



(51) International Patent Classification:

C12N 9/14 (2006.01) C12N 9/42 (2006.01)
C12N 9/24 (2006.01) C12P 19/00 (2006.01)

(21) International Application Number:

PCT/CA2012/000270

(22) International Filing Date:

26 March 2012 (26.03.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2,735,607 29 March 2011 (29.03.2011) CA
2,772,388 22 March 2012 (22.03.2012) CA

(71) Applicant (for all designated States except US): **LIGNOL INNOVATIONS LTD.** [CA/CA]; 101-4705 Wayburne Drive, Burnaby, British Columbia V5G 3L1 (CA).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **BERLIN, Alex** [CA/CA]; c/o 101-4705 Wayburne Drive, Burnaby, British Columbia V5G 3L1 (CA).

(74) Agents: **POLONENKO, Daniel** et al.; Gowling Lafleur Henderson LLP, Box 30, 2300-550 Burrard Street, Vancouver, British Columbia V6C 2B5 (CA).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: FLOCCULANTS FOR ENZYME RECOVERY AND RECYCLING

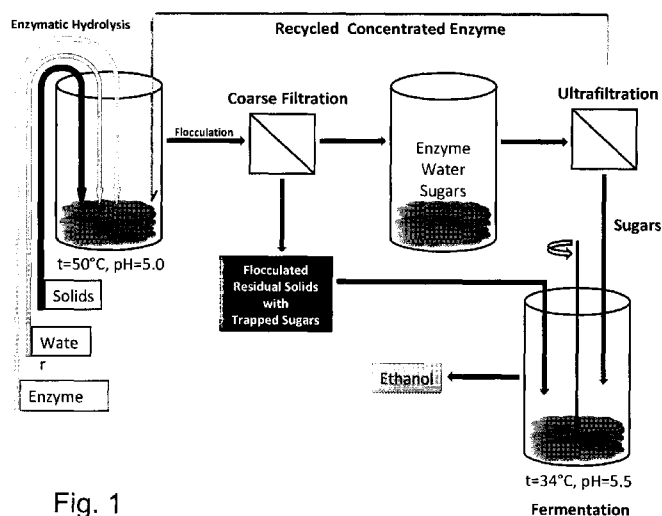


Fig. 1

(57) Abstract: A composition for separation and recovery of hydrolytic enzymes from saccharified hydrolysates. The composition comprises flocculants for flocculating particulates. When added to saccharified hydrolysates, the composition flocculates the particulates thereby separating a clarified supernatant that comprises the hydrolytic enzymes. The compositions comprise one or more of cationic polyacrylamides, lignins, lignin derivatives, sulfonated lignin derivatives and mixtures thereof. Suitable lignin derivatives can be recovered from organosolv pretreatment of lignocellulosic feedstocks.

TITLE: FLOCCULANTS FOR ENZYME RECOVERY AND RECYCLING

FIELD

This disclosure relates to recovery of enzymes such as lignocellulose-degrading enzymes. For example, the present disclosure relates to the recovery of hydrolytic enzymes from hydrolysate mixtures. More particularly, this disclosure relates to use of flocculants for recovery of enzymes from hydrolysates. This disclosure further relates to the recycling of flocculated enzymes. This disclosure also relates to flocculant compositions for enzyme recovery, and to methods and systems using the flocculant compositions for recovery and recycling of hydrolytic enzymes.

BACKGROUND

Biochemical transformation of lignocellulosic biomass into fermentable carbohydrates useful for cellulosic ethanol production is currently considered to be one of the most promising technological routes for commercial biorefining of lignocellulosics into sustainable fuels and chemicals. The advantages include the potential for high product yields and selectivity of fermentable monosaccharides such as hexoses (glucose, mannose, galactose) and pentoses (xylose and arabinose), and/or oligosaccharides, cellobiose, xylobiose, xylotriose, cellotriose, etc., from the enzymatic hydrolysis of the cellulosic constituents of biomass by glycanases and other hydrolases or non-catalytic proteins. However, exceedingly high commercial costs for lignocellulose-degrading enzymes remain a major deterrent to current commercialization efforts for lignocellulosic biorefinery technologies. Consequently, considerable research efforts have recently been focused on reducing the cost of lignocellulose-degrading enzymes and on improving their hydrolytic performance. For example, the United States Department of Energy (US-DOE) set the goal of, by the year 2012, to bring the cost of enzymes per gallon of cellulose ethanol produced, to 10 cents. The US-DOE has granted over \$50Million USD toward this goal during the last eight years, to industrial, academic, and government research organizations with the mandate to decrease lignocellulose-degrading enzyme costs and/or improve enzymatic efficiency. Similar efforts in Europe are being supported by the European Commission through their NILE program (i.e., New Improvements for Ligno-cellulosic Ethanol). These significant funding programs have accelerated scientific research efforts that assessed the feasibility of

different approaches to improve the specific activity and operational performance of lignocellulose-degrading enzymes by enhancement of substrate reactivity by pretreatment and post-pretreatment methods, increased enzyme stability, tailoring of enzyme specificity, and reduced enzyme end-product inhibition. However, the continuing high costs associated
5 with the enzymatic hydrolysis of cellulosic substrates at current pre-commercial pilot plant throughput volumes underscore the significant need for further incremental reductions in the costs of lignocellulose-degrading enzymes.

One strategy recently considered was the recovery and recycling of the enzymes. However, all of the results available in the public domain are based on experiments run under
10 conditions that are irrelevant from an industrial prospective because of the very low substrate consistencies used, i.e., with less than 5% substrate solids in the reaction mixtures in combination with very high enzyme loadings that far surpassed current loadings used in commercial systems.

