Biological Upgrading of Wastes from the Pulp and Paper Industry

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Abstract

A process for biological upgrading of recycled paper sludge (RPS) was developed based on the enzymatic hydrolysis of major sludge components (cellulose and xylan) with commercial enzymes and fermentation of the resulting sugars into ethanol or lactic acid with adequate microbial strains. The process was implemented performing both steps sequentially (SHF) or simultaneously (SSF).

Cellulosic and hemicellulosic fractions of RPS were completely converted by enzymatic hydrolysis (using Celluclast®1.5L with Novozym®188) into the constitutive glucose and xylose.

Ethanol was produced from the RPS hydrolysate by the yeast *Pichia stipitis* CBS 5773. A slightly higher conversion yield was attained on SHF process, corresponding to an ethanol concentration of 19.6 g L^{-1}, but 179 hours were needed. The SSF process was completed after 48 hours of incubation allowing the production of 18.6 g L^{-1} of ethanol from 178.6 g L^{-1} of dried RPS, corresponding to an overall conversion yield of 51% of the available carbohydrates on the initial substrate.

Maximum production of lactic acid (LA) with *Lactobacillus rhamnosus* ATCC 7469 was obtained by performing the SSF process: 73 g L^{-1} of LA was achieved, corresponding to a maximum productivity of 2.9 g L^{-1} h^{-1}, with 0.97 g LA produced per g of carbohydrates on initial sludge.

The present results demonstrate the feasibility of the biological conversion of the ultimate waste obtained in the paper recycling loop into a biofuel (bioethanol) or an important chemical intermediate (LA, precursor of bioplastics), under the concept of a multi-purpose biorefinery.

Introduction

Industrial production of pulp and paper is very demanding in terms of energy and raw material consumption, and thus pulp producers make every effort to optimise the use of energy and raw material, as much as possible, in a sustainable manner. The ultimate ambition is to achieve a zero-waste paper mill on a wood-based biorefinery concept.

The pulp and paper industry generates about 80 million tons of solid waste every year, and only about 42% of the waste is recycled [1]. Indeed wastewater treatment units of paper mills produce a large quantity of sludge, which represents currently a major concern of the paper industry. This solid paper sludge is mostly disposed off by combustion or by landfilling. In terms of combustion treatment, paper sludge containing more than 60% water by weight should be incinerated in combustors with an auxiliary fuel. Landfilling is expected to decrease because of the limitations of existing capacities, costs, and environmental concerns. To be used as a compost, toxic materials must be removed [2]. Therefore it is urgent to find an alternative disposal solution for this waste. Since this paper sludge has a high content of polysaccharides (mainly cellulose), it could be further processed to obtain compounds with high added value produced from sugars. This conversion requires the polysaccharides on sludge to be broken down into the constitutive monomers and the released sugars to be

fermented [3]. The common methods for degradation of a polysaccharide to the monomers consist of acid hydrolysis and enzymatic hydrolysis. Enzymatic hydrolysis is often preferable since it can not only economize energy on account of the relatively mild reaction conditions, but also avoid using toxic and corrosive chemicals [4]. The enzymatic hydrolysis and fermentation steps can be performed as separate hydrolysis and fermentation (SHF) or as simultaneous saccharification and fermentation (SSF). The SSF process offers various advantages over SHF such as the use of a single-reaction vessel for both steps (allowing process integration with the consequent reduction on capital cost), rapid processing time, reduced end-product inhibition of hydrolysis and increased productivity [5].
Inspired in the biorefinery ecosystem proposed by Ohara [6], in the present study the ultimate waste obtained in the paper recycling loop, i.e. recycled paper sludge (RPS), was evaluated as feedstock for lactic acid and ethanol production. In a biorefinery based on lactic acid fermentation and ethanol fermentation, ethanol is used as fuel for transportation and lactic acid is polymerised to form polylactate, which is used as a plastic. Lactic acid and ethanol are esterified to produce ethyl lactate, which is used as a biodegradable solvent.

Experimental

Feedstock

The present study used pressed recycled paper sludge (RPS) consisting of the solids resulting from the wastewater treatment facility of a local paper recycling mill (Renova, Torres Novas, Portugal). The as-received sludge contained calcium carbonate that rendered the resulting suspensions alkaline. Therefore, RPS was neutralised with hydrochloric acid prior to use. The initial composition of RPS was determined to be (in mass percentage of the oven-dried sludge): 29.3% ash, 3.5% fat, 4.8% protein, 20.4% lignin, 34.1% cellulose and 7.9% xylan.

