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Production of Ethanol and Feed by High Dry Matter Hydrolysis and Fermentation of Palm Kernel Press Cake

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Abstract Palm kernel press cake (PKC) is a residue from palm oil extraction presently only used as a low protein feed supplement. PKC contains 50% fermentable hexose sugars present in the form of glucan and mainly galactomannan. This makes PKC an interesting feedstock for processing into bioethanol or in other biorefinery processes. Using a combination of mannanase, β-mannosidase, and cellulases, it was possible without any pretreatment to hydrolyze PKC at solid concentrations of 35% dry matter with mannose yields up to 88% of theoretical. Fermentation was tested using *Saccharomyces cerevisiae* in both a separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) setup. The hydrolysates could readily be fermented without addition of nutrients and with average fermentation yields of 0.43±0.02 g/g based on consumed mannose and glucose. Employing SSF, final ethanol concentrations of 70 g/kg was achieved in 216 h, corresponding to an ethanol yield of 70% of theoretical or 200 g ethanol/kg PKC. Testing various enzyme mixtures revealed that including cellulases in combination with mannanases significantly improved ethanol yields. Processing PKC to ethanol resulted in a solid residue enriched in protein from 17% to 28%, a 70% increase, thereby potentially making a high-protein containing feed supplement.

Keywords Bioethanol · Cellulase · Galactomannan · Mannanase · *Saccharomyces cerevisiae*

Introduction

World annual production of palm oil for the period March 2008 to 2009 exceeds 43 million metric tons [1], and palm oil is one of the largest sources of cooking oil in the world [2].

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Recent demands for biofuels and the possibility of using plant oils to produce biodiesel have further increased the global demand for plant oils. This has caused a substantial increase in the palm oil production especially in Indonesia with a doubling of the production from 2000 to 2008, and with an expected further increase in coming years [1].

Due to the large amounts of palm fruit being processed annually, it is important to optimize the use of present-day production of palm oil and the by-products or waste streams generated [3]. It has been suggested that residues such as empty fruit bunches and palm press fiber can be used as solid fuel for incineration or converted into various liquid biofuels [4]. Another residue is palm kernel press cake (PKC) coming from the pressing and extraction of oil from palm kernels. Based on current production of palm oil, it can be estimated that around 4.5 million tons of PKC are available, mainly in Malaysia and Indonesia. PKC contains around 15% protein and is, at present, only used as a feed supplement [3, 5]. However, the large fiber content and low concentrations of relatively low nutritional value protein limit the use and efficiency of PKC as feed, especially in broiler feeds [5, 6]. Berepubo et al. [6] suggest that a maximum of 15% PKC can be incorporated into feed for broilers.

One way to maximize the use and value of PKC is to convert the carbohydrates in PKC into various products in a biorefinery process [3]. Unlike press cakes from other oil seeds, PKC has a high content of carbohydrates, around 50% [7]. Sugars in PKC are mainly hexoses such as glucose, galactose, and mannose [8–10], which can potentially be fermented by traditional yeast *Saccharomyces cerevisiae* into, e.g., ethanol with a theoretical yield of 0.51 g ethanol/g hexose sugar. Utilizing carbohydrates in PKC for ethanol production would result in a final product enriched in protein and with reduced fiber content, which could be a more attractive feed supplement compared with traditional PKC. The demand for bioethanol as a biofuel has also been growing in recent years. In addition, an integrated biodiesel process combining ethanol production with biodiesel production by substituting methanol for transesterification by ethanol has also been suggested [4, 7]. These factors all make a feedstock such as PKC interesting in a biorefinery concept.

The main carbohydrate in PKC is mannan or galactomannan. Galactomannan consists of a linear (1→4) linked β-D-mannopyranose backbone with (1→6) linked α-D-galactopyranose side groups. In PKC, the galactose substitution is 12–20% [8–10]. The linearity in mannan results in a predominantly crystalline structure, much similar to cellulose [8, 11, 12]. Although crystalline in structure, it has been demonstrated that galactomannan is fairly easy to digest by endomannanases and therefore more accessible to enzymes compared with cellulose [13]. Recent work in our laboratory also indicates that a high-temperature pretreatment similar to what is used for lignocellulosic materials is not a prerequisite for enzymatic hydrolysis of PKC. The ability to enzymatically hydrolyze PKC without a high-temperature pretreatment step is an advantage since it significantly reduces cost and makes the process more economically feasible. Another advantage is that the protein in PKC, which makes the residues valuable as a feed product, is preserved. In addition, the absence of a pretreatment process avoids the potential formation of degradation products such as acetic acid, furans, and phenolic compounds [13–15]. These inhibitors are known to affect the fermentation efficiency of ethanol-producing microorganisms such as *S. cerevisiae* [16–18].

An important economic consideration is also the ability to operate the process at high solid concentrations, which results in higher final ethanol content in the fermentation broth. At ethanol concentrations above 4% (*w*/*w*), energy requirement for ethanol recovery is

reduced significantly [19, 20]. Operating at high solid concentrations has previously been a technical challenge during enzymatic hydrolysis due to mixing difficulties [21]. The use of new reactor designs, such as gravimetric mixing based on a free fall system in a horizontal-placed cylindrical reactor with paddles, has resulted in significant advantages over previous systems and enabled processing of lignocellulosic materials up to 40% dry matter (DM) [22, 23].