The kinetics of the enzymatic hydrolysis of insoluble cellulosic substrates by
15 cellulases do not follow simple Michaelis-Menten behaviour (Zhang et al., 1999, Substrate heterogeneity causes the nonlinear kinetics of insoluble cellulose hydrolysis, *Biotechnol. Bioeng.* 66:35-41). Specifically, increasing the dosage of cellulase in a hydrolysis reaction does not provide a linearly dependent increase in the amount of glucose produced in a given time. There is also a significant decrease in the rate of reaction as cellulose hydrolysis
20 proceeds.

Inhibition of enzymes by the products of the reactions they catalyze has long been recognized by those skilled in these arts. The nature of product inhibition may be competitive, as product competes with substrate to form the same interactions with the enzyme, but other forms of inhibition are possible. Indeed, due to the insoluble nature of
25 cellulose and the challenges it poses as a substrate in kinetic studies, there have been many conflicting reports as to the nature of inhibition in the cellulase system (Holtzapple et al , 1990, *Biotechnol. Bioeng.* 36:275-287). The cellobiohydrolases are subject to inhibition by their direct product, cellobiose, and to a lesser degree by the glucose produced by the further hydrolysis of the cellobiose by β -glucosidase. One technique for reducing cellulase inhibition
30 is to increase the amount of β -glucosidase in the system (U.S. Patent No. 6,015,703), as cellobiose is more inhibitory to cellulases than glucose (Holtzapple et al , 1990, *Biotechnol. Bioeng.* 36:275-287; Teleman et al, 1995). Inhibition can be mitigated by altering the primary

sequence of the protein using DNA mutagenesis guided by rational design or applied randomly. For example, rational design was used to target the Y245 residue in Cel5A, an endoglucanase, for mutagenesis, which resulted in an increase in its cellobiose inhibition constant (U.S. Publication No. 2003/0054535).

- 5 All of these problems contribute to the current lack of viable enzyme recovery and recycling processes and systems for conversion of lignocellulosic substrates into fermentable monosaccharides and/or oligosaccharides.

SUMMARY

- The exemplary embodiments of the present disclosure relate to compositions,
10 methods, and systems for recovery of enzymes from suspensions comprising saccharified hydrolysates and solids comprising spent or nearly spent cellulosic feedstocks. Suitable flocculant materials are exemplified by polyelectrolytes such as polyacrylamides. Suitable polyacrylamides are cationic polyacrylamides. Other suitable flocculant materials include
15 lignins, lignin derivatives, and lignin-containing polymers such as lignosulfonates. Suitable lignin derivatives include lignin derivatives recovered from organosolv pulping of lignocellulosic feed stocks. Such organosolv lignin derivatives may be sulfonated prior to their use as flocculants for recovery of clarified supernatants comprising enzymes from saccharified hydrolysates and spent solids slurries.

BRIEF DESCRIPTION OF THE DRAWINGS

- 20 The present disclosure will be described in conjunction with reference to the following drawings, in which:

- Figure 1 is a schematic flowchart showing a portion of a biorefining system for processing lignocellulosic feedstocks, wherein a pretreated cellulosic feedstock is saccharified by enzymatic hydrolysis after which, the enzymes are recovered from
25 saccharified hydrolysates by flocculation and membrane filtration and then recycled for saccharification;

Fig. 2 is a chart showing the effects of different concentrations of a flocculant on enzyme hydrolytic activity in a buffered saccharification system;

Fig. 3 is a chart showing the effects of different concentrations of a flocculant on enzyme hydrolytic activity in a non-buffered saccharification system;

Figs. 4(A) and 4(B) are charts showing the hydrolytic performance of a commercial cellulase preparation during three cycles of recovery and recycling by flocculation, (A) shows a time course of glucose accumulation, and (B) shows the percentage of glucan converted to glucose over time; and

Figs. 5(A) and 5(B) are charts showing the effects of recovery of enzymes from saccharified hydrolysates on subsequent fermentation of glucose, (A) shows a time course percentage of glucan converted to glucose concurrently with ethanol production, and (B) shows the percentage conversion of glucose to ethanol over time.

DETAILED DESCRIPTION

The exemplary embodiments of the present disclosure relate to compositions, methods, and systems for recovery of enzymes from suspensions comprising saccharified hydrolysates and solids comprising spent or nearly spent cellulosic feedstocks. The exemplary compositions comprise materials selected for recovery from saccharified hydrolysates, of clarified supernatants comprising enzymatic proteins exemplified by endo- β -1,4-glucanases, cellobiohydrolases, cellulases, hemicellulases, β -glucosidases, β -xylosidases, xylanases, α -amylases, β -amylases, pullulases, esterases, and mixtures thereof.

Suitable flocculant materials are exemplified by polyelectrolytes such as polyacrylamides. Flocculants are chemicals that promote flocculations by causing colloids and other suspended particles in liquids to aggregate. The preferred flocculants for enabling the recovery and recycling of enzymes present in hydrolysates produced by enzymatic hydrolysis, are exemplified by long-chain polymer flocculants, such as modified polyacrylamides. Flocculants such as aluminium sulfate, iron(II)sulfate and other flocculants containing heavy or transition metals are not suitable since these metal cations are strong inhibitors of lignocellulose-degrading enzymes. However, other suitable flocculants for recovery and recycling of enzymes from reaction mixtures are further exemplified by linear polysaccharides such as chitosan, lignin-derivatives containing amino groups, other commercial flocculants such as Nalco's flocculant products 71303, 9907, 8181, 9908, 9909. Suitable polyacrylamides are cationic polyacrylamides exemplified by OPTIMER[®] 7192

PLUS (OPTIMER is a registered trademark of Nalco Chemical Company Corp., Naperville, IL, USA). Other suitable flocculant materials include lignins, lignin derivatives and lignin-containing polymers such as lignosulfonates exemplified by REAX[®] 85A (REAX is a registered trademark of MeadWestvaco Corp. Richmond, VA, USA). Suitable lignins include lignin derivatives recovered from organosolv pulping of lignocellulosic feed stocks referred to hereinafter as organosolv lignins. Such organosolv lignins may be sulfonated prior to their use as flocculants for recovery of enzymes from saccharified hydrolysates.

