Title: METHOD OF IMPROVING PALM OIL YIELDS FROM PALM FRUIT OR PALM FRUIT LIQUID

Abstract: A method of improving palm oil yields is provided, which method comprises: admixing an enzyme composition comprising cellulase activity with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed comprises at most 40% water content w/w; incubating the admixture for between 0.5 to 8 hours (preferably 4-8 hours) at a temperature of between 50 °C to 95 °C; and separating the oil from other components of the admixture. Claims are also directed to a palm oil phase, a sludge phase or an aqueous phase obtainable by the method. Claims are also directed to the use of the aqueous phase as a fermentation medium, or to a method for producing a fermentation product comprising fermenting a feedstock comprising said aqueous phase and recovering said fermentation product, for instance biofuel.
TITLE

METHOD OF IMPROVING PALM OIL YIELDS FROM PALM FRUIT OR PALM FRUIT LIQUID

CROSS-REFERENCE TO RELATED APPLICATIONS


FIELD OF THE INVENTION

The present invention relates to a method for the treatment of palm fruit or a portion thereof or palm fruit extract (or pressed palm fruit extract also known in the industry as "pressed liquid" or "pressed oil") with at least a cellulase. The method results in a decrease in oil in sediment, a decreased viscosity of the oil obtained from the palm fruit or portion thereof or palm fruit extract (e.g. pressed palm fruit extract), improved palm oil yields, and/or an increase in fermentable sugars in the aqueous phase.

BACKGROUND

Palm oil obtained from oil palm (*Elaeis guineensis*) is commercially important edible oil. Palm oil has been a prominent fat and oil resource for the food industry due to several advantageous properties, such as high productivity, low price, high thermal and oxidative stability and plasticity at room temperature. Additionally, compared with other vegetable oils, palm oil is a rich source of the anti-oxidant vitamin E.

In 2012, the world production of palm oil was 50 million ton/year, which in quantity makes it the most important vegetable oil produced. It is estimated that 4-8% of palm oil is lost during processing. This loss can be split up as follows: 0.8-1% is lost in the palm mesocarp fibre, 1.5-2.7% is lost from the empty fruit bunch (EFB); and more than about 1% is lost in oil mill effluent (Ho et al., JAOCS, Vol. 69, No. 3 (1992)). The percentage calculation is based on fresh fruit bunches.

The processing of palm oil is complicated and extensive. Figure 1 shows flow diagram for palm oil processing. Production of crude palm oil is conducted by a series of unit operations starting with a sterilization of the fresh fruit bunch (FFB). After sterilization, the fruits are stripped from the bunch and digested. During the digestion, the palm fruit is
disintegrated and oil released from the mesocarp. After digestion, the crude oil is separated from fiber by pressing the digested fruits.

The pressed palm oil contains crude palm oil, water and solid debris. Crude palm oil is separated under the influence of gravity from the pressed liquid by clarification in a clarification tank. Crude palm oil that is discharged from the presses is highly viscous. Thus, separation of the oil from the solid and water is difficult without the addition of dilution water. Hot water is therefore added to the pressed liquid to dilute it prior to or during clarification. This typically occurs using a temperature range of 80-90 °C. The dilution provides a barrier causing the heavy solid to settle to the bottom of the clarification tank while the lighter oil droplets rise through the sludge phase to the top when heat is applied. In practice it has been found that dilution with water such that 38% to 40% of the mixture is crude oil is best for good separation in the clarification tank. Any remaining available oil post clarification is removed by centrifugation. The centrifuge sludge is a viscous liquid containing water, about 0.5-1.5% oil, and about 5-10% non-oil solids. For each ton of oil produced, 1-1.5 ton of centrifuge sludge is produced. The centrifuge sludge thus presents a substantial loss of oil. During these initial processing steps, 90-92% of the theoretical palm oil amount is obtained, but there is a significant oil loss to the fiber (e.g. press cake) during processing as well as oil loss during downstream processing.