The purpose of this work was to demonstrate the ability to produce ethanol and a high-protein residue under conditions that would be industrially relevant, i.e., at high solid concentrations and no high-temperature pretreatment. Enzymatic hydrolysis and fermentation was studied using various process designs such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Various enzymes such as cellulases, β-glucosidase, α-galactosidase, and protease were used in combination with the two main enzyme activities endomannanase and β-mannosidase to investigate whether these auxiliary enzyme activities would help increase conversion of the carbohydrates and thereby the yield of ethanol.

Materials and Methods

Raw Materials and Initial Processing

Industrial standard PKC was supplied from P.T. Musim Mas, Indonesia. The DM content was around 96%. PKC was sieved through a 1-mm aperture mesh and the remainder ground using a Braun coffee grinder for 10 s and again sieved. This was repeated twice. Material that did not pass through the mesh after the third grinding was discarded, and this generally amounted to about 8% by weight of the PKC as received. The composition of the sieved material was on a dry matter basis: glucose 8.0±0.5%, xylose 1.8±0.2%, arabinose 1.4±0.1%, galactose 2.3±0.1%, mannose 41.7±1.9%, klason lignin 10.2±1.3%, protein 16.8±0.3%, and ash 4.4±0.1% (sugars reported as anhydrous form). In addition, PKC contains 5–10% residual oil (not determined), which could be observed floating on top of the slurry after the hydrolysis stage.

Enzymes

Depending on the type of experiment, various enzyme preparations were used and the dosage was varied accordingly. The applied enzyme preparations were monocomponent endomannanase [24], monocomponent β-mannosidase [24], cellulase preparation [24], β-glucosidase (Novozym 188), protease (Alcalase 2.4 L), and α-galactosidase. All enzymes were kindly provided by Novozymes, Bagsværd, Denmark. Filter paper activity was determined at pH 4.8 according to [25]. Mannanase activity was measured at pH 5.0 according to [26] using locust bean gum (Sigma G-0753) as substrate. The activity of β-glucosidase, β-mannosidase, and α-galactosidase was measured at pH 5.0 using 5 mM *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, and *p*-nitrophenyl-α-D-galactopyranoside, respectively, in 50 mM sodium citrate buffer as substrate. The assay was performed in microtiter plates by incubating 20 μl of sample with 100 μl of substrate solution at 50 °C for 15 min in a Grant Scientific QBD2 heating block. The reaction was terminated by addition of 120 μl stop solution (0.5 M glycin/NaOH buffer, pH 10.0, 2 mM ethylenediaminetetraacetic acid) and absorbance read at 405 nm. Enzyme activities in the various preparations are given in Table 1.

Table 1 Enzyme activities in enzyme preparations given as average±1 standard deviation (n=3).

Enzyme	Mannanase (nkat/ml)	β-Mannosidase (nkat/ml)	α-Galactosidase (nkat/ml)	Filter paper activity (FPU/ml)	β-Glucosidase (nkat/ml)
Endomannanase	62,500±3,800	0	0	0	0
β-Mannosidase	1,270±80	2,830±120	130±0	0	4±1
Cellulase	5,400±200	6±0	20±1	162	1,022±29
β-Glucosidase	380±30	85±5	550±40	0	6,040±550
Protease[a]	nd	nd	nd	nd	nd
α-Galactosidase	270±10	0	14,300±500	nd	30±2
30:10:1 mix[b]	46,200	690	32	4	26

nd Not determined

[a] Protease activity not measured. The activity as given by the supplier was 2.4 AU/ml (AU - Anson unit)

[b] Mixture of endomannanase, β-mannosidase, and cellulase in 30:10:1 volumetric ratio as used in experiments. Activity calculated based on individual enzyme preparations

For most experiments, endomannanase, β-mannosidase, and cellulase were mixed in the volume ratio 30:10:1, respectively, based on previous experiments at low consistency (5% DM). The enzyme activities in the mixture are given in Table 1. Three different enzyme loadings were tested—"low", "medium", and "high"—and the total amount of added enzyme were 61.5, 123, and 184.5 ml/kg PKC, respectively. To test the effect of auxiliary enzymes, enzymes were used according to Table 2.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed in 100-ml polyethylene bottles. PKC was added to give between 15 and 25 g of dry PKC per bottle (resulting in 30% to 50% final DM) along with 1 M sodium citrate buffer, pH 5, to give a concentration of 50 mM sodium citrate in the final mixture. MilliQ water was then added to the bottles to give a total reaction mass of 50 g after addition of enzymes. The bottles were tightly shut and then pasteurized at 80 °C for 1 h. After cooling to room temperature, the required amount of enzyme was added

Table 2 Dosage of enzymes and enzyme activities (calculated from the activities in the individual enzyme preparations) in experiment with auxiliary enzymes.