Some embodiments of the present disclosure relate to methods for saccharification of cellulosic outputs recovered from lignocellulosic feedstocks pretreated with one or more of organosolv pretreatment, strong acid hydrolysis, kraft pulping, and the like. The cellulosic pulps are commingled with one or more enzymes selected for their hydrolytic performance characteristics. Suitable enzymes are exemplified by endo- β -1,4-glucanases, cellobiohydrolases, cellulases, hemicellulases, β -glucosidases, β -xylosidases, xylanases, α -amylases, β -amylases, pullulases, esterases, and mixtures thereof. It is within the scope of this disclosure that at least one of the enzymes added to the high-consistency cellulosic substrate, may be a genetically-modified enzyme. Enzymatic hydrolysis of the cellulosic substrate results in production of: (i) a supernatant, also commonly referred to as a hydrolysate or a reaction mixture, comprising sugars, enzymes and particulate matter, and (ii) spent solids slurry that may comprise unhydrolysed cellulose, lignins, and enzymes bound to the cellulose and/or lignins.

The supernatant is then separated from the spent solids. A selected flocculant is commingled with the supernatant for recovery of particulate matter thereby providing a clarified supernatant comprising sugars and enzymes. The spent solids slurry can be directly commingled with a flocculant to separate additional supernatant from the spent solids. The additional supernatant also comprises sugars and enzymes. It is optional to dilute the recovered spent solids slurry with a suitable diluent exemplified by water, prior to commingling with the flocculant. The recovered clarified supernatant and additional clarified supernatant are suitable for recycling for saccharification of fresh cellulosic pulp feedstocks. If so desired, the recovered clarified supernatant and the additional clarified supernatant can be combined prior to recycling.

Suitable concentrations of flocculants for recovering clarified supernatants from supernatants and spent solids slurries are in the range of 10–1000 ppm, 25–500 ppm, 50–250

ppm, 75–125 ppm, 85–100 ppm. The clarified supernatants may be recovered by centrifugation. Alternatively, the flocculated particulates and solids may be allowed to separate from the supernatants and allowed to settle thereby clarifying the supernatants. The recovered clarified supernatants comprising the cellulytic enzymes may be recycled into fresh
5 cellulosic pulp substrates for continued saccharification. The recovery and recycling of enzymes using flocculant compositions may be employed in batch saccharification systems and in continuous saccharification systems.

Some aspects of the present disclosure relate to commingling the enzymes recovered in clarified supernatants with fresh enzymes to adjust and/or increase the specific activity of
10 the commingled enzymes.

Other exemplary embodiment pertain to biorefinery systems for processing lignocellulosic feedstocks to produce cellulosic pulps that are subsequently saccharified and fermented to produce short-chain alcohols using batch or continuous batch or continuous SHF systems, single-tank SSF systems or alternatively single-tank HSF systems wherein at
15 least a portion of the saccharification enzymes are recovered by flocculation and are recycled for continued saccharification. An exemplary system according to one embodiment of the present disclosure for post-saccharification recovery by flocculation and filtration, and then recycled for additional saccharification of fresh cellulosic pulp streams, is shown in Fig. 1. The recovered enzymes in clarified supernatants may be commingled with fresh enzymes
20 and/or with bound enzymes recovered with spent solids.

In the present description, a number of terms are used, the following definitions are provided to facilitate understanding of various aspects of the disclosure. Use of examples in the specification, including examples of terms, is for illustrative purposes only and is not intended to limit the scope and meaning of the embodiments of the invention herein. Numeric
25 ranges are inclusive of the numbers defining the range. In the specification, the word “comprising” is used as an open-ended term, substantially equivalent to the phrase “including, but not limited to,” and the word “comprises” has a corresponding meaning.

The invention includes all embodiments, modifications and variations substantially as hereinbefore described and with reference to the examples and figures. It will be apparent to
30 persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims. Examples of such

modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way.

EXAMPLES

Example 1:

5 CELLIC[®] Ctec2 enzyme suspensions were prepared in 50 mL volumes of: (i) distilled water, and (ii) in a pH 5.0, 50 mM sodium citrate buffer at a concentration of 0.1 mg/10 mL (CELLIC is a registered trademark of Novozymes A/S, Krogshoejveg 36, DK-2880, Bagsvaed, Denmark). A cationic polyacrylamide, OPTIMER[®] 7192 PLUS, was added to subsamples of distilled water, distilled water containing the enzyme, buffer, and buffer
10 containing the enzyme. Protein concentrations in the subsamples were measured by the Bradford (Coomassie Blue) assay. The data in Table 1 indicate that recovery of enzymes in clarified supernatants by flocculation of particulates, did not affect the enzymatic protein concentrations.

Table 1:

	[Enzyme] 0.1 g / 10 mL	OPTIMER [®] 7192-PLUS 50 ppm	Protein mg / mL	Protein %
Water	+	-	0.56	100
	-	+	0.00	0
	+	+	0.47	85
Buffer	+	-	0.65	100
	-	+	0.00	0
	+	+	0.63	97

15 Example 2:

3.6-L reaction vessels were loaded with 3.0L of a 50-mM potassium citrate buffer pH 5.5 to which was added a CELLIC[®] Ctec2 enzyme suspension comprising 15 mg protein per g glucan and 2 ppm of the antibiotic Lactrol[®] made in 75% ethanol w/w (Lactrol is a registered trademark of Phibro Animal Health Corp., Fort Lee, USA). 4% solids comprising a
20 cellulosic pulp produced by organosolv pretreatment of an aspen feedstock was added at 4-h intervals to a total solids content of 16. The hydrolysis reaction temperature was maintained

at 50° C with mixing at 250 rpm with marine impeller blades. The reaction mixtures were sampled for glucose analysis every 24 hours over a 5-day period.