Enzymatic hydrolysis trials

A sample of RPS was suspended in 0.05 M sodium citrate buffer, pH 5.5, for an initial consistency of 3% or 7.5% (w/v), expressed in terms of total carbohydrate mass, and it was steam sterilised by autoclaving (at 121ºC, 1 atm, for 15 min). Sludge suspension was incubated with the filter-sterilised enzyme solution at 50ºC or 35ºC in an orbital shaker (150 rev min^{-1}). Aseptic conditions were maintained throughout the experiments.
Enzymatic hydrolysis was performed with a previously selected mixture of two commercial enzyme preparations (from Novozymes, Denmark): Celluclast® 1.5L (exhibiting an FPase activity of 14.7 U mL^{-1} and an endo-β-xylanase activity of 228.7 U mL^{-1}) and Novozym® 188 (exhibiting an FPase activity of 0.6 U mL^{-1} and an endo-β-xylanase activity of 854.9 U mL^{-1}). This mixture was applied at different enzyme loadings. For each enzyme tested, a control enzyme mixture was subjected to the same assay conditions but in the absence of sludge. The hydrolysates obtained, after residual solid removal by filtration (through a membrane-filter of 0.45 μm pore size), were analysed for sugar profiles by HPLC.

Fermentation studies

Microorganisms

The yeast *Pichia stipitis* CBS 5773, obtained from Centraalbureau voor Schimmelcultures (CBS, Baarn) culture collection, was used for ethanol production, and *Lactobacillus rhamnosus* ATCC 7469, obtained from the American Type Culture Collection, was used in lactic acid fermentation studies.

General conditions

Experiments were performed in 500 mL Erlenmeyer flasks containing 100 mL (for ethanol) or 200 mL (for lactic acid) of culture medium, which were inoculated with 5% (v/v) of a

microbial suspension obtained from a 15-h growth. Cultivations were carried out in duplicate at 30ºC (for ethanol) or 37ºC (for lactic acid) with 150 rev min^{-1} (orbital shaking). Collected samples were analysed in terms of pH and cell growth, and then filtered for HPLC analysis of the cell-free broth obtained.

Separate hydrolysis and fermentation (SHF)

A filter-sterilised hydrolysate produced from RPS (on an initial consistency of 7.5% (w/v)) was used as fermentation medium. This hydrolysate was used with no supplement addition for ethanol production. On lactic fermentation, the RPS hydrolysate was supplemented with all the components (except glucose) of de Man Rogosa and Sharpe (MRS) broth (on the same level) and calcium carbonate (at a 30 g L^{-1} concentration, for buffering effect).
For comparative purposes, control fermentations were firstly run on YMP (for ethanol) and and MRS (for lactic acid) media supplemented with reagent-grade xylose, glucose and cellobiose mixed at the same concentrations present in the sludge hydrolysate used for the SHF process, with addition of calcium carbonate in case of lactic acid production.

Simultaneous saccharification and fermentation (SSF)

A steam-autoclaved (121ºC, 1 atm for 15 min) suspension of sludge in sodium citrate buffer pH 5.5, supplemented with all the components (except glucose) of MRS broth with calcium carbonate (for lactic fermentation), was used as SSF medium. SSF experiments were started by addition of the filter-sterilised enzyme solution and inoculation with the microorganism (in the same amounts used to obtain the hydrolysate for SHF and in its subsequent fermentation).

Analytical methods

Cell growth was directly monitored by measuring the optical density of the culture broth samples at 600 nm.
The commercial enzyme preparations applied were previously characterised for catalytic activity at the conditions used for the enzymatic hydrolysis of RPS. Filter paper activity (FPase), describing the cellulolytic activity, was assayed using Whatman number 1 filter paper as substrate. Enzyme activity was expressed in international units (U), as the amount of enzyme required to release 1 μmol of glucose reducing equivalent per minute under the assay conditions. Reducing sugars were estimated by the dinitrosalycilic acid (DNS) method [7]. Sugars (glucose, xylose and cellobiose), ethanol and organic acids (lactic, acetic and propionic acids) were measured by high-performance liquid chromatography using a Waters LC1 module 1 plus (Milford, MA) instrumentation equipped with a two-serial differential refractive index/ultraviolet detector, the latter being set at a fixed wavelength of 280 nm (for hydroxymethylfurfural and furfural detection). An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) was used, operating at 50ºC with 0.005 M H_2SO_4 as mobile phase at a flow rate of 0.4 mL min^{-1}.

