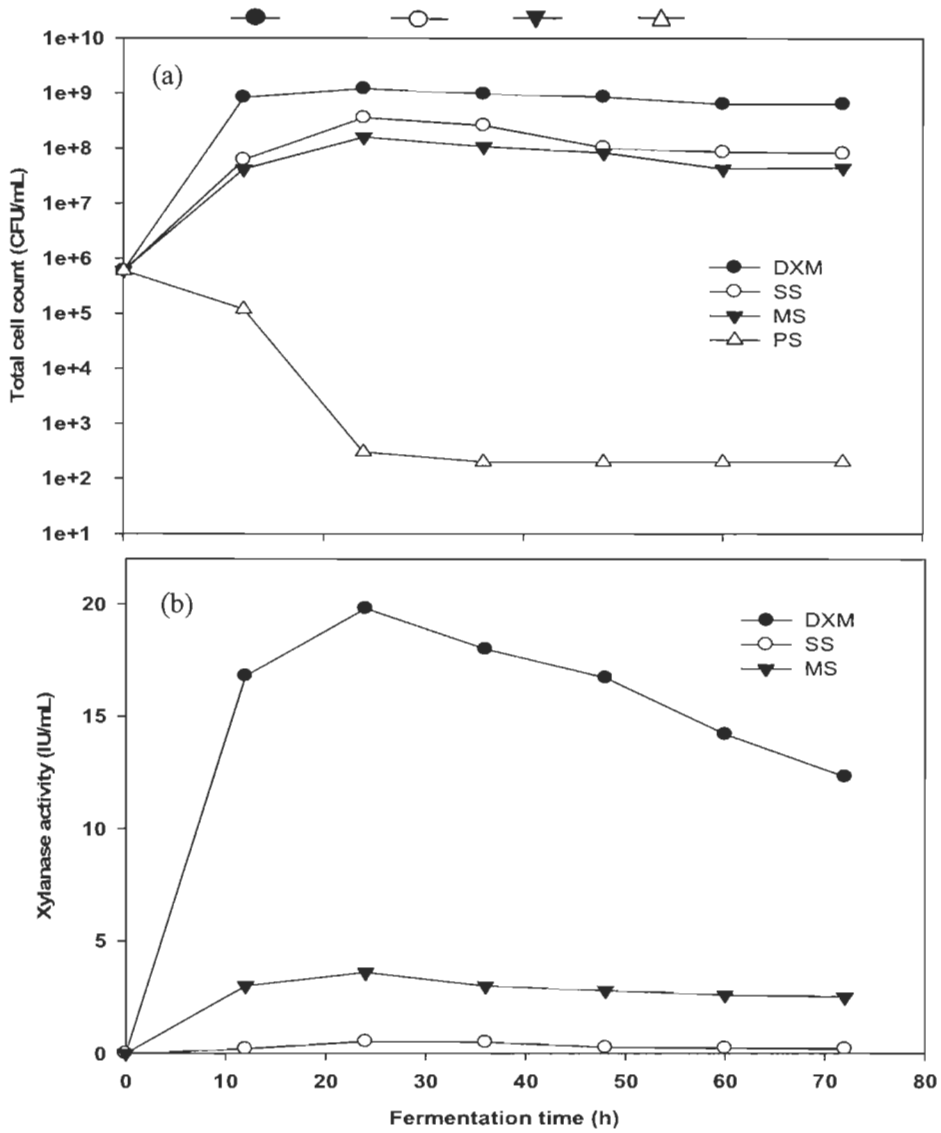

Fig 1 Profile of (a) *B. pumilus* growth and (b) and xylanase production during shake-flask fermentation in defined xylan medium (DXM, primary sludge (PS), secondary sludge (SS) and mixed sludge (MS)

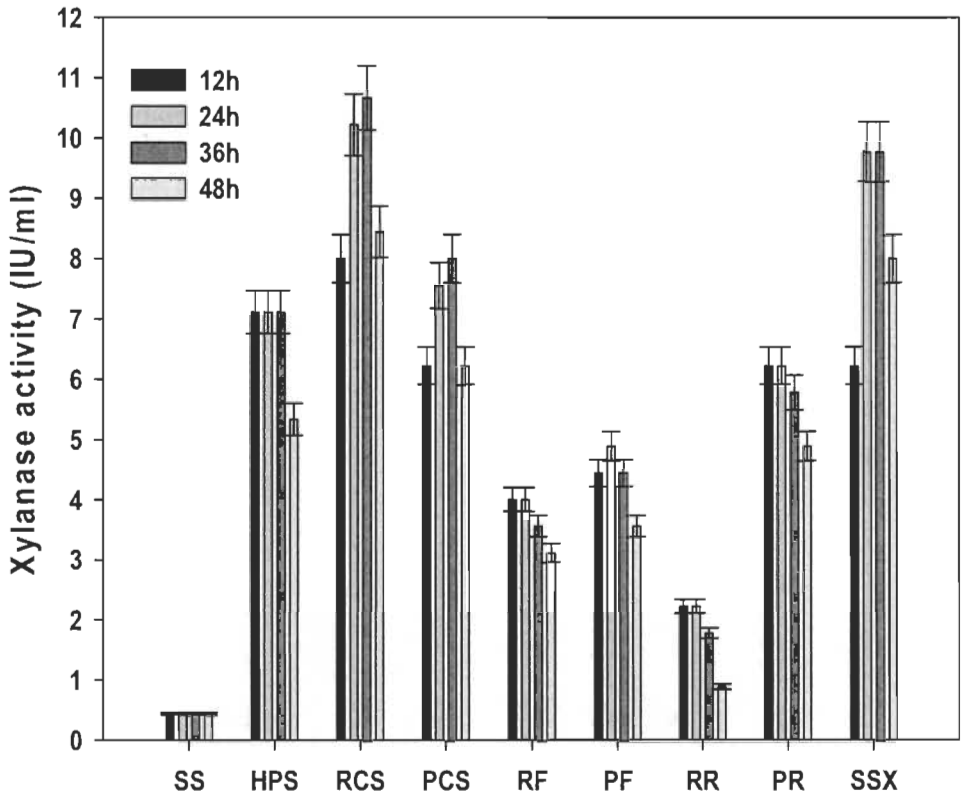

Fig 2 Xylanase activity in pulp and paper secondary sludge (SS) based medium supplemented with lignocellulosic residues; hydrolyzed primary sludge (HPS), untreated and extrusion-pretreated corn stover (RCS and PCS), untreated and extrusion-pretreated flax (RF and PF), and untreated and extrusion-pretreated reed canary grass (RR and PR) and commercial beechwood-extracted xylan (SSX)