After 5 days of hydrolysis, the hydrolysate mixture was adjusted to pH 6.0 with 1.0M potassium hydroxide. The following concentrations of OPTIMER[®] 7192-PLUS flocculant was added to separate reaction mixtures; (i) 0 – control, (ii) 20 ppm, (iii) 50 ppm, and (iv) 125 ppm. The reaction mixtures with the flocculant were gently stirred with a glass stir rod over a period of 15 minutes. The flocculated solids were left to settle at room temperature for 15 minutes. Each supernatant was then transferred and the remaining flocculated solids was filtered through a Buchner funnel with a polypropylene multi-film membrane (coarse pore size: 20 µm, permeability: 2.5 cubic metre/m²/min, Type S2191-L1, Batch: 165157, supplied by Tamfelt). The supernatant and the filtrate from the Buchner funnel filtration were then filtered through a coarse glass 600 mL Buchner funnel with a coarse fritted disc (Fisher, cat# 10-358-T, pore size 40 to 60 µm). The final filtrate was ultra-filtered with the help of a tangential filtration system equipped with NOVASET[®]-LS 10 KDa polyethersulfone membrane filter cassettes (NOVASET is a registered trademark of Stora Enso Oyj Corp., Helsinki, Finland). The recovered enzyme concentrates and ultrafiltrates were separated using this system. Initially, the ultra-filtration membranes were washed with water, then with 50 mM potassium citrate buffer (pH 5.5). After ultra-filtration, the filter membranes were washed with 0.5 M sodium hydroxide solution, then with water and then with 0.05% sodium azide. The membranes were stored at 4° C in sodium azide solution.

The recovered filtrates comprising enzymes were then added to fresh 3.6-L vessels containing 3.0 L fresh buffer and 16% cellulosic pulp. The hydrolysis reaction temperatures were maintained at 50° C with mixing at 250 rpm with marine impeller blades. The reaction mixtures sampled for glucose analysis every 24 hours over a 96-h period. The data in Fig. 2 show that the rates of saccharification were in the reaction vessels receiving recovered enzymes were identical to the unflocculated controls.

Example 3

The study outlined in Example 2 was repeated substituting tap water pH-adjusted to 5.5 with 10% aqueous ammonia solution, for the 50-mM potassium citrate buffer. After 5 days of hydrolysis, the hydrolysate mixture was adjusted to pH 6.0 with 1.0M potassium hydroxide. The following concentrations of OPTIMER[®] 7192-PLUS flocculant was added to

separate reaction mixtures; (i) 0 – control, (ii) 20 ppm, (iii) 50 ppm, and (iv) 125 ppm. The reaction mixtures with the flocculant were gently stirred with a glass stir rod over a period of 15 minutes. The flocculated solids were left to settle at room temperature for 15 minutes. Each supernatant was then transferred and the remaining flocculated solids were recovered and processed as described in Example 2.

The recovered enzymes were then added to fresh 3.6-L vessels containing 3.0 L fresh water and 16% cellulosic pulp. The reaction mixtures were made up with 7.5 mg per gram glucan of fresh enzyme. The hydrolysis reaction temperatures were maintained at 50° C with mixing at 250 rpm with marine impeller blades. The reaction mixtures sampled for glucose analysis every 24 hours over a 96-h period. The data in Fig. 3 show that the rates of saccharification were in the reaction vessels receiving recovered enzymes were identical to the controls.

Example 4

The study outlined in Example 3 was repeated with the enzymes recovered by flocculation and recycling for three consecutive cycles. At the completion of each saccharification cycle, the enzymes were recovered by flocculation with 80 ppm Nalco OPTIMER® 7192 PLUS. Each recycled batch of enzymes was made-up with fresh CELLIC® CTec2 enzymes (7.5 mg/g glucan). Fig. 4(A) shows glucose concentration (g/L) versus enzymatic hydrolysis time at 50° C, 16% TS, 150 rpm. Fig. 4(B) shows glucan-to-glucose conversion (% theoretical yield) versus enzymatic hydrolysis time at 50° C, 16% TS, 250 rpm.

The clarified hydrolysates, i.e., hydrolysates recovered from the first saccharification cycle and the second saccharification cycle were subsequently fermented. THERMOSACC® (THERMOSACC is a registered trademark of Lallemand Specialities Inc. Milwaukee, WN, USA) was added to the hydrolysate at a concentration of 0.25 g/Kg, urea was added at a concentration of 1g/L, and AYF1000 and AYF1200 were added at concentrations of 0.25% each. Fermentation was carried out at 34° C and 150 rpm. 0.25g/Kg Thermosacc with addition of 1g/L urea, and 0.025% of AYF1000 + AYF1200 each. The data in Figs. 5(A) and 5(B) show that recovery of enzymes from the hydrolysates by flocculation, did not affect the rates of fermentation of the amounts of ethanol produced.

What is claimed is:

1. A composition for separation of hydrolytic enzymes from hydrolysates produced during saccharification of cellulosic feedstocks thereby enabling recovery and recycling of the hydrolytic enzymes, said composition comprising a flocculant.
2. The composition of claim 1, wherein the flocculant comprises a lignin derivative.
3. The composition of claim 1, wherein the flocculant comprises a sulfonated lignin derivative.
4. The composition of any of claims 2 and 3, wherein the lignin derivative is an organosolv lignin.
5. The composition of claim 1, wherein the flocculant comprises a cationic polyacrylamide.
6. The composition of claim 1, wherein the flocculant comprises a mixture of cationic polyacrylamides.
7. The composition of claim 1, wherein the flocculant comprises a chitosan.
8. The composition of claim 1, wherein the flocculant comprises a mixture of two or more of a lignin derivative, a cationic polyacrylamide, and a chitosan.
9. Use of a composition comprising a flocculant for separation of hydrolytic enzymes from a saccharified hydrolysate.
10. Use according to claim 9, wherein the hydrolytic enzymes are saccharification enzymes useful for hydrolysing cellulosic feedstocks.