Results and discussion

Enzymatic hydrolysis of RPS

The results obtained for the set of enzymatic saccharification trials using several conditions (temperature, residence time and substrate and enzyme loadings), which are shown on Table 1, are compared in terms of sludge conversion through the calculation of the degree of saccharification (DS). DS was based on the total sugar concentration in the final hydrolysate (corrected for concentration in the control assay) relatively to the content of polysaccharides (potential glucose and xylose) in the initial substrate.
Firstly, the hydrolysis was run applying Celluclast® 1.5L at the optimal conditions for activity of the enzyme preparation: 50ºC and pH 5.5. In this trial, RPS hydrolysis was apparently limited by the low enzyme thermostability at 50ºC. The following saccharification

experiments were performed at a lower temperature of 35ºC, allowing reasonable enzymatic hydrolysis activities during an extensive period of incubation with, in addition, a lower energy demand. In these trials, with Celluclast® 1.5L applied on a lower FPase dosage of 25 U g^{-1} carbohydrate, total solubilisation of RPS carbohydrates (DS = 100%) was observed after 72 hours. Monosaccharides (glucose and xylose) were the main products obtained but cellobiose was also present in the hydrolysate as well as minor amounts of xylobiose. Therefore, the hydrolysis mixture was supplemented with an excess of Novozym® 188, a cellobiase (β-glucosidase), in order to maximise the sludge conversion to monosaccharides (glucose) and to prevent cellobiose accumulation minimising product inhibition. Since in this assay no xylobiose was also detected in the hydrolysate, Novozym® 188 has also apparently contributed to complete hydrolysis of xylan to xylose.

Table 1. *Enzymatic hydrolysis yields (expressed as Degree of Saccharification, DS) and major carbohydrate products obtained from RPS in different conditions.*

T	**Sludge consistency**	**Celluclast® 1.5L**	**Novozym® 188**	**Incubation time**	**Liberated sugars for hydrolysate** (g L^{-1})				**DS**
(ºC)	% (w/v)	($U/g_{carbohydrates}$)	($U/g_{carbohydrates}$)	(h)	**Glc**	**G_2**	**Xyl**	**X_2**	(%)
50	3.0	120	0.0	144	9.9	1.6	1.9	0.0	45
35	3.0	25	0.0	72	22.6	1.8	4.9	0.7	100
35	3.0	25	1.0	72	24.4	0.0	5.6	0.0	100
35	7.5	10	0.4	120	51.1	6.3	12.0	0.0	92

Glc, glucose; **G_2**, cellobiose; **Xyl**, xylose; **X_2**, xylobiose.

The complete hydrolysis achieved in these experiments confirmed that the polysaccharides in RPS are much more susceptible to enzymatic digestion than those in traditional native lignocellulose feedstocks avoiding the need for sludge pre-treatment.
Despite the high extent of hydrolysis of sludge polysaccharides, relatively low sugar concentrations were obtained in the previously described saccharification experiments since low concentrations of substrate were used. Therefore, in order to maximise product concentrations in the hydrolysate to be used for subsequent fermentation, prolonged hydrolysis (for 120 hours) of sludge on an initial consistency of 7.5% (w/v) was performed. From the HPLC analysis of all the hydrolysates, it could be confirmed that biological inhibitory compounds (sugar-degradation products, such as furfural and hydroxymethylfurfural, or lignin-degradation products) were not produced by enzymatic action on this sludge, which constitutes an important feature for the subsequent fermentation.