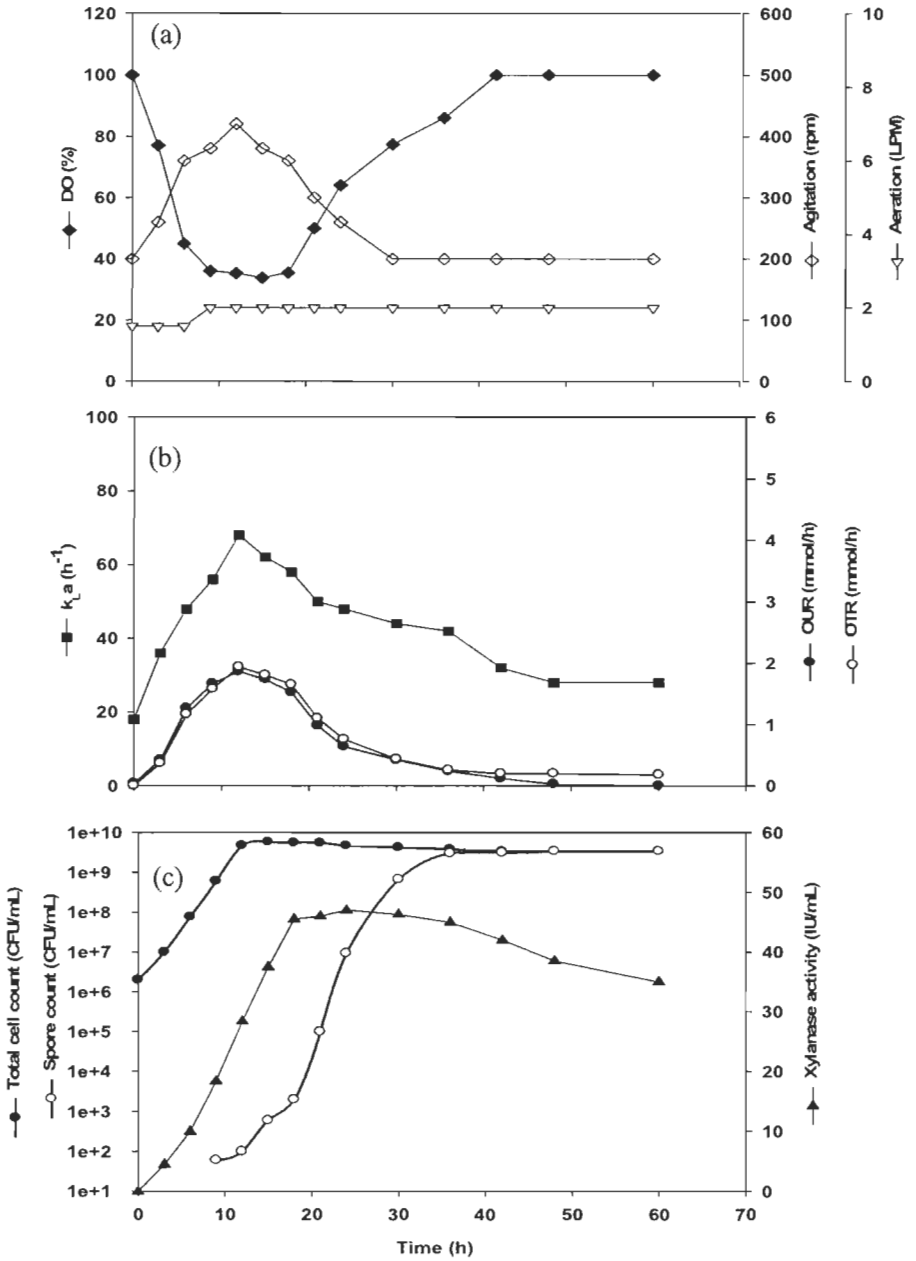

Fig 3 (a) Dissolved oxygen (DO), agitation, aeration (a), (volumetric oxygen transfer coefficient (kLa), oxygen transfer rate (OTR), oxygen uptake rate (OUR) (b), total cell and spore counts, xylanase production (c) during bioreactor fermentation using DXM as semi-synthetic culture medium.