Denotation	Dosage of enzyme (ml/kg PKC)	Mannanase (nkat/g PKC)	β-Mannosidase (nkat/g PKC)	α-Galactosidase (nkat/g PKC)	Filter paper activity (FPU/g PKC)	β-Glucosidase (nkat/g PKC)
S	45 endomannanase + 5 β-mannosidase	2,819	14.1	0.7	0.0	0.02
S + C	S+ 1.5 cellulase	2,827	14.1	0.7	0.24	1.55
S + C + B	S+ 1.5 cellulase + 5 Novozym 188	2,829	14.6	3.4	0.24	31.8
S + P	S+ 10 Alcalase 2.4 L[a]	2,819	14.1	0.7	0.0	0.02
S + G	S+ 5 α-galactosidase	2,821	14.1	72	0.0	0.17
M	45 endomannanase + 15 β-mannosidase + 1.5 cellulase	2,827	42.4	0.7	0.24	1.55

[a] Protease activity not measured. The activity as given by the supplier was 2.4 AU/ml (AU – Anson unit)

thereby giving the final reaction mass of 50 g per bottle. To prevent the cap loosening and causing leakage during long reaction times, electrical tape was wrapped several times around the lid and the top of the bottle. The bottles were put in a heated (50 °C) rotating drum which rotated at 30 rpm. The 80-cm-diameter drum was equipped with wooden paddles that lifted and dropped the plastic bottles during rotation, mimicking the gravimetric mixing as reported by Larsen et al. [23] and Kristensen et al. [27]. Most experiments were performed in duplicate, although some were done in triplicate. Times were varied according to the experimental design.

Hydrolysis was terminated by boiling the bottles for 10 min. Some bottles were used to measure the amount of solubilized material. The total content was transferred to 50-ml falcon tubes and centrifuged at 4,200×*g* for 10 min. The liquid was decanted and kept for sugar analysis on high-performance liquid chromatography (HPLC). The solids were washed three times with MilliQ water. The pellet was dried at 105 °C for 48 h. The dried solids were weighed and the amount of solubilized material calculated from the original content. The density of the liquid was measured using the volume and weight of the liquid. Together with the determined amount of solid, this can be used to calculate the corrected sugar concentration in experiments with high solid concentration [27]. This correction is only needed when the sugars are measured from the decanted liquid. From other bottles, the content of sugars released was measured by taking a representative whole slurry sample and diluting with eluent to 10 g and then measured by HPLC [27]. Due to this procedure, the concentrations are consequently reported in the unit grams per kilogram of slurry and not as grams per liter.

Separate Hydrolysis and Fermentation

Enzymatic hydrolysis was performed as described above. After 96, 144, or 192 h of hydrolysis at 50 °C, the bottles were removed and cooled to room temperature. The whole contents of the bottles were transferred to sterile falcon tubes and centrifuged for 10 min at 4,200×*g*. The liquid was decanted off to new sterile falcon tubes. The weight of the liquid was recorded, and a liquid sample was taken for HPLC analysis. The tubes were incubated with dry baker's yeast (*S. cerevisiae*) from the supermarket (Malteserkors, Lallemand, Denmark) corresponding to 2 g/l. To allow venting of carbon dioxide, a needle was pierced on the cap. The tubes were placed in an Ecotron shaking incubator at 32 °C, shaking at 180 rpm. After 72 h of fermentation, bottles were removed, sealed with new caps, and stored frozen at −20 °C until analysis by HPLC.

Simultaneous Saccharification and Fermentation

Prior to the SSF experiments, the samples were hydrolyzed as described above. This part of the hydrolysis was then called the prehydrolysis stage of the SSF. After prehydrolysis, the bottles were removed from the drum and cooled. Dry baker's yeast (*S. cerevisiae*) from the supermarket (Malteserkors, Lallemand, Denmark) was added corresponding to 7 g dry yeast/kg of initial DM. To allow venting of carbon dioxide, a needle was pierced on the cap. The bottles were then placed in an Ecotron incubator shaker at 32 °C for up to 168 h while shaking at 180 rpm. At regular intervals, bottles were removed, sealed with new caps, and frozen at −20 °C until analysis by HPLC. One set of bottles was used for each time point during the experiment. For HPLC, a representative whole slurry sample was diluted with eluent to 10 g and then analyzed by HPLC [27].

Analysis of PKC Before and After Processing

The PKC dry matter content was determined using a Sartorius MA 30 moisture analyzer at 105 °C. The carbohydrate and klason lignin content of PKC was measured using the strong acid hydrolysis procedure developed at NREL by Sluiter et al. [28], and sugars were determined by HPLC. Since PKC was obtained dry, no further drying was done before the analysis. Total ash content was determined by incineration at 550 °C for 3 h.

Protein content was measured by the Kjeldahl method. Destruction was performed by taking 2 g of material, mixing with 30 ml of 96% sulfuric acid, 3.5 g K_2SO_4, and 0.4 g $CuSO_4 \cdot 5H_2O$ and incubating at 360 °C until all material had dissolved. Distillation was performed on a Kjeltec 1002 distillation unit, and titration with 0.015 N HCl was performed using a Metrohm 716 DMS Titrino titrator. A conversion factor of 6.25 between nitrogen and protein was used. PKC was measured as supplied without additional drying. Before analysis of residues from hydrolysis and fermentation, the sample was centrifuged at 4,200×*g* for 10 min, and the liquid was kept for protein analysis. Solids were washed once with water and dried at 50 °C for 24 h.