11. Use according to claim 9, wherein the hydrolytic enzymes are recovered in a clarified supernatant separated from the saccharified hydrolysate.
12. A method for recovery and recycling of hydrolytic enzymes from a hydrolysate saccharified from a cellulosic feedstock, the method comprising:
 - (a) commingling the saccharified hydrolysate with a flocculant composition selected for flocculating particulates;
 - (b) separating from the flocculated particulates, a clarified supernatant comprising recovered hydrolytic enzymes;
 - (c) recovering the clarified supernatant; and
 - (d) recycling the clarified supernatant into a fresh cellulosic feedstock for hydrolysis thereof.
13. The method according to claim 12, additionally comprising making up the recovered hydrolytic enzymes with fresh enzymes prior to recycling.
14. The method according to claim 13, wherein the flocculant composition is the composition of claim 1.

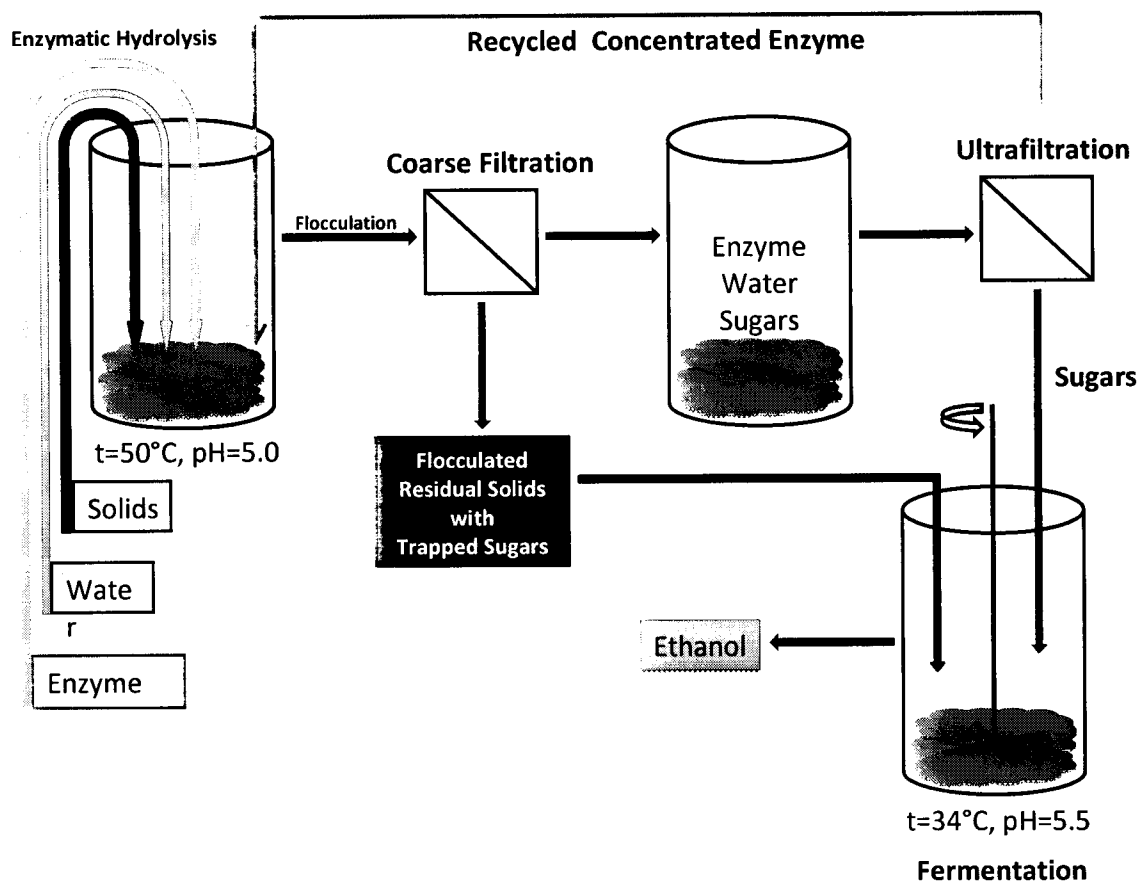


Fig. 1

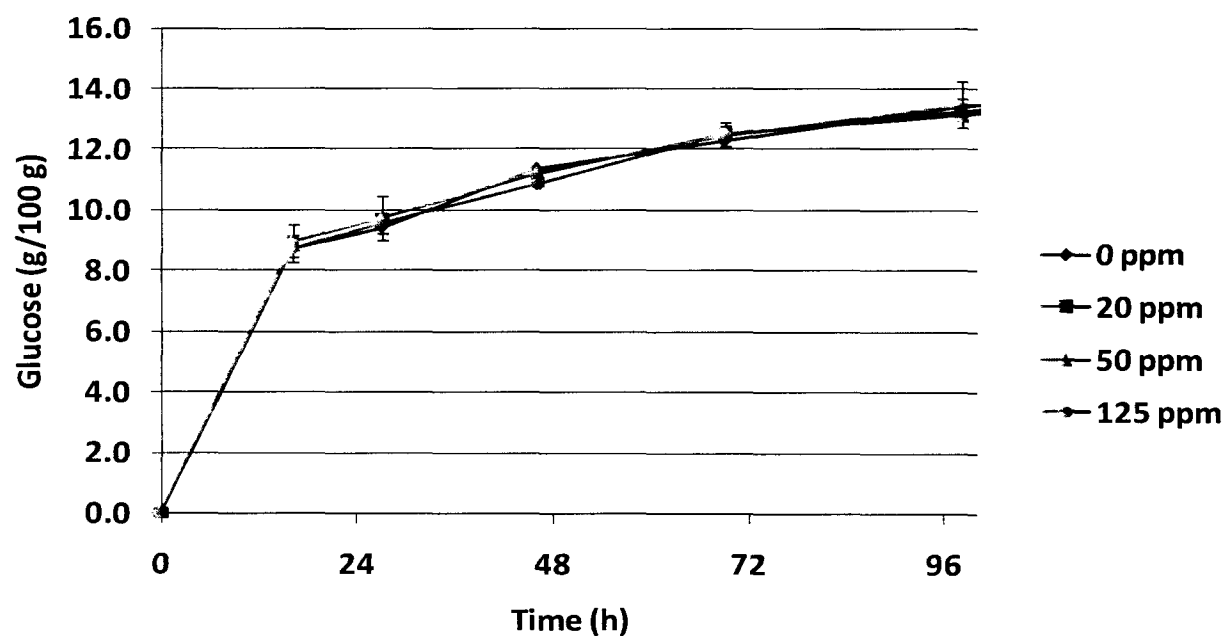


Fig. 2

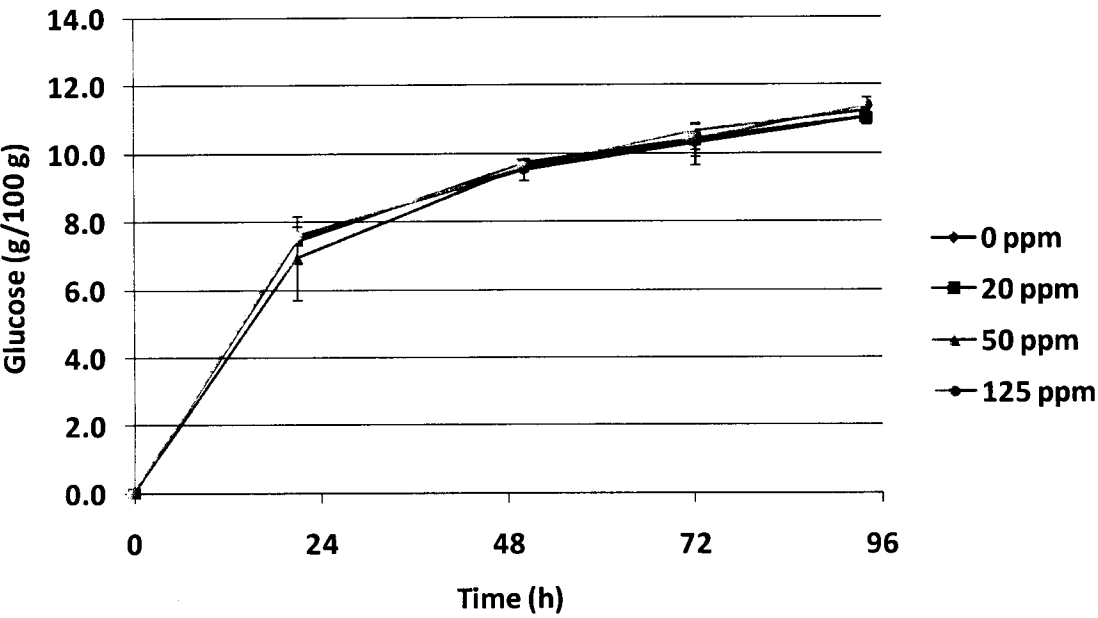
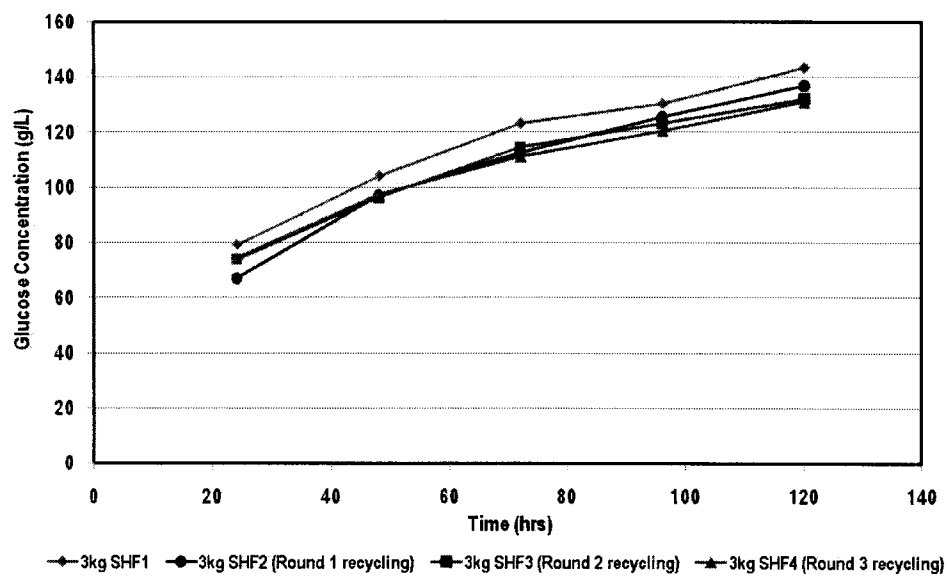


Fig. 3

(A)



(B)