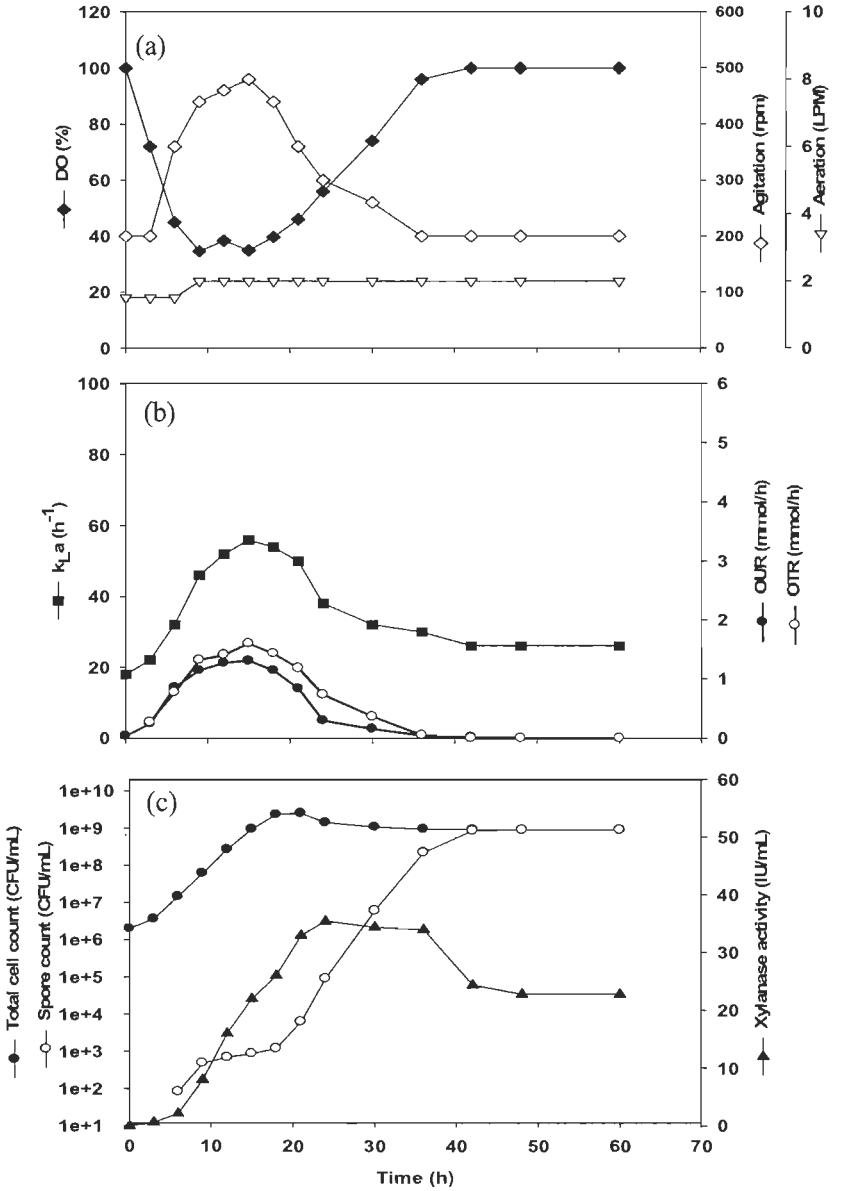

Fig 4 (a) Profile of dissolved oxygen (DO), agitation, aeration, (b) volumetric oxygen transfer coefficient (kLa), oxygen transfer rate (OTR), oxygen uptake rate (OUR), (c) Total cell and spore counts, xylanase production during bioreactor – scale fermentation in SSX

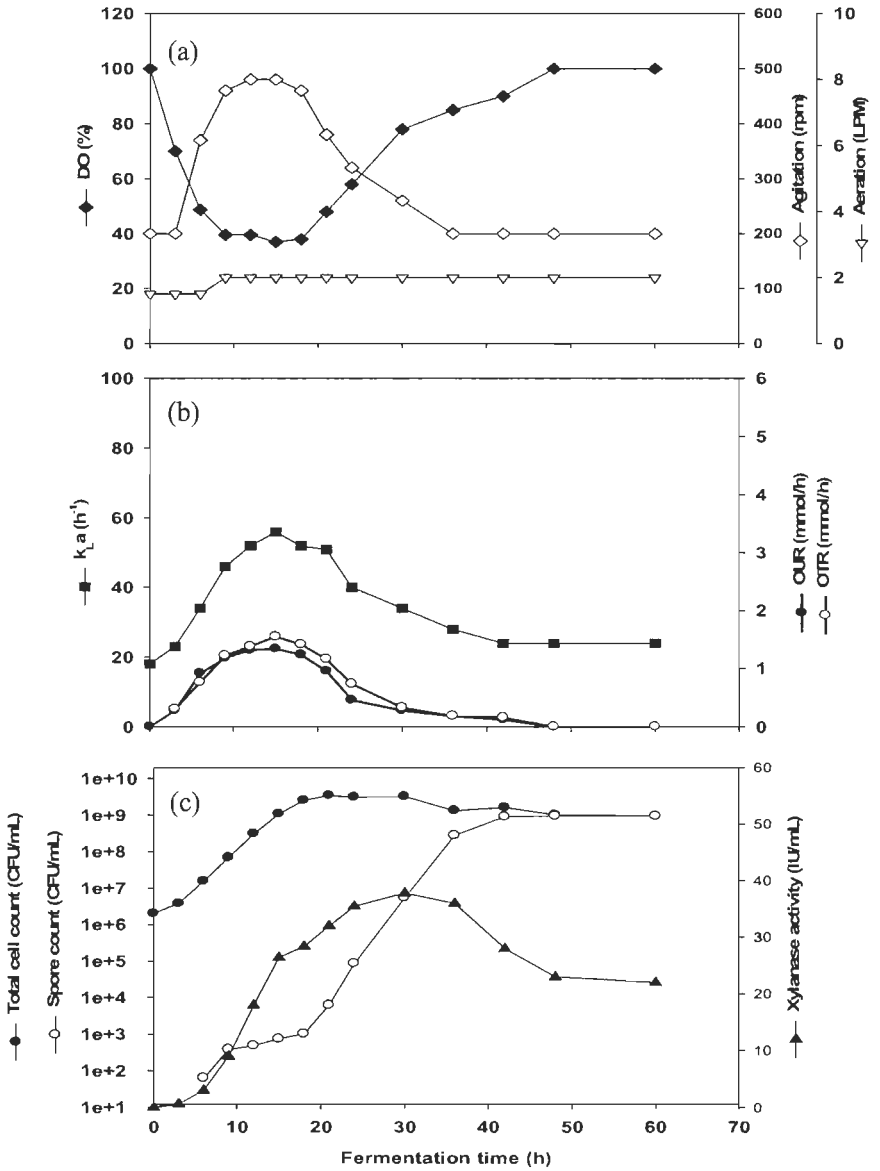

Fig 5 (a) Profile of dissolved oxygen (DO), agitation, aeration, (b) volumetric oxygen transfer coefficient (kLa), oxygen transfer rate (OTR), oxygen uptake rate (OUR), (c) Total cell and spore counts, xylanase production during bioreactor – scale fermentation in SSC