HPLC Analysis

Separation and quantification of arabinose, galactose, glucose, mannose, and xylose were done using a Dionex BioLC system with a CarboPac PA1 column (4×250 mm) and pulsed amperometric detection. The separation was performed at 25 °C using gradient elution with KOH at 1.05 ml/min and the following profiles: 0–30 min 2 mM KOH, 30–35 min linear increase from 2 to 25 mM, 35–40 min 25 mM, 40–50 min 2 mM with 30 min at 2 mM KOH. Samples were appropriately diluted in MQ-water and filtered through a 0.2-μm filter prior to analysis.

The amount of glucose, mannose, lactic acid, acetic acid, and ethanol was measured using a Dionex Summit HPLC system with a Phenomenex Rezex RHM column and a Shimadzu refractive index detector. Separation was performed at 80 °C with a flow rate of 0.6 ml/min of 5 mM H_2SO_4. This column does not separate mannose, galactose, and xylose. All sugars assigned as the mannose peak was assumed to be mannose although small amounts of xylose and galactose might also be present. Samples were appropriately diluted in MQ-water and filtered through a 0.2-μm filter prior to analysis.

Results and Discussion

Hydrolysis of PKC at High Dry Matter

PKC was hydrolyzed using a mixture of endomannanase, β-mannosidase, and a cellulase preparation with a volume ratio of 30:10:1. This ratio was based on preliminary experiments performed at low solid concentrations (5% DM). Three different enzyme loadings (low, medium, and high, corresponding to 61.5, 123, and 184.5 ml of enzyme mixture per kilogram of PKC, respectively) were tested. At the low enzyme loading, the enzyme activities corresponded to 3 FPU/g cellulose, 6,809 nkat mannanase/g mannan, and 102 nkat β-mannosidase/g mannan (Table 1).

Hydrolysis was followed for 192 h, and using the highest enzyme loading, it was possible to reach 88% conversion of mannan into mannose and 69% of the glucan/cellulose into glucose in 192 h (Fig. 1). This corresponded to a final concentration of 142 g/kg of

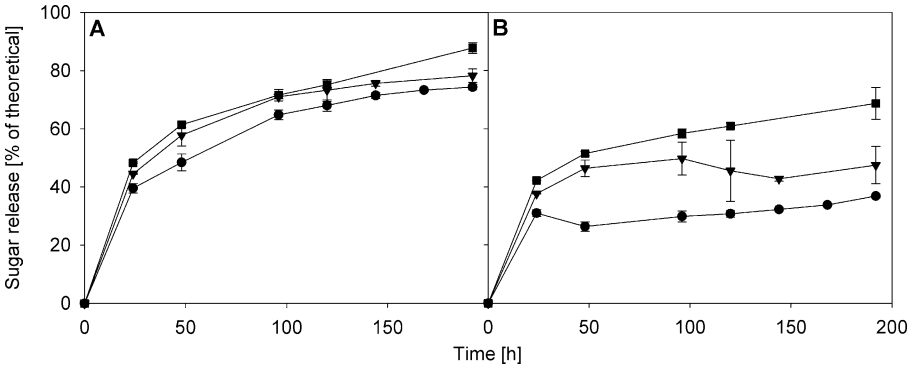

Fig. 1 Release of mannose (**a**) and glucose (**b**) during enzymatic hydrolysis of PKC at 35% DM at three different enzyme loadings 61.5 (*circle*), 123 (*inverted triangle*), and 184.5 ml/kg (*square*) of PKC using a 30:10:1 mixture of endomannanase/β-mannosidase/cellulase. Release calculated relative to the maximum theoretical based on content of mannose and glucose in PKC. *Error bars* indicate ±1 standard deviation (n=2)

mannose and 21 g/kg of glucose (concentrations are reported as grams per kilogram of slurry and not liter due to the high content of solids [27]). In total, the amount of fermentable hexose sugars corresponded to 467 g/kg PKC. Compared with various other oil seed cakes such as soy, peanut, and canola that have also been tested for ethanol production, it is possible with PKC to obtain reasonably high amounts of fermentable sugar per kilogram of processed material and without using a costly pretreatment step [7].

Reducing the enzyme loading to one third (from high to low) reduced the conversion yield of mannose by 15% and glucose of 46%. This equals 376 g of mannose and glucose produced per kilogram of PKC. Interestingly, at low and medium enzyme loading, the hydrolysis rate of cellulose was significantly decreased after 24–48 h (Fig. 1b). These loadings correspond to 3 and 6 FPU/g cellulose, respectively, which are realistic loadings also used for other lignocellulosic materials [23]. However, the more pronounced effect observed on the cellulose hydrolysis might be due to inhibition of the cellulases. After 24 h, the concentration of glucose was 9.5 g/kg using the medium enzyme loading, but the concentration of mannose was 64.1 g/kg. Although not as potent an inhibitor as glucose, it has been reported that mannose at 100 g/l inhibited cellulases by 45% [29], which is approximately the liquid concentration after 48 h of hydrolysis at the low enzyme loading. The β-glucosidase activity relative to filter paper activity is also of relevance due to removal of the potent cellulose inhibitor cellobiose by β-glucosidases. In this study, the ratio between β-glucosidase activity and filter paper activity was 6.4 nkat/FPU. A previous study found that increasing the ratio from 6.6 to 20 nkat/FPU resulted in a significant improvement in cellulose hydrolysis, mainly due to removal of cellobiose that otherwise inhibited the cellulases [30].