It is known to use enzymes in the processing of vegetable oils. Enzymes such as phospholipases or lipid acyltransferases have been used to increase the oil yield in enzymatic and/or water degumming of oil with a high content of phospholipids (see US Patent No. 6,001,640 and International Patent Application Publication Nos. WO2006/008508 and WO2009/081094). In these reactions the enzyme is added to water-degummed edible oils, crude edible oils or semi-crude edible oils comprising relatively high amounts of a non-hydratable phosphorus ranging from about 50 ppm to about 3000 ppm. In the flow diagram shown herein in Figure 1 the type of oil which would have been produced in such processes is designated "crude palm oil". In any event degumming of palm oil is often not essential as the level of non-hydratable phosphorus can be naturally low in this product, especially in comparison to other vegetable oils. For example the phosphorus content of palm oil is about 15-30 ppm which is very low when compared to e.g. corn oil (250-800 ppm), cottonseed (400-1000 ppm), rapeseed (200-1400 ppm), soya (400-1200 ppm) or sunflower (200-500 ppm). Traditionally, palm oil is produced by pressing the oil out of the palm mesocarp without use of organic solvents.
SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method of improving palm oil yields and/or decreasing oil in sediment or sludge and/or increasing fermentable sugars in the aqueous phase, which method comprises:

i. admixing an enzyme composition comprising cellulase activity with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed comprises at most 40% water content w/w;

ii. incubating the admixture for between 0.5 to 8 hours at a temperature of between 50 to 95 °C; and

iii. separating the oil from other components of the admixture.

In a further aspect the present invention provides use of an enzyme composition comprising cellulase activity in combination with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed with the enzyme composition comprises at most 40% water content w/w in the manufacture of palm oil for improving palm oil yield, for improving separation of palm oil from the sludge phase, for producing an aqueous phase with improved fermentable sugars, or for improving the speed of oil separation.

In a yet further aspect the present invention provides a palm oil phase, a sludge phase or an aqueous phase obtainable (preferably obtained) by the method according to the present invention.

In another aspect the present invention provides the use of an aqueous phase obtained (preferably obtained) by the method of the present invention as a fermentation medium.

The present invention yet further provides a method for producing a fermentation product comprising fermenting a feedstock comprising said aqueous phase (or a portion thereof) obtainable (preferably obtained) by the method according to the present invention and recovering said fermentation product.

BRIEF DESCRIPTION OF THE DRAWINGS

Various embodiments will now be described, by way of example only, with reference to accompanying drawings, in which:

Figure 1 shows a flow diagram for Palm Oil processing.

Figure 2 shows pressed palm liquid after enzyme treatment and centrifugation.
Figure 3 shows the effect of LAMINEX® BG2, -Super 3G, -750 and -C2K on residual oil in sediment after centrifugation.

Figure 4 shows the effect of LAMINEX® BG2, -Super 3G, -750 and -C2K on amount of dry sediment after centrifugation.

Figure 5 shows the effect of LAMINEX® BG2, -Super 3G, -750 and -C2K on water in sediment after centrifugation.

Figure 6 shows the effect of LAMINEX® BG2, -Super 3G, -750 and -C2K on content of glucose in water phase after centrifugation.

Figure 7 shows the effect of water dilution at different temperatures on viscosity of PALM_2.

Figure 8 shows the effect of water dilution at different temperature on viscosity of PALM_2 treated with 0,5% LAMINEX® BG2.

Figure 9 shows oil separation of samples from Table 7 after 10 minutes.

Figure 10 shows the effect of water dilution at different temperature on viscosity of PALM_1.

Figure 11 shows the effect of water dilution at different temperature on viscosity of PALM_1 treated with 0,5% LAMINEX® BG2.

Figure 12 shows oil separation of samples from Table 9 after 10 minutes.