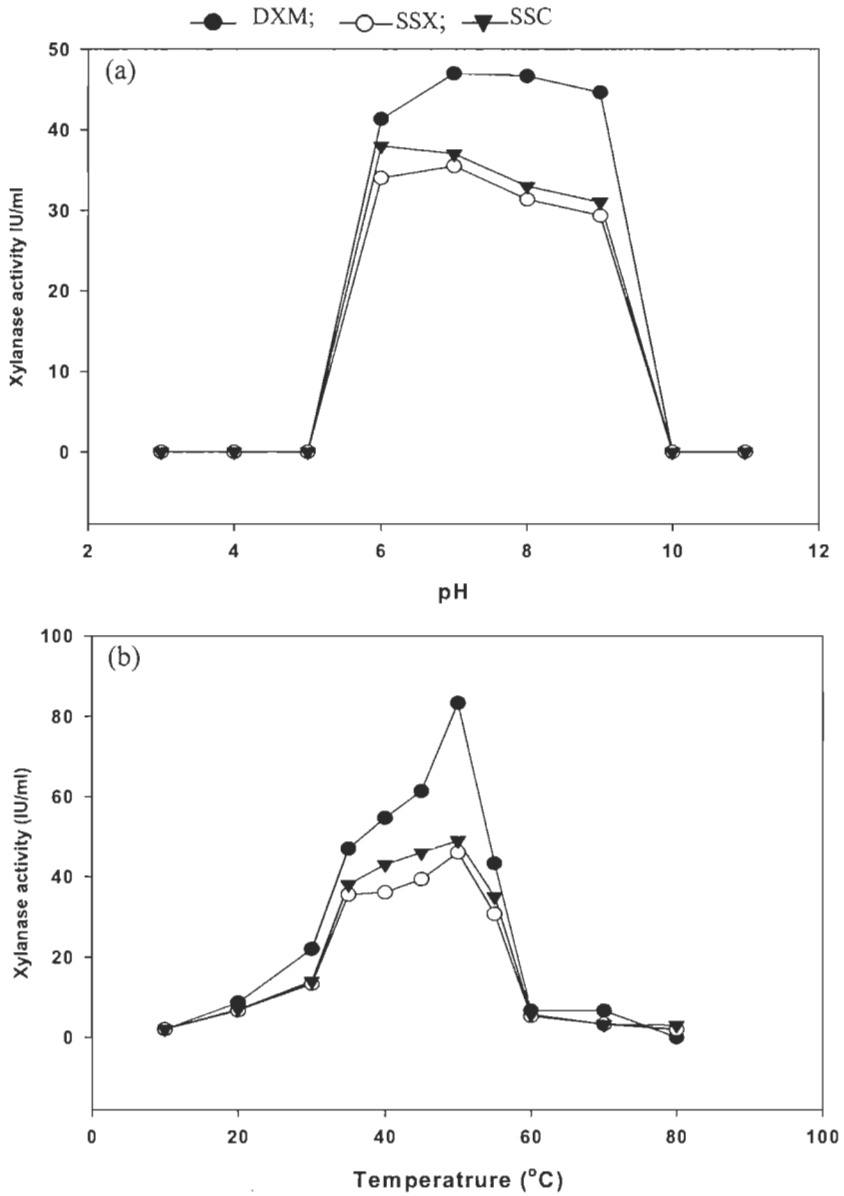

Fig 6 Effect of (a) pH and (b) temperature on xylanase activity

Chapter 7. Discussion

Fermentation of individual Bl, Bs and Bp using pulp and paper industry wastewater sludges as raw material was carried out to evaluate their growth and cellulase production. Primary, secondary and mixed sludges supported the growth of Bl and Bs, whereas Bp sustained in secondary and mixed sludges. The highest cell counts of all three strains were obtained at mixed sludge for Bl, Bs and at secondary sludge for Bp that were 1.2 x 109, 9.6 x 108 and 3.6 x 108 CFU/mL, respectively. The results of cellulase activity test on agar plates along with measurement of reducing sugars from enzymatic hydrolysis shown that Bl and Bs produced extracellular cellulase responsible for depolymerization of cellulose only and do not secrete the complete enzyme complex to convert cellulose into monosugars. More specifically, cellulases produced in PPS by Bl had a higher thermal stability, which increase their range of applications. The supplementation of inorganic and organic nitrogen in primary sludge stimulated Bl to growth. The exponential phase of Bl cultured was achieved after 24h of fermentation while it was achieved after 36h in primary sludge without adding nitrogen sources. Higher Bl cell and spore counts were obtained in primary sludge with added yeast extract (1.4×10^9 and 8.4×10^8 CFU/mL, respectively), followed by peptone (1.4×10^9 and 8.0×10^8 CFU/mL, respectively), soya flour (9.8×10^8 and 3.2×10^8 CFU/mL , respectively), and ammonium chloride (6×10^8 and 8.4×10^7 CFU/mL, respectively) compared to primary sludge without added nitrogen source (4×10^8 and 1.6×10^7 CFU/mL, respectively). Thus, mixed sludge and primary sludge with added nitrogen were proved to be an alternative culture medium for cellulase production of Bl.