Enzymes hydrolyzing mannan are most likely also inhibited at the high mannose concentrations during the last part of the hydrolysis (over 130 g/kg after 96 h). The inhibition constant for mannose has for different β-mannosidases been reported in the range of 0.2–5.5 mM [31, 32], which indicates that inhibition should be expected at the concentrations obtained in this study. Despite product inhibition, close to complete hydrolysis was obtained—although only after very long time of incubation and at a very high enzyme loading, which is not economically feasible.

Higher solid concentrations are theoretically advantageous due to the lower costs for distillation [20], but too high solid concentrations might not be feasible to process. To further evaluate the effect of working at very high solid concentrations on the hydrolysis performance, a series of experiments were conducted in the range from 30% to 50% DM (Fig. 2). By increasing the solid concentration from 30% to 50% DM, the mannose concentration after 48 h increased by 37% (both enzyme loadings), and just over 100 g/kg was obtained at 50% and the medium enzyme loading (123 ml/kg PKC; Fig. 2). However, increasing the initial solid concentration from 30% to 50% resulted in a 17% decrease in the conversion of mannan into mannose after 48 h, irrespective of the enzyme loading used (61.5 or 123 ml/kg PKC). A similar trend was also observed after 96 h of hydrolysis (data not shown). These results are comparable to what has also been found on other substrates such as filter paper and wheat straw when testing the hydrolysis in the range from 5% to 40% solid concentration [22, 27].

Unlike lignocellulosic substrates, which require a pretreatment and therefore water addition, PKC is obtained in a dry form and could potentially be processed at even very high solid concentrations. Due to the gravimetric mixing principle employed, it was possible to operate at solid concentrations up to 50% DM. However, even after 120 h of hydrolysis, the mass was still like a thick paste and would be difficult to handle in any normal equipment in an ethanol plant. At 30% to 40% DM, the mass was a liquid with suspended particles after 48–96 h of hydrolysis. Combined with the decreasing efficiency of the enzymes due to the high product inhibition, it was decided to use 35% DM in all subsequent experiments. At this solid concentration, the final theoretical ethanol concentration is 9.8% (*w*/*w*) based on glucose and mannose, which well above 4–5% (*w*/*w*) usually reported as lower limit for a feasible distillation [20, 33].

Separate Hydrolysis and Fermentation

Ethanol production from PKC in a SHF setup was tested by doing hydrolysis for 96, 144, or 192 h after which the solids were removed. The sugars in the liquid fraction were then

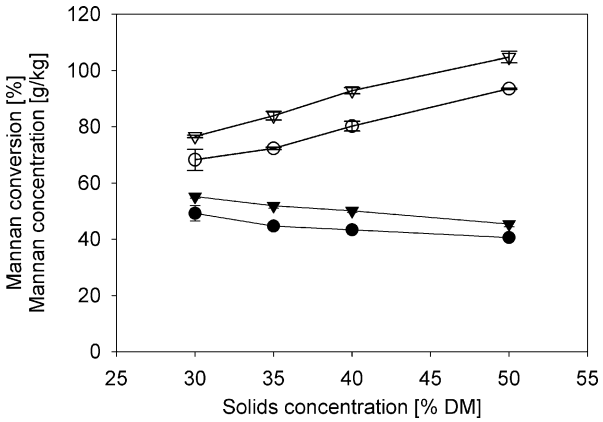

Fig. 2 Mannan conversion (*filled symbols*) and mannose concentration (*open symbols*) after 48 h as function of initial solid loading at two different enzyme loadings 61.5 ml (*filled circle*, *open circle*) and 123 ml/kg (*filled inverted triangle*, *open inverted triangle*) of PKC using a 30:10:1 mixture of endomannanase/β-mannosidase/cellulase. Conversion is calculated relative to the maximum theoretical based on content of mannose in PKC. *Error bars* indicate ±1 standard deviation (n=2)

fermented by yeast *S. cerevisiae* for 72 h. Increasing the length of the hydrolysis phase obviously had a positive effect on final ethanol concentration and thereby also the amount of ethanol that could be produced from PKC (Table 3). The experiment was performed by removing the solids before onset of the fermentation, and residual glucan and mannan in the solids could therefore not be utilized. Using a 192-h hydrolysis stage and low enzyme loading of 61.5 ml/kg PKC resulted in 139 g ethanol being produced per kilogram of PKC with a final ethanol concentration of 83 g/l. Doubling the enzyme loading to 123 ml increased the yield by 7% to 150 g/kg PKC, which is 53% of theoretical maximum. Operating a process with high solid concentrations as SHF seems not feasible as very long hydrolysis time or high enzyme loadings are required due to the severe product inhibition of the enzymes. Even then only modest conversion yields are obtained.