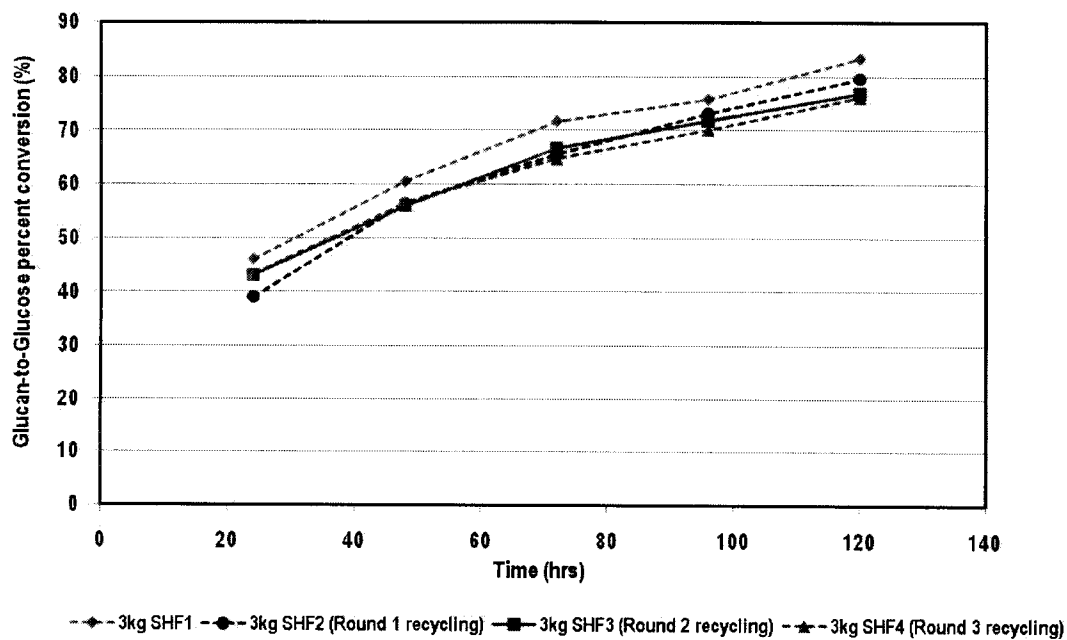
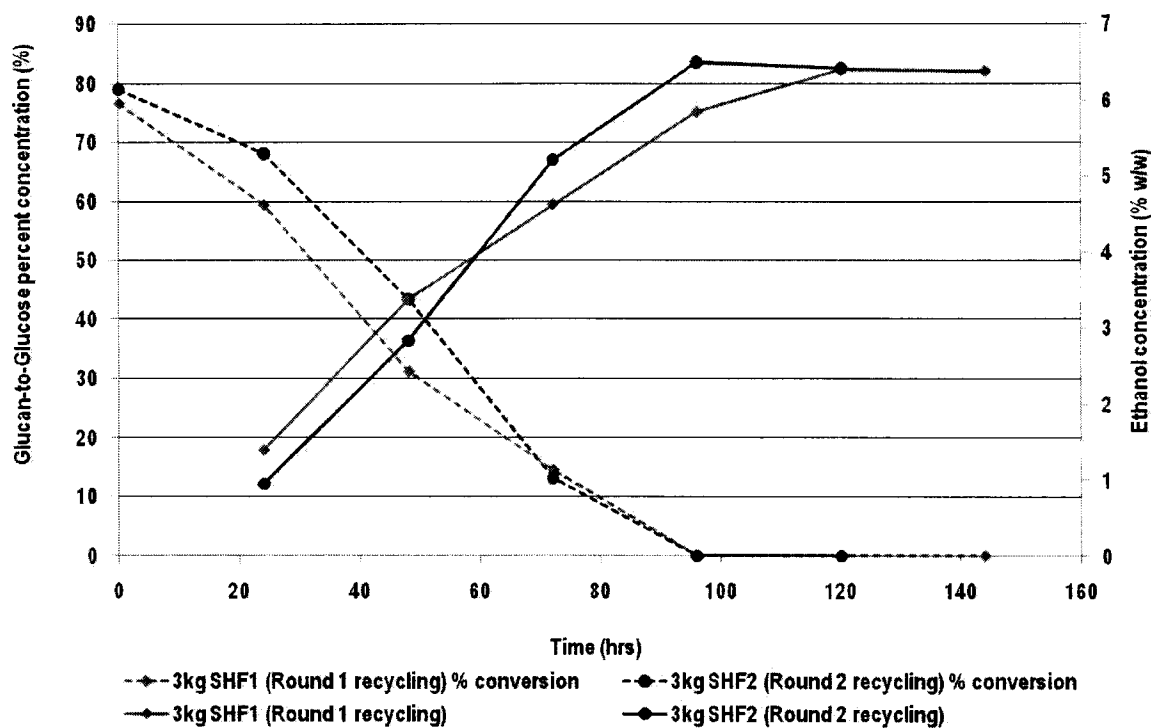


Fig. 4

(A)



(B)