The production of cellulase and hemicellusases of *Trichoderma reesei* in PPS was also carried out. The fungi grew in pellet form and produced extracellular cellulases (CMCase, β-glucosidase) and hemicellulases (xylanse, β-xylosidase) in all primary, secondary and mixed sludges. The highest enzyme activity (CMCase) of enzymes produced in mix sludge, secondary sludge and primary sludge, obtained after seven days

of fermentation, were 7.3, 4.8, and 1.5 IU/ml, respectively. Modification of sludges including the (1) PS – SS mixing ratio of 1:1 (v/v), (2) substituting NH_4SO_4 as inorganic nitrogen source, and (3) increasing solids concentration, was an effective way to improve enzyme production. Crude enzyme mixture produced from PPS was efficient to hydrolyse various biomasses, from highly purified CMC, Avicel, beech wood extracted xylan to complex corn crop residues (mainly composed of corn stover) and primary PPS into monosugars. Addition of Tween 20 as surfactant enhanced saccharification, especially for the hydrolysis of corn crop residues containing lignin. In conclusion, PPS can be used as low cost and adequate fermentation substrates of *Bacillus* spp. and *Trichoderma reesei* to produce saccharolytic enzymes.

To evaluate the production of xylanase, fermentation of Bp using PPS as culture media was conducted in shake flasks and bioreactor. Secondary sludge was shown to be a adequate medium for Bp growth, whereas primary sludge was a better culture medium for xylanase production. Mixing primary (PS) and secondary (SS) sludges at 2 SS: 1 PS (w/w), having 15 g/L solids concentration, resulted in the highest cell concentration of 2×10^8 CFU/mL and the highest xylanase activity of 3.8 IU/mL in shake flask fermentation. Supplementation with corn stover as xylanase inducer in secondary sludge gives the highest xylanase activity (10.7 IU/mL). In bioreactor, cell concentration and xylanase level obtained in sludge with added xylan (2.5×10^9 CFU/mL and 35.5 IU/mL, respectively) or corn stover (3.4×10^9 CFU/mL and 37.8 IU/mL, respectively) were very high in comparison with the defined xylan medium (5.8×10^9 CFU/mL and 47.0 IU/mL, respectively). The Bp – based xylanase produced from paper sludge is stable in neutral – to – alkaline pH at 50 o C and cellulse – free that offered a potential application of the enzyme for biobleaching (biological bleaching) in pulp and paper industry. Also, the enzyme was tested to be able to degrade commercial xylan, corn stover and primary sludge to monosugars, mainly xylose. As a result, utilisation of secondary sludge as basic culture medium added corn stover as enzyme inducer was shown to be efficient and less expensive for xylanase production compared to commercial xylan – based medium.

Chapter 8. Conclusions

8.1. Conclusions

This work allowed to elucidate the feasibility of using PPS as fermentation substrate for growth and enzyme production of *Trichoderma reesei* and *Bacillus* spp. Primary, secondary and mixed sludges were used as low cost, even negative cost, culture media. The modification of PPS including the adjustment of solid concentration and ratio of C/N as well as the supplementation of lignocellulosic biomass as enzyme inducers are efficient techniques to make PPS an adequate culture medium for microbial growth and their enzyme production.

The conclusion of this work are:

- Primary, secondary and mixed sludges can be used as raw material for growth and enzyme production of *Trichoderma reesei* and *Bacillus* spp.. Among three types of paper sludge, the mixed sludge is the best culture medium for production of cellulases and xylanase for which the primary sludge served as cellulase inducer and the secondary sludge as nutrient source;
- Supplementation of nitrogen sources to PPS is required to increase the yield of *Trichoderma reesei* and *Bacillus* spp. in terms of growth and enzyme production;
- Lignocellulosic residues can be alternated to commercial substrates for enzyme induction in fermentation of paper sludge;

  Bl and *Bs* were able to produce endoglucanase responsible for cellulose depolymerization, but not a complete cellulase enzyme complex to convert cellulose into monosugars;
- *Bp* was able of producing xylanase in PPS. This enzyme is active at neutral – to – alkaline pH at 50°C and almost cellulase-free. The best period for xylanase recovery was between the end of exponential phase and early of stationary phase of growth;

- Corn stover was a cost-effective cellulose inducer rather than commercial xylan for xylanase production when added to secondary sludge;

- *Trichoderma reesei* produced a mixture of cellulases and xylanases that were effective to hydrolysis pretreated corn stover and primary paper sludges into fermentable sugars;

- Addition of Tween 20 as surfactant enhanced hydrolysis of lignocellulosic biomass by the enzyme mixture produced from PPS. The Tween 20 addition was more effective on lignin-containing substrates.

This work proposed an economical way to produce cellulases and xylanases using PPS as raw material. Use of secondary sludge as culture medium along with addition of lignocellulosic residues as enzyme synthesis inducer enhances the yield of cells, spores and enzymes and reduces the cost production of the enzymes. In a biorefining context, lowering the cost of enzymes could be advantageous and encourage their uses. Consequently, PPS can be beneficially used to get value added products through fermentation technologies. This can help the pulp and paper industry to seize an opportunity to become a biorefinery or be partner with a biorefinery and then contribute to the emergence of the new bioeconomy.

8.2. Contributions

The scientific papers published and submitted on the results of the thesis are the main contributions of this thesis. However, it is important to underline that the results caught the interest of Agrosphere, a company in Quebec province of Canada founded by La Coop Profid'Or and Ferme Olivier Lépine. Agropshere is looking to produce locally cellulolytic enzymes for its future community-scale biorefinery in Lanaudière region (QC, Canada) to produce cellulosic sugars and bioethanol from local corn crop residues. In their biorefinery plan, there is a cellulosic sugars production line involving the use of cellulolytic enzymes. As this thesis demonstrated that these sludges can support very

well cellulolytic enzyme production, the company then included in its business plan the possibility of producing its enzymes in the region to reduce the cost and also the GHG emission related to their use. In fact, the paper sludges used in this thesis work were sampled at the Kruger mill at Crabtree (QC, Canada), which is about at 30 km of the targeted site of the future Agrosphere biorefinery.