Removal of solids before the fermentation is allowed for the calculation of fermentation performance assuming that only minor amounts of oligosaccharides were present in the liquid and that these would not contribute to the ethanol yield in the fermentation step. No statistical difference was found for the fermentation yield based on consumed sugar (glucose and mannose). The yield was between 0.41 and 0.44 g ethanol/g sugar, or 80–86% of theoretical (Table 3). Although yields are lower compared with results found from controlled fermentations on defined media, it is at the level found in the corn ethanol production in very high gravity fermentations [34], resembles the experiment performed in this study. High initial sugar concentrations, other osmolytes, and high final ethanol concentration can cause osmotic stress of yeast and affect fermentation performance [34]. Often, supplementations with nutrients, especially nitrogen in the form of amino acids, have proven beneficial in alleviating the negative effect of inhibitors and stress on fermentation performance [35, 36]. In this study, no nutrients were added, indicating that PKC likely contains not only sufficient amounts of nutrients but also low amounts of inhibitory compounds. Since PKC could be utilized without pretreatment, many of the degradation products causing problems in fermentation of lignocellulosic hydrolysates were avoided [13, 14].

Simultaneous Saccharification and Fermentation

A SSF setup was tested with enzyme loadings similar to those used in the SHF experiment. Due to constraints when working at high solid concentration in laboratory scale, it was

Table 3 Results after SHF of PKC at 35% DM.

Hydrolysis time[a] (h)	Enzyme loading[b] (ml/kg PKC)	Final ethanol concentration[c] (g/l)	Fermentation efficiency[d] (%)	Ethanol yield (g/kg PKC)
96	61.5	63.4±3.9	80±1	122±6
96	123	75.8±5.5	83±3	145±11
144	61.5	77.1±6.1	86±3	135±11
144	123	84.1±1.3	85±4	147±2
192	61.5	82.6±7.3	83±2	139±13
192	123	92.6±0.6	82±3	150±2

[a] Hydrolysis time before separation of liquid and solids. Fermentation of liquid fraction 72 h

[b] Total enzyme loading using a 30:10:1 mixture of endomannanase/β-mannosidase/cellulase

[c] Concentration given in grams per liter as solids were removed before the fermentation

[d] Fermentation efficiency calculated on basis of consumed glucose and mannose assuming a theoretical ethanol yield of 0.51 g/g consumed sugar

necessary to include a prehydrolysis step. In the prehydrolysis, the material was liquefied using the gravimetric mixing principle that enables efficient mixing of lignocellulosic materials at even very high dry matter. In the laboratory setup used in this study, prehydrolysis is performed in tightly closed bottles, which is incompatible with the CO_2 production during fermentation. After liquefaction, SSF was performed in a normal shaking incubator. If in a larger scale or employing specially designed reactors, yeast inoculation could be done from the start [22].

In this study, the effect of prehydrolysis time was investigated using 24 or 48 h of prehydrolysis. Using low enzyme loading of 61.5 ml/kg PKC resulted in 64 and 78 g/kg of mannose being released during the prehydrolysis for 24 and 48 h, respectively (Fig. 3). No difference was observed in the amount of glucose released after 24 or 48 h. Seventy-two hours after inoculation with yeast, all mannose and glucose were consumed, and the concentration remained low throughout the SSF period. The ethanol concentration continued to increase during the SSF period with an almost constant rate of 0.27 g/kg h (24 h prehydrolysis) and 0.24 g/kg h (48 h prehydrolysis). The final ethanol yields after 168 h of SSF were 164 and 169 g/kg PKC using 24 and 48 h of prehydrolysis, respectively (Table 4). The experiment was also performed at a twice higher enzyme loading (123 ml/kg PKC) with final yields of 190 and 200 g/kg PKC using 24 and 48 h of prehydrolysis, respectively (Table 4).

Unlike the case when using the lower enzyme loading (61.5 ml/kg PKC), at the medium enzyme loading (123 ml/kg PKC), the fermentation rate seemed to decline in the last part of the SSF period, especially with 48 h of prehydrolysis. This indicates that final yields around 200 g/kg PKC are likely to be expected. This corresponds to 70% of maximum theoretical yield based on glucose and mannose and assuming a fermentation yield of 0.51 g/g. However, the fermentation yield in this SSF experiment is likely similar to the yields obtained in the SHF experiment. Base on the average fermentation yield in the SHF experiment (0.43±0.02), a final ethanol yield of 200 g/kg PKC corresponds to a glucan and mannan conversion of 84%.

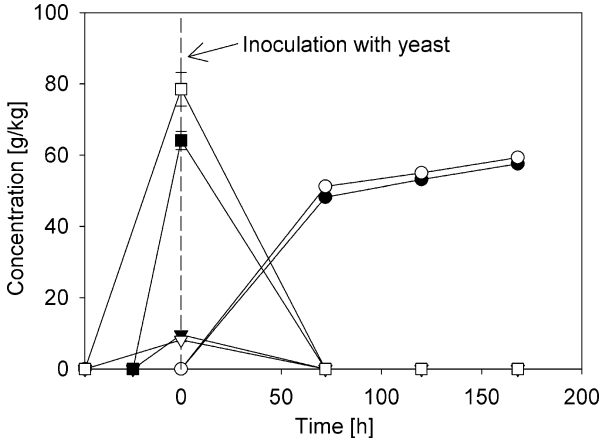

Fig. 3 SSF of PKC at 35% DM using 24 h (*filled symbols*) or 48 h (*open symbols*) of prehydrolysis showing mannose (*open square*, *filled square*), glucose (*open inverted triangle*, *filled inverted triangle*), and ethanol concentration (*open circle*, *filled circle*). The *dotted line* indicates the time point for addition of yeast. The experiment was performed at an enzyme loading of 61.5 ml/kg of PKC using a 30:10:1 mixture of endomannanase/β-mannosidase/cellulase. *Error bars* indicate ±1 standard deviation (n=2)

Table 4 Results after SSF of PKC at 35% DM.