Figure 13 shows the effect of water dilution at different temperature on viscosity of PALM_8.

Figure 14 shows the effect of water dilution at different temperature on viscosity of PALM_8 treated with 0,5% LAMINEX® BG2.

Figure 15 shows oil separation of samples from Table 11 after 10 minutes.

Figure 16 shows pressed palm liquid samples from Table 13 after reaction and separation for 10 minutes. A white line indicates a boundary between sludge and oil.

Figure 17 shows % Oil separated after 10 minutes settling of samples from Table 13.

Figure 18 shows the effect of enzyme treatment on oil in dry sediment after centrifugation of samples from Table 13.

Figure 19 shows the effect of enzyme treatment on water content in wet sediment after centrifugation of samples from Table 13.

Figure 20 shows the effect of enzyme treatment on amount of dry sediment after centrifugation of samples from Table 13.

Figure 21 shows the effect of LAMINEX® BG2 and water on viscosity of pressed palm liquid from Table 17.

Figure 22 shows the effect of LAMINEX® BG2 and water dilution on oil in sediment from samples in Table 17.
DETAILED DESCRIPTION

A seminal finding of the present invention is that treatment of palm fruit or a portion thereof or palm fruit extract (e.g. pressed palm fruit extract also known in the industry as "pressed liquid" or "pressed oil" or "pressed palm liquid") with celulase to reduce oil in sediment/sludge and/or increase oil yields is enhanced by ensuring that the palm fruit or a portion thereof or palm fruit extract (e.g. pressed palm fruit liquid) comprises at most 40% water content w/w.

Conventionally even when enzymes have been used in the treatment of palm fruits or pressed palm oil liquid a significant amount of water is used in the enzyme reaction or to dilute the palm fruit or palm fruit extract or pressed palm liquid prior to admixing with the enzyme. The present inventors have surprisingly shown that adding water in to the enzyme reaction or prior to mixing the palm fruit or palm fruit extract or pressed palm liquid with the enzyme such that the water content in the reaction mixture is greater than about 40% w/w has a negative impact on the oil separation and particularly has as negative impact on the oil in sediment/sludge. In particular, significantly less oil is recovered from the sediment/sludge phase when dilution above 40% water content in addition to enzymes are used compared with use of enzymes whilst the water content is maintained at less than or equal to about 40% water content w/w.

In addition the method of the present application also advantageously reduce the viscosity of crude palm oil allowing easier and/or faster and/or more efficient separation of crude palm oil, e.g. from sludge.

Based on these findings there is provided a method of improving palm oil yields and/or decreasing oil in sediment or sludge and/or increasing fermentable sugars in the aqueous phase, which method comprises:

i. admixing an enzyme composition comprising celulase activity with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed comprises at most 40% water content w/w;

ii. incubating the admixture for between about 0.5 to about 8 hours at a temperature of between about 50 to about 95 °C; and

iii. separating the oil from other components of the admixture.

In a further aspect the present invention provides use of an enzyme composition comprising celulase activity in combination with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed with the enzyme composition comprises at most 40% water content w/w in the manufacture of palm oil for improving palm oil yield, for improving separation of palm oil from the sludge.
phase, for producing an aqueous phase with improved fermentable sugars, or for improving the speed of oil separation.

In one embodiment the incubation may be between about 1 hour and about 8 hours.
In one embodiment the incubation may be between about 4 hours and about 8 hours.

In one embodiment the temperature may be between 50 to 90 °C, suitably between about 55 to about 65°C.

In some embodiments the admixture may be incubated at about 70 °C to about 95 °C, more preferably at about 80 to about 95 °C.

In one embodiment said palm fruit or portion thereof or palm fruit liquid when admixed with said enzyme composition comprises at most 38% water content w/w, suitably at most 35% water content w/w, suitably at most 30% water content w/w.

In one embodiment palm fruit liquid is a pressed palm fruit liquid.