8.3. Future works

Using PPS to produce enzymes or other microbial derivatives is very promising. It is recommended to pursue the research on this topic and focus on:

- Cultivating *Bl, Bs* and *Trichoderma* reesei in PPS in bioreactor. Fermentation in bioreactor with control of parameters (pH, agitation, aeration) would improve yield of the microorganisms as shown in the results of fermentation of Bp;
- Employ enzyme producer microorganisms isolated from PPS and other lignocellulosic residues. These microorganisms are well adapted to growth in PPS. When cultivated as monoculture with optimized fermentation technique, we may expect high yield of cell spore and enzyme production;
- To improve the yield of saccharification of lignocellulosic biomass using the enzymes produced from PPS, it is recommended to characterize the enzymes as follow:
    - Study on the properties of enzymes (thermostability, pH range, resistance to inhibitors) for saccharification;
    - Characterize types of enzymes and optimize enzyme dosage used to hydrolyze pretreated lignocellulosic biomass.

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Appendix I

Characteristics of pulp and paper making process

Table AI.1 Typical processes of pulp and paper making

Process	Description
Pulping	In chemical pulping the yields are only about 45–55%, mechanical pulping uses about 80–95% of the fiberwood.
Mechanical	This is manufactured by mechanical defibration using a variety of mechanical procedures
	Stone groundwood: this is manufactured from round logs in a grinder.
	Refiner mechanical pulp: this is manufactured by the mechanical defibration of wood chips in a disc refiner.
Chemical	The wood chips are cooked with appropriate chemicals in an aqueous solution at an elevated temperature and pressure to break chips into a fibrous mass.
	Kraft process: The woodchips are cooked in a solution of sodium hydroxide (NaOH) and sodium sulfide (Na_2S). This process is widely used.
	Sulfite process: The wood chips are cooked in a mixture of sulfurous acid (H_2SO_3) and bisulfide ions (HSO_3^-) to dissolve lignin.
Chemo-mechanical	The raw material is first treated chemically and then subjected to drastic mechanical treatment to separate the fibers. The strength of the pulp is relatively better than the pulp from the mechanical pulping alone.
Thermo-mechanical	This process involves steaming the raw materials under pressure for a short period, prior to and during refining. The thermo-mechanical process is further modified using chemicals during the steaming stage, and the process is called chemi-thermomechanical pulping.
Papermaking:	The paper making operation consists of two parts; one is stock

	preparation by treating the pulp to the required degree of fitness and the other is paper making where the treated pulp is passed through continuous moulds/wires to form sheets.
Fine paper:	Fine papers are intended for writing, typing, and printing purposes. They may be white or colored, are made from bleached kraft or sulfite softwood pulps, and may contain hardwood pulp for smoothness and opacity.
Tissue paper:	Sanitary tissues are made from bleached sulfite or kraft pulps and are soft, bulky, absorbent, and moderately strong. Wrapping tissues are used for wrapping clothes, flowers, etc. and are made of bleached kraft or sulfite softwood pulps that impart high strength. Tracing tissue is a dense, transparent grade of paper that is used for tracing.
Paperboard	Included Linerboard, corrugating media, tubes, drums, milk cartons, recycled board used in shoe and cereal boxes, roofing felt, fiberboard, etc.

Table AI.2 Principal characteristics of Primary sludge (values in average) [11]

Parameter	Process					
	Kraft pulping	**Deinking**	**Mechanical pulping**	**Mechanical / sulfite-bisulfite**	**Semichemical pulping**	**Board / Fine paper / Tissue**
Convenient parameter (mg/kg, dry weight)	N = 9	N= 3	N= 14	N = 16	N = 1	N = 9
pH	8.3	7.8	5.95	5.45	7.4	7.9
Total Organic mater (%)	69.4	47.2	87.1	94.4	90.7	77.2
Initial relative humidity (%)	74.5	57.6	71	73	53	73.5
Residual humidity (%)	11.6	35	35.3	21.1	33.5	37.6
General compounds (mg/kg, dry weight)						
Total Kjeldhal Nitrogen	556	1305	1427	1095	2302	1314
Nitrogen – NH_3	N.D.	5.5	N.D.	N.D.	N.D.	N.D.
Nitrogen – NO_2 and NO_3	15	15.5	5	12.5	5.5	6
Total inorganic phosphorus	17	22.5	44	10.5	350.5	23.5
Total phosphorus	247	698	256	118.5	529.5	251
Elementary Ratio C/N	424	82	280.5	342	145.5	182

Metals (mg/kg, dry weight)						
Calcium (Ca)	22500	93800	3250	5600	16166	6975
Cobalt (Co)	1	2.7	1	0.25	1	2
Iron (Fe)	1655	1665	1300	1300	1383	1360
Magnesium (Mg)	857	1070	540	408	780	695
Manganese (Mn)	277.5	25.5	125	170	445	72.5
Potassium (K)	453.5	300	335	310	813.5	202
Sodium (Na)	2750	1100	600	750	1816	635
N Number of fabrication N.D. non detectable						

Table AI.3 Principal characteristics of Secondary sludge (values in average) [11]

Parameter	Process					
	Kraft pulping	**Deinking**	**Mechanical pulping**	**Mechanical / sulfite-bisulfite**	**Semichemical pulping**	**Board / Fine paper / Tissue**
Convenient parameter (mg/kg, dry weight)	N = 3	N = 3	N= 1	N = 1	N = 1	
pH	7.6	6.1	7.6	8.6	6.9	-
Total Organic mater (%)	64	57.2	56.1	83.3	81.1	-
Initial relative humidity (%)	94	88.7	89	-	94	-
Residual humidity (%)	6.8	6.8	89.7	48	6.5	-
General compounds (mg/kg, dry weight)						
Total Kjeldhal Nitrogen	7817	23500	23375	63920	35395	-
Nitrogen – NH_3	80	2165	2534	595	344	-
Nitrogen – NO_2 and NO_3	8	n.d.	61	31.35	180	-
Total inorganic phosphorus	27	1722	5276	2265	2800	-
Total phosphorus	590	10085	11540	24310	4880	-
Elementary Ratio C/N	39	13	10.2	7.3	517	-