Prehydrolysis time[a] (h)	Total time[a] (h)	Enzyme loading[b] (ml/kg PKC)	Final ethanol concentration (g/kg)	Ethanol yield (g/kg PKC)
24	192	61.5	57.6±0.5	164±2
48	216	61.5	59.3±1.1	169±3
24	192	123	65.5±1.2	190±3
48	216	123	70.1±0.6	200±2

[a] Prehydrolysis was performed at 50 °C, and total time includes time for prehydrolysis and SSF

[b] Total enzyme loading using a 30:10:1 mixture of endomannanase/β-mannosidase/cellulase

In the SHF experiment, maximum ethanol yield was 150 g/kg PKC in a total of 264 h. Using SSF, it was possible to reach 200 g/kg PKC within a total of 216 h. This clearly reveals that at high solid concentrations only SSF will be a feasible process configuration due to severe product inhibition of enzymes during the late stage of hydrolysis. Furthermore, only a minor difference was observed in ethanol yield between 24 and 48 h of prehydrolysis. Using the rather constant ethanol production rate of 0.24 to 0.27 g/kg h to extrapolate the ethanol yield after a total of 216 h with 24 h prehydrolysis gives 170 g/kg PKC. This is the same yield as obtained with 48 h prehydrolysis. Even at 32 °C, reasonable hydrolysis rates can thus be obtained. Employing SSF therefore reduces overall time compared with SHF and reduces energy savings due to lower process temperature, especially if prehydrolysis time is reduced.

The SSF experiment also revealed that PKC seems readily fermentable by traditional yeast (*S. cerevisiae*). No accumulation of glucose or mannose was observed, even during long incubations at high solid concentrations, indicating good viability of the yeast. In fermentation of pretreated lignocellulosic materials, loss of viability has been observed during prolonged fermentations likely due to the stress imposed by inhibitors and accumulating ethanol [22].

Effect of Auxiliary Enzyme Activities

The previous experiments were performed with an enzyme composition determined on the basis of experiments at low dry matter (5% DM). Operating at high dry matter (35%) might alter the specifications for the enzyme mixture. As galactomannan is the main carbohydrate in PKC, mannanases are the key enzyme activities needed for saccharification. As a basis, a mixture of only endomannanase and β-mannosidase was therefore used in the experiment (termed "standard"). In addition, it was tested if a lower amount of β-mannosidase could be used in order to reduce the cost of the enzyme mixture. The enzyme mixture used in the previous experiments had a ratio between β-mannosidase activity and endomannanase activity of 66 based on the 1:3 volume ratio (Table 1). The standard mixture therefore had a volume ratio of 1:9 (activity ratio 200) between β-mannosidase and endomannosidase. The possibility to improve hydrolysis and saccharification by addition of other enzyme activities than endomannanase and β-mannosidase was tested in an SSF experiment with 24 h of prehydrolysis. Additional enzymes were added according to Table 2, which also shows the enzyme activities in the various mixtures.

Release of glucose in the 24-h prehydrolysis period was clearly increased by addition of the cellulase preparation to the standard mixture (S), thereby ending at the same level as in the medium loading (M) used in previous experiments with similar cellulase loading

(Fig. 4). As already mentioned, the β-glucosidase activity was maybe not sufficient in the cellulose mixture. Increasing the ratio between β-glucosidase activity and filter paper activity from 6.7 to 131 nkat/FPU improved the cellulose hydrolysis even further. Compared with previous experiments at low dry matter, the effect of β-glucosidase is likely to be more pronounced at high dry matter due to stronger product inhibition at the higher sugar concentrations. A 5-fold increase in glucose yield compared with only endomannanase and β-mannosidase could therefore be obtained. Final ethanol yields were also improved by cellulase addition due to the extra glucose released. The release of mannan was on the contrary not affected by cellulase addition. This proves that the minor mannanase activity measured in the cellulase preparations (Table 2) was not enough to improve hydrolysis of galactomannan and that glucans or celluloses were not obstructing the access of mannanases to the substrate.

Mannose from PKC is present as galactomannan with (1→6) linked α-galactose side groups. Efficient and complete hydrolysis of galactomannan is thus depending on these side groups being removed as they will otherwise hinder access of the mannanases and β-mannosidases to the mannan backbone. However, addition of an α-galactosidase in this study did not result in improved mannose yields after 24 h prehydrolysis or final ethanol yield (Fig. 4). Either the selected α-galactosidase did not work on a substrate such as PKC or the minor α-galactosidase activity already present in the β-mannosidase preparation (Table 1) was enough to remove α-galactose side groups under these conditions.