SHF studies

The purpose of this work was to produce ethanol and lactic acid using sugars derived from recycled paper sludge as an alternative and inexpensive carbon source. *Pichia stipitis* CBS 5773 was used in ethanol production studies since this yeast has been described to possess the ability to convert both glucose and xylose into ethanol [8], an essential aspect for the economic feasibility of the process. Lactic acid was produced by *Lactobacillus rhamnosus* ATCC 7469, which is reported to provide high productivities and yields. The parameters measured during the course of the cultivations were cell growth, sugars consumption and ethanol and lactic acid production, along with accumulation of possible by-products. Indeed acetic and propionic acids were always the major by-products accumulated during lactic fermentation. The time-course profiles obtained for all the fermentation experiments are represented in Figures 1 and 2. For comparison terms, fermentative parameters concerning the ethanol and lactic acid production for all the cultivations performed in this work are summarised on Tables 2 and 3.

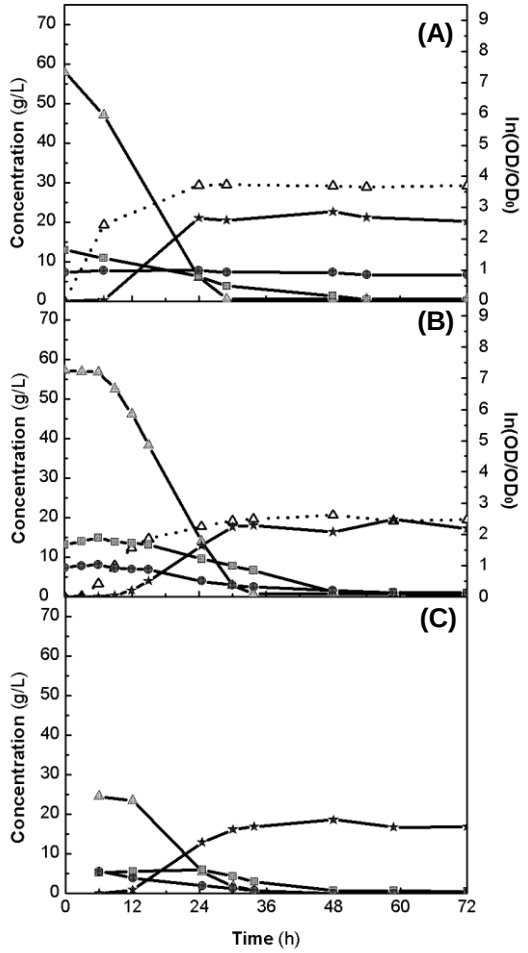

Figure 1. Time-course of the fermentation with *P. stipitis*: Panel A, Control fermentation; Panel B, SHF process; Panel C, SSF process. -▲-, glucose; -■-, xylose; -●-, cellobiose; -★-, ethanol concentrations; ··△··, growth.

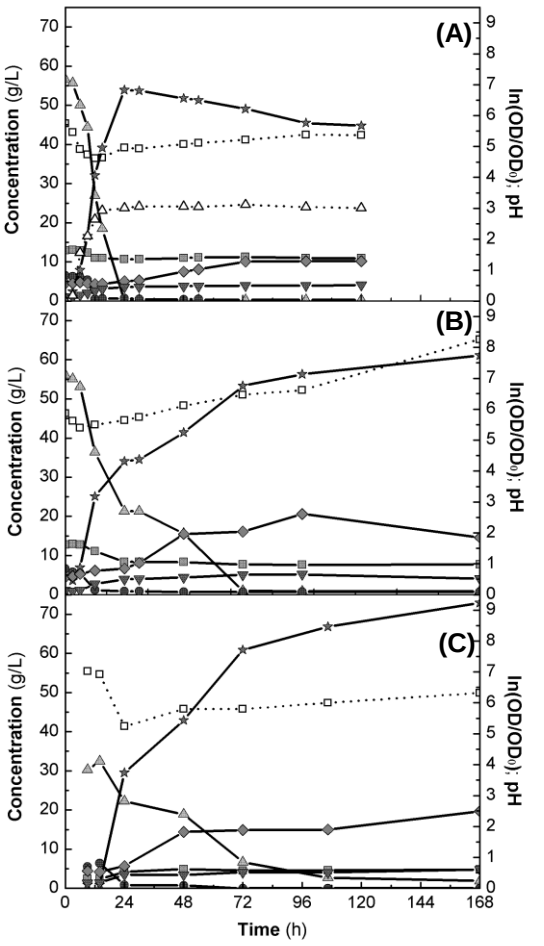

Figure 2. Time-course of the fermentation with *L. rhamnosus*: Panel A, Control fermentation; Panel B, SHF process; Panel C, SSF process. -▲-, glucose; -■-, xylose; -●-, cellobiose; -★-, lactic acid; -◆-, acetic acid; -▼-, propionic acid concentrations; ··□··, pH; ··△··, growth.