Metals (mg/kg, dry weight)						
Calcium (Ca)	45550	14000	16000	10750	21775	-
Cobalt (Co)	3	4	10	0.5	3	-
Iron (Fe)	4900	5055	15000	520	3480	-
Magnesium (Mg)	1900	1525	6200	1000	2400	-
Manganese (Mn)	660	207	2200	135	1705	-
Potassium (K)	575	1440	5000	2800	2840	-
Sodium (Na)	1525	3390	2700	26000	6500	-
N Number of fabrication N.D. non detectable						

Appendix II

Microorganisms Used

Table AII.1 General information about microorganisms used in our research

Strain	Interested products/application	Basic condition of growth
B. licheniformis	Antibiotic bacitracin, proteases, pectate lyases, lipases and polysaccharide degrading enzymes.	30 – 37°C , pH 7 - 7.5, obligate aerobe
B. pumilus	D-ribose, antibiotic, Vaniline, pectinase, cellulase and xylanse	30 – 37°C , pH 7 - 7.5, obligate aerobe
B. subtilis	Riboflavin, biosurfactants, amylase, protease, cellulase and lipase.	30 – 37°C , pH 7 - 7.5, obligate aerobe
T. reesei	Cellulases, heterologous protein production/	20 - 25°C, pH 4.5 - 5, typical aerobe.

Appendix III

Buffer Systems

Table AIII.1 Useful buffer system

Buffering system	**buffering pH range 25°C**
Hydrochloric acid/ Potassium chloride	1.0 - 2.2
Glycine/ Hydrochloric acid	2.2 - 3.6
Potassium hydrogen phthalate/ Hydrochloric acid	2.2 - 4.0
Citric acid/ Sodium citrate	3.0 - 6.2
Sodium acetate/ Acetic acid	3.7 - 5.6
Potassium hydrogen phtaalate/ Sodium hydroxide	4.1 - 5.9
Disodium hydrogen phthalate / Sodium dihydrogen orthophospate	5.8 - 8.0
Dipotassium hydrogen phthalate / Potassium dihydrogen orthophospate	5.8 - 8.0
Potassium dihydrogen orthophosphate / sodium hydroxide	5.8 - 8.00
Barbitone sodium / Hydrochloric acid	6.8 - 9.6
Tris (hydroxylmethyl) aminomethane / Hydrochloric acid	7.0 - 9.00
Sodium tetraborate/ Hydrochloric acid	8.1 - 9.2
Glycine/ Sodium hydroxide	8.6 - 10.6
Sodium carbonate/ Sodium hydrogen carbonate	9.2 - 10.8
Sodium tetraborate/ Sodium hydroxide	9.3 - 10.7
Sodium bicarbonate / Sodium hydroxide	9.60 - 11.0
dium hydrogen orthophosphate / Sodium hydroxide	11.0 - 11.9
Potassium chloride/ Sodium hydroxide	12.0 - 13.0

Appendix IV

Proof of Submission for Article 1

De :	onbehalfof+jamlam+wiley.com@manuscriptcentral.com pour le compte de jamlam@wiley.com
A :	thanhtung.lai@uqtr.ca; Barnabé, Simon
Cc :	thanhtung.lai@uqtr.ca; Barnabé, Simon; thi.thanh.ha.pham@uqtr.ca; Adjalle, Kokou; Brouillette, François
Objet :	Manuscript submitted to Letters in Applied Microbiology - LAM-2016-0876
Date :	25 avril 2016 11:13:37

25-Apr-2016

Dear Mr. Lai

The following manuscript has been successfully submitted to Letters in Applied Microbiology.

Manuscript ID: LAM-2016-0876
Title: Use of pulp and paper sludge as a raw material for Bacillus spp. fermentation to produce cellulolytic enzymes for biorefining activities
Authors: Lai, Thanh-Tung; Pham, Thi-Thanh-Ha; Adjallé, Kokou; Brouillette, François; Barnabé, Simon

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Thank you for your submission to Letters in Applied Microbiology.

Kind regards

Ms. Catherine Hyland
Letters in Applied Microbiology

Appendix V

Published Article - Article no.2

Waste Biomass Valor
DOI 10.1007/s12649-016-9686-y

ORIGINAL PAPER

Production of *Trichoderma Reesei* RUT C-30 Lignocellulolytic Enzymes Using Paper Sludge as Fermentation Substrate: An Approach for On-Site Manufacturing of Enzymes for Biorefineries

Thanh Tung Lai[1] · Thi Thanh Ha Pham[1] · Kokou Adjallé[1] · Daniel Montplaisir[1] · François Brouillette[1] · Simon Barnabé[1]

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Abstract Different types of pulp and paper sludge were used as raw materials for the production of a cocktail of lignocellulolytic enzymes of *Trichoderma reesei* RUT C-30 (*T. reesei* RUT C-30). The fungus were grown in pellets to produce cellulases (carbomethylcellulase, β-glucosidase) and hemicellulases (xylanase, β-xylosidase) in three types of sludge: primary (PS), secondary (SS) and mixed sludge (MS). The highest carbomethylcellulase activities obtained after 7 days of fermentation were 7.3, 4.8, and 1.5 IU/ml in MS, SS, and PS, respectively. Sludge modification such as the mixing SS and PS at 1:1 (v/v) ratio, the addition of ammonium sulfate as an inorganic nitrogen source, and the increase of the solids content were shown to improve enzyme production. The crude enzyme mixture obtained from the sludge samples showed a synergistic effect to hydrolyze various biomasses into monosugars. The tested biomasses included highly purified CMC, xylan from birch wood and lignocellulosic materials (corn stover and primary pulp and paper sludge). The addition of a surfactant (polysorbate 20) to the enzyme cocktail enhanced the saccharification efficiency of the sludge, in particular the hydrolysis of the corn stover which contains lignin. The obtained results contribute to the assessment of the feasibility of on-site low cost enzyme production at paper mills generating the sludge, or for neighboring or local biorefineries.