The standard mixture had a 3-fold lower loading of β-mannosidase activity compared with the medium mixture used in the other experiments in this study. The standard mixture with addition of cellulase (S + C) is comparable to the medium mixture (M) used in the previous experiments except for a lower β-mannosidase activity (Table 2). Comparing these two in Fig. 4 reveals that the higher amount of β-mannosidase statistically results in higher mannose yield after 24 h and also final ethanol yield. Like it was found for the β-glucosidase activity, β-mannosidase activity also seems to be a critical activity when operating at high solid concentrations. There is therefore a limit to which extent the β-mannosidase activity can be reduced without affecting yields.

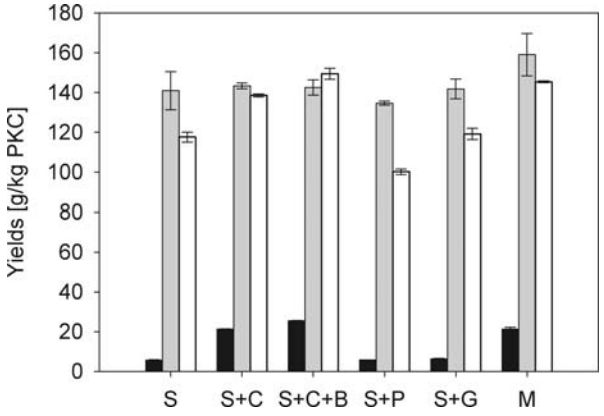

Fig. 4 Effect of auxiliary enzyme activities on glucose (*black*) and mannose (*gray*) yields after 24 h prehydrolysis and final ethanol yields (*white*) after 168 h of SSF. The enzymes were endomannanase and β-mannosidase (*S*), S + cellulase (*S*+*C*), S + C + β-glucosidase (*S*+*C*+*B*), S + protease (*S*+*P*), S + α-galactosidase (*S*+*G*), endomannanase, β-mannosidase, and cellulase (*M*). The amounts used are listed in Table 2. *Error bars* indicate ±1 standard deviation (n=3)

PKC is rich in protein, which could limit the access of enzymes to the carbohydrates depending on the actual localization. Addition of a protease was therefore tested, but no positive effect was observed (Fig. 4). The lowest yields were actually obtained with this mixture. In addition, the degradation of protein will result in increased solubilization of protein, which is less beneficial if the solids are to be used as a protein-rich feed product.

There is obviously a potential to further improve the hydrolysis and minimizing enzyme costs by optimizing the ratio between specific enzyme activities. However, the test revealed that endomannanase, β-mannosidase, and cellulase are the most important activities.

Protein Recovery for Feed Purpose

An important part of the process is also the recovery of protein in the solid residues after hydrolysis and fermentation as PKC is presently sold as animal feed. The protein content of the starting material was 17% protein. After 192 h of SSF, the protein content in the washed residue was 28.5±0.3%. This is a 70% increase in the protein content. The liquid fraction after fermentation contained 9 g/l of protein showing that some protein is solubilized during the process. Concentrating the liquid and mixing it with the solid residue to make a product similar to distiller's dry grain with solubles (DDGS) could increase the protein content in the final product to around 34%. This would make it comparable to the protein content in DDGS [37], whereas the normal PKC is only a low-protein feed additive.

The fact that 94% of the sugars in PKC are hexoses (glucose, galactose, and mannose) that can be fermented to ethanol by traditional yeast (*S. cerevisiae*) is advantageous. *S. cerevisiae* is classified as a generally regarded safe organism, and feed products such as DDGS are already on the market. Processing of PKC to make ethanol and a high-protein feed should therefore not impose any problems with regard to legislation and usage.

An important factor is also the presence of lignin, which is up-concentrated in the process as well. It was found that the initial processing step removing some of the larger nutshell particles lowered the lignin content from around 15% to 10% (data not shown). Previously, it has been demonstrated that lignin is likely present in nutshell particles that are not efficiently separated from the kernel during the palm kernel processing [38]. Nutshell separation could most likely be improved if more attention was on the value of the final feed product. Besides improving feed quality, lowering the lignin content could also have other processing benefits. It is well known that enzymes are nonproductively adsorbed onto lignin [21]. Lignin removal by simply improving the fractionation of shell and kernel could thus lower enzyme requirements.

Conclusion

Using PKC as a feedstock for bioethanol production seems promising as PKC has a high content of hexose sugars (50% of total weight). It was shown that, even without any pretreatment step, which is a costly step necessary for lignocellulosic materials, it was possible with the medium enzyme loading to obtain 84% conversion of mannan and glucan in 216 h using an SSF process and with a final ethanol yield of 200 g ethanol/kg of PKC. The process could be operated at very high solid concentrations, up to 50% DM if required, using the appropriate reactor designs. However, using 35% DM resulted in final ethanol concentrations up to 70 g/kg (or 80 g/l excluding the residual solids), which is well above the 4–5% (*w*/*w*) level where distillation becomes energetic and economical viable. Under these conditions and without addition of nutrients, the sugars were readily metabolized by

traditional yeast *S. cerevisiae* with average fermentations yields of 0.43±0.02 g/g. The ability to utilize PKC for ethanol production using only modest process conditions (low temperatures) and *S. cerevisiae* as fermenting organism is advantageous since it preserves the protein in the solid residues, which could be used as a high-value feed product.

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