The fermentation of the sludge hydrolysate (containing 56.2 g L^{-1} glucose, 12.9 g L^{-1} xylose and 6.8 g L^{-1} cellobiose) was compared with that of commercial media supplemented with reagent-grade glucose, xylose and cellobiose at the same concentrations. Similar behaviour was observed for cell growth, sugars assimilation and product (ethanol and LA) formation on both experiments (Figures 1A and B; 2A and B), meaning that the hydrolysate could be used as carbon source and it did not produce any significant inhibitory effect on microbial growth. However, a slight delay on the beginning of the exponential phase is observed for growth on hydrolysate and sugars assimilation on this medium is somewhat slower. This observation might indicate the need for some period of adaptation to growth on hydrolysate.

Both microbial strains readily consumed glucose to produce ethanol and lactic acid, along with cell growth. Cellobiose has also been depleted by *L. rhamnosus* though it was consumed at a slightly lower rate than that of glucose assimilation. This fact confirms an important feature, that has already been described in literature [5,9].concerning the ability of *L. rhamnosus* to convert both glucose and cellobiose to lactic acid, allowing an overall bioconversion process that could avoid Novozym® 188 as supplier of cellobiase additional to that contained in the Celluclast 1.5L cellulase complex. Cellobiose consumption was not

detected in the control fermentation with *P. stipitis*. This difference might arise from the absence of cellobiase activity able to convert cellobiose to glucose. In the SHF process, the commercial enzymes added in the previous hydrolysis step (displaying cellobiase activity) remain active during the fermentation course. Thereby, the total consumption of cellobiose observed for the SHF process with *P. stipitis* might be a result from its hydrolysis to glucose and subsequent fermentation.
Xylose was fermented by *P. stipitis* only after an initial period (about 12 h) and at a slower rate than that of glucose assimilation. This observation is in agreement with previous reports stating that *P. stipitis* grown in glucose/xylose mixtures ferments preferentially glucose, since xylose assimilation is competitively inhibited by glucose [8,10,11].
Conversely, *L. rhamnosus* was unable to completely assimilate xylose. It is observed a very limited consumption of this pentose and with a low lactic acid yield since, according to literature [12], it is converted via a heterofermentative pathway yielding high titres of acetic acid as co-product.

Table 2. *Final ethanol concentration, productivity and yields obtained in the different experiments with P. stipitis.*

Experiment	**F.T.** [a]	C_{max} [b] ($g\ L^{-1}$)	$Y_{P/S}$ [c] ($g\ g^{-1}$)	$Q_{P\ max}$ [d] ($g\ L^{-1}\ h^{-1}$)
A) Control	48	22.6	0.33	1.2
B) SHF process	59	19.6	0.26	0.9
C) SSF process	48	18.6	---	1.0

[a] **F.T.** - Fermentation time corresponding to maximum ethanol concentration ([b] C_{max}).
[c] $Y_{P/S}$ - Product yield (ethanol produced in terms of total consumed sugars) calculated at F.T.
[d] $Q_{P\ max}$ - Maximum ethanol productivity, which corresponds to the maximal slope in the plot of product concentration vs time, calculated not taking into account the residence time of the enzymatic hydrolysis step that precedes the fermentation in the SHF process.

Table 3. *Final lactic acid concentration, productivity and yields obtained in the different experiments with L. rhamnosus.*

Experiment	**F.T.** [a]	C_{max} [b] ($g\ L^{-1}$)	$Y_{P/S}$ [c] ($g\ g^{-1}$)	$Q_{P\ max}$ [d] ($g\ L^{-1}\ h^{-1}$)
A) Control	24	53.9	0.82	4.0
B) SHF process	168	61.1	0.91	3.0
C) SSF process	168	72.9	---	2.9

[a] **F.T.** - Fermentation time corresponding to maximum lactic acid concentration ([b] C_{max}).
[c] $Y_{P/S}$-Product yield (lactic acid produced in terms of total consumed sugars) calculated at F.T.
[d] $Q_{P\ max}$ - Maximum lactic acid productivity, which corresponds to the maximal slope in the plot of product concentration vs time, calculated not taking into account the residence time of the enzymatic hydrolysis step that precedes the fermentation in the SHF process.