✉ Simon Barnabé
Simon.barnabe@uqtr.ca

[1] Lignocellulosic Materials Research Centre, Université du Québec à Trois-Rivières, 3351, Boul. des Forges, Trois-Rivières G9A 5H7, Canada

Keywords Cellulase · Enzyme · On-site manufacturing · Paper sludge · Lignocellulosic biomass · *Trichoderma reesei* · Xylanase

Introduction

Lignocellulosic biomass is a renewable and abundant source of carbohydrates that can be converted into simple sugars to produce biofuels and biochemical through microbial fermentation technologies. Agricultural and industrial residual biomasses are an interesting alternative to crop biomass as secondary generation feedstock for biorefinery that would not contribute to the global food crisis. However, high amounts of various lignocellulolytic enzymes are required to convert complex lignocellulosic materials into fermentable sugars. This particular situation contributes to the high cost of industrial scale biofermentation processes. Several solutions are being investigated to minimize enzyme consumption. Among them, engineering of cellulase enzymes with high hydrolytic efficiency and yield, biomass pretreatment, and alternate raw materials for enzyme production are particularly promising [1–3].

Various lignocellulosic residues have already been successfully used as carbon source and cellulase inducers in the cultivation of cellulolytic bacteria and fungi. Lignocellulolytic enzymes with high specific activities were obtained [4–6]. However, competition for the biomass supply could arise from the fact that the same material, which contains high amounts of cellulose, would be used for the production of enzymes and also as a feedstock for cellulosic ethanol manufacturing. This could result in both enzyme and ethanol yield reductions [7]. Waste streams from agricultural and forestry industries, such as wastewaters and wastewater sludge, contain carbohydrates potentially capable of

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Appendix VI

Published Article - Article no.3

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ORIGINAL PAPER

Strategies for Using Pulp and Paper Sludges as Culture Media for Xylanase Production with *Bacillus pumilus*

Thanh Tung Lai[1] · Thi Thanh Ha Pham[1] · Kokou Adjallé[1] · François Brouillette[1] · Simon Barnabé[1]

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Abstract Fermentation of *Bacillus pumilus* (*B. pumilus*) using different pulp and paper sludges as culture media were performed in this work to produce at lower cost industrial enzymes such as xylanases. Secondary sludge was shown to be a suitable alternative culture medium for *B. pumilus* growth, while primary sludge may serve as xylanases inducer. Mixing primary (PS) and secondary sludges (SS) at 1PS:2SS (w/w) ratio having 15 g/L total solids concentration resulted in the highest cell concentration of 2×10^8 CFU/mL and the highest xylanase activity of 3.8 IU/mL under shake flask fermentation. Other lignocellulosic biomasses were tested as potential xylanase inducers. Addition of corn stover to SS showed the highest xylanase activity (10.7 IU/mL). When using a 7 L bioreactor, total cell concentration and xylanase activity obtained in the secondary sludge medium supplemented with commercial xylan (2.5×10^9 CFU/mL and 35.5 IU/mL, respectively) and corn stover (3.4×10^9 CFU/mL and 37.8 IU/mL, respectively) were comparative to a semi-synthetic based medium (5.8×10^9 CFU/mL and 47 IU/mL, respectively). The xylanase activity of *B. pumilus* produced in paper sludge is stable at pH 6–9 at 50 °C that offered a potential application of the enzyme for biobleaching in pulp and paper industry.

Keywords *Bacillus pumilus* · Xylanase · Pulp and paper sludge · Lignocellulosic biomass

✉ Simon Barnabé
simon.barnabe@uqtr.ca

[1] Université du Québec à Trois-Rivières, 3351, boul. des Forges, Trois-Rivières G9A 5H7, Canada

Introduction

The global forest, paper and packaging industry has been under pressure over the last years. The financial situation of the pulp and paper sector was dramatically affected by economic downturns, weak markets, intense competition, environmental regulations, and increasing production costs. The industry must develop new and creative strategies to reduce costs, improve margins and adapt to new regulations. Such strategies could be the application of biotechnology to increase production yields, reduce input cost, energy consumption and pollution generation. One example is the use of enzymes in the pulp and paper mills to mitigate pitch deposits [1, 2] or to convert waste streams into valuable coproducts like C5 sugars or building block molecules [3, 4]. In this study, the potential of the xylanase enzyme to generate interesting coproducts and be part of a value added strategy pulp and paper mills is investigated.

Xylanases are hydrolytic enzymes well known to catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan of hemicellulose. They find applications in various industrial processes: food (fruit and vegetable processing, brewing, wine production, baking), animal feed, starch, textile, bioremediation. One current application of xylanases in pulp and paper is the prebleaching of kraft pulp to minimize the use of harsh chemicals. Xylanases are also useful for the production of second generation biofuels. They are commonly used as accessory enzymes, to supplement multi-enzyme cocktails, in some biofuel production processes in bioethanol fermentation. They have a synergistic effect with cellulases in the hydrolysis of the lignocellulosic biomass to simple sugars [5–7].

Various microorganisms, including bacteria, fungi, yeasts, and actinomycetes can produce multiple xylanases [5, 6, 8, 9]. Although fungi are considered as host producers of xylolytic