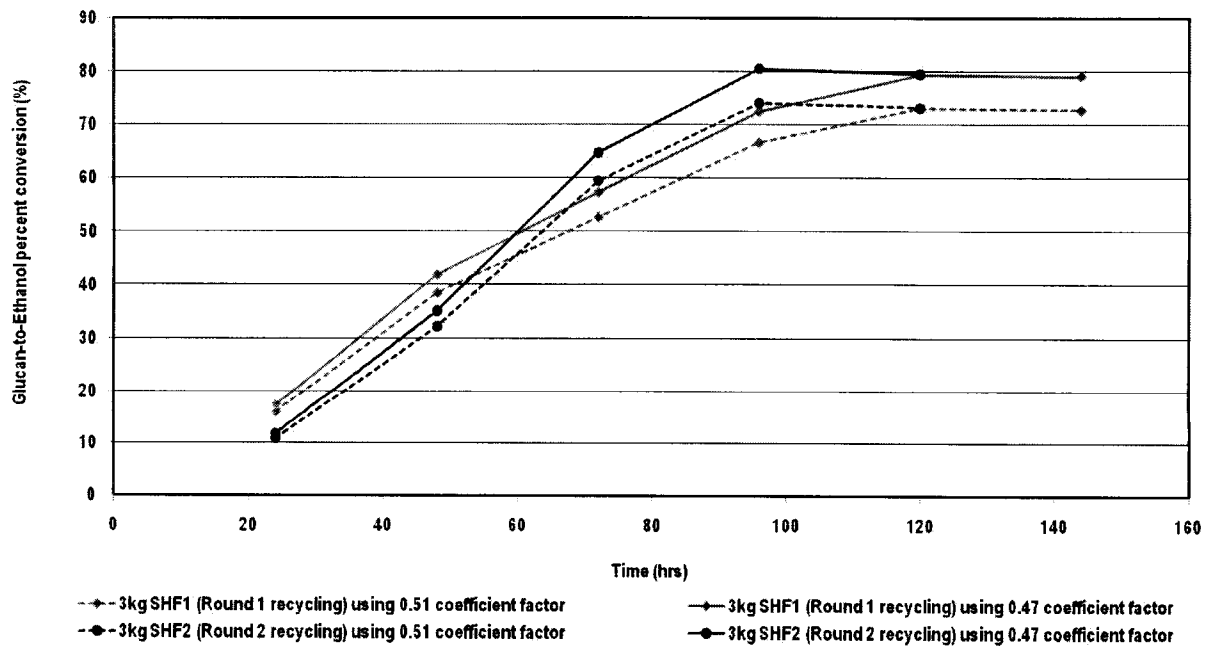


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000270

A. CLASSIFICATION OF SUBJECT MATTER IPC: C12N 9/14 (2006.01) , C12N 9/24 (2006.01) , C12N 9/42 (2006.01) , C12P 19/00 (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC: C12N 9/14 (2006.01) , C12N 9/24 (2006.01) , C12N 9/42 (2006.01) , C12P 19/00 (2006.01) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Canadian Patent Database, TotalPatent, Scopus, Medline; Search terms: flocculant, flocculation, lignin, polyacrylamide, chitosan, hydrolase, enzyme, glucanase, cellulase, saccharification, lignocellulose, feedstocks, ethanol, recovery, reuse, purification, recycling, reutilization														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>TANAKA, M. ET AL.: "Removal of lignin and reuse of cellulases for continuous saccharification of lignocelluloses", BIOTECHNOLOGY AND BIOENGINEERING, 20 September 1988 (20-09-1988) vol. 32, no. 7, pages 897-902, ISSN: 0006-3592 see the whole document, especially Figures 2 and 5 and page 900, left column, paragraph 2</td> <td>1, 2 and 7-14</td> </tr> <tr> <td>X</td> <td>LIU, H. ET AL.: "Study on preparation and application in flocculants of modified lignin", MODERN APPLIED SCIENCE, February 2011 (02-2011) vol. 5, no. 1, pages 205-208, ISSN:1913-1844 see the whole document</td> <td>1, 3 and 4</td> </tr> <tr> <td>X</td> <td>WANG, Y. ET AL.: "Application of a novel cationic polyacrylamide as flocculant in treatment of papermaking wastewater", June 2010 (06-2010) BIOINFORMATICS AND BIOMEDICAL ENGINEERING, 2010 4th INTERNATIONAL CONFERENCE, pages 1-4, ISSN: 2151-7614 see the whole document</td> <td>1, 5 and 6</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	TANAKA, M. ET AL.: "Removal of lignin and reuse of cellulases for continuous saccharification of lignocelluloses", BIOTECHNOLOGY AND BIOENGINEERING, 20 September 1988 (20-09-1988) vol. 32, no. 7, pages 897-902, ISSN: 0006-3592 see the whole document, especially Figures 2 and 5 and page 900, left column, paragraph 2	1, 2 and 7-14	X	LIU, H. ET AL.: "Study on preparation and application in flocculants of modified lignin", MODERN APPLIED SCIENCE, February 2011 (02-2011) vol. 5, no. 1, pages 205-208, ISSN:1913-1844 see the whole document	1, 3 and 4	X	WANG, Y. ET AL.: "Application of a novel cationic polyacrylamide as flocculant in treatment of papermaking wastewater", June 2010 (06-2010) BIOINFORMATICS AND BIOMEDICAL ENGINEERING, 2010 4th INTERNATIONAL CONFERENCE, pages 1-4, ISSN: 2151-7614 see the whole document	1, 5 and 6
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	TANAKA, M. ET AL.: "Removal of lignin and reuse of cellulases for continuous saccharification of lignocelluloses", BIOTECHNOLOGY AND BIOENGINEERING, 20 September 1988 (20-09-1988) vol. 32, no. 7, pages 897-902, ISSN: 0006-3592 see the whole document, especially Figures 2 and 5 and page 900, left column, paragraph 2	1, 2 and 7-14												
X	LIU, H. ET AL.: "Study on preparation and application in flocculants of modified lignin", MODERN APPLIED SCIENCE, February 2011 (02-2011) vol. 5, no. 1, pages 205-208, ISSN:1913-1844 see the whole document	1, 3 and 4												
X	WANG, Y. ET AL.: "Application of a novel cationic polyacrylamide as flocculant in treatment of papermaking wastewater", June 2010 (06-2010) BIOINFORMATICS AND BIOMEDICAL ENGINEERING, 2010 4th INTERNATIONAL CONFERENCE, pages 1-4, ISSN: 2151-7614 see the whole document	1, 5 and 6												
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents :</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention													
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family													
"O" document referring to an oral disclosure, use, exhibition or other means														
"P" document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 11 June 2012 (11-06-2012)		Date of mailing of the international search report 29 June 2012 (29-06-2012)												
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Sandra Hurley (819) 934-7934												

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000270

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OLEMPСКА-BEER, Z.: "Alpha-amylase from Bacillus licheniformis containing a genetically engineered alpha-amylase gene from B. licheniformis (thermostable)", 61st JECFA-CHEMICAL AND TECHNICAL ASSESSMENTS OF FOOD ADDITIVES (CTA), 2004, pages 1(6)-6(6) see the whole document, especially pages 4(4) and 5(5)	1
A	KNUTSEN, J. AND DAVIS, R.: "Cellulase retention and sugar removal by membrane ultrafiltration during lignocellulosic biomass hydrolysis", APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, 2004, vol. 113-116, pages 585-599, ISSN: 0273-2289 see the whole document	1-14