Lactic acid yields achieved in *L. rhamnosus* cultures are near 1 $g\ g^{-1}$ (Table 3). Since theoretical yields from the three consumed cellobiose, glucose and xylose are 1.0, 1.0 and 0.6 g LA per g of substrate, respectively, this observation indicates that little of the substrate is used for cell growth and maintenance, and almost all is converted to product, as desired.

SSF studies

Once demonstrated the feasibility of producing ethanol and lactic acid from RPS hydrolysate, experiments were carried out simultaneously performing the enzymatic hydrolysis and the microbial fermentation steps. In SSF, samples were collected only after a short period of incubation (6 h for ethanol and 9 h for LA) due to the high initial thickness of the suspension. In SSF (Figures 1C and 2C), after the initial cultivation period, no further glucose accumulation was detected, indicating that microbial cells were metabolically active during the entire course of the fermentation, keeping glucose concentrations near zero for process times longer than 48 and 72h for ethanol and LA production, respectively. This also means that enzymatic hydrolysis of cellulose was the rate-limiting step for most of the duration of the SSF process, as already observed by other authors [9,13-15].

When comparing SSF and SHF profiles (Figures 1C and 2C versus 1B and 1C, respectively), it is observed a delay on the beginning of product formation on SSF experiments relatively to SHF, due to the need for a previous accumulation of glucose from RPS hydrolysis on the SSF medium. After this initial delay, production occurs with maximum volumetric production rates ($Q_{P\,max}$ on Tables 2 and 3) comparable to those obtained on SHF processes.

In order to allow the comparison between SHF and SSF, results were expressed as a percentage of an overall theoretical yield calculated by assuming that all the potential glucose and xylose in the sludge starting material is available for fermentation with a yield of 0.51 g ethanol and 1.0 g lactic acid g^{-1} glucose (or xylose). On this basis: the highest ethanol yield was equivalent to approximately 54% and 51% conversion; and the highest lactic acid yield was equivalent to approximately 81% and 97% conversion of the available carbohydrates in the initial sludge, respectively for SHF and SSF. Thereby it was demonstrated that the SSF process is more efficient than the SHF for RPS conversion into lactic acid and it allows process integration since both steps are performed in the same vessel. Moreover, the fermentation step on SHF was preceded by a prolonged period of 5 days of enzymatic hydrolysis that must be taken into account for the total residence time, with the correspondent decrease of the real productivity. Conversely, in ethanol production, the extent of carbohydrate conversion into monosaccharides in the SSF process is slightly lower than the one obtained in the enzymatic hydrolysis prior to fermentation in the SHF process. This may appear to be in disagreement with the reduction in end-product inhibition of β-glucosidase that occurs in the course of the SSF process as a result of the rapid fermentation of the released glucose [16]. This observation might be explained by the difference in process temperatures. The SSF process was carried out at the temperature selected for the fermentation step, 30ºC, whereas the hydrolysis of RPS in the SHF was performed at 35ºC, which corresponds to higher enzyme activities.

Conclusions

Bench-scale batch experiments were successfully conducted for conversion of RPS to ethanol and lactic acid by simultaneous saccharification and fermentation, justifying work prosecution in order to improve the SSF process to make it cost effective. Future research might include the development of a membrane-recycle bioreactor enabling simultaneous product recovery and recycling of enzymes and microbial cells, increasing the capacity and yield by operation at higher cell densities and avoiding product inhibition. The bioconversion of RPS might also be conducted under fed-batch SSF conditions, with periodic addition of fresh substrate, so as to overcome the limit to high substrate concentrations imposed by mixing constraints and providing higher SSF yields at lower enzyme loading, together with higher product concentrations.

It is important to emphasize that the main advantage of the proposed processes lies in the use of a waste material as substrate. This biotechnological procedure has thus a double profit

because an environmental pollution problem is relieved along with the production of a commercial product via fermentation using an economic nutrient as carbon source. In conclusion, recycled paper sludge represents a potential resource to be used by other industries to obtain useful value-added products under a biorefinery concept.

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