The oil may be separated from the sediment and/or aqueous phase by any method known in the art. By way of example the oil may be separated by gravity (e.g. by clarification). In one embodiment the oil may be separated by centrifugation instead of gravitation separation or in addition to separation by gravity.

In a preferable embodiment the separation (e.g. centrifugation) results in a triple phase composition comprising an oil layer, an aqueous layer and a substantially solid sediment (or sludge) layer.

The aqueous phase may be enriched in soluble fermentable sugars.

In one embodiment the sludge phase produced by the method of the present invention has less dry solids compared with the sludge phase from a control (non-enzyme treated sample).

The present invention yet further provides the use of an aqueous phase obtained preferably obtained) by the method of the present invention as a fermentation medium.

The present invention yet further provides a method for producing a fermentation product comprising fermenting a feedstock comprising said aqueous phase (or a portion thereof) obtainable (preferably obtained) by the method according to the present invention and recovering said fermentation product.

The feedstock used in accordance with the present invention may in addition to the aqueous phase further comprise empty palm fruit bunches or processed empty palm fruit bunches. The term processed empty palm fruit bunches means empty palm fruit bunches which have undergone one or more processing steps, e.g. milling or chemical treatment, e.g. acid treatment or alkaline treatment.

A further surprising finding is that the resulting aqueous phase after separation from the oil, e.g. by centrifugation, is enriched in fermentable sugars. The inventors have found that
this aqueous phase can be used either alone or in combination with empty palm fruit
bunches or processed empty palm fruit bunches as a substrate for fermentation, e.g. in the
production of a fermentation product such as biofuel (e.g., ethanol or butanol). The empty
palm fruit bunches may be pretreated prior to use for fermentation. For example, the empty
palm fruit bunches may be milled and/or chemically treated by alkaline ammonia treatment or
acid pretreatment.

The fermentation product in accordance with the present invention may be a compound
selected from the group consisting of: an alcohol (e.g. a biofuel (e.g. ethanol, butanol or a
combination thereof), an antibiotic, an antimicrobial, a bioinsecticide, a solvent, a
polyhydroxyalkanoate and an organic acid, e.g. gluconic acid, ascorbic acid intermediates,
succinic acid, citric acid, acetic acid, lactic acid, butyric acid or propionic acid as well as other
fermentation end-products, including but not limited to, 1,3-propanediol, acetone, and
glycerol. In accordance with the present invention, the conversion of a substrate for
fermentation to an end product involves the use of an enzymatic conversion by a known
enzyme to the desired end-product using known enzymatic conversion methods. For
example, in some embodiments, the conversion of glucose to a desired end product (e.g.,
propanediol, succinic acid, gluconic acid, lactic acid, amino acids, antimicrobials, ethanol,
butanol, ascorbic acid intermediates and/or ascorbic acid) is accomplished by the addition of
an amount of an enzyme known to convert glucose to the specified end product desired. For
example, enzymes useful for the conversion of a sugar to 1,3-propanediol may include, but
are not limited to, enzymes produced by E. coli and other microorganisms. For example
enzymes useful for the conversion of a sugar to lactic acid include, but are not limited to,
those produced by Lactobacillus and Zymomonas. Enzymes useful for the conversion of a
sugar to ethanol include, but are not limited to alcohol dehydrogenase and pyruvate
decarboxylase. Enzymes useful for the conversion of a sugar to ascorbic acid intermediates
include, but are not limited to glucose dehydrogenase, gluconic acid dehydrogenase, 2,5-
diketo-D-gluconate reductase, and various other enzymes. Enzymes useful for the
conversion of a sugar to gluconic acid include, but are not limited to glucose oxidase and
catalase.

The desired end-product can be any product that may be produced by the enzymatic
conversion of the substrate to the end-product. For example, gluconate can be converted
from glucose by contacting glucose with glucose dehydrogenase (GDH). In addition,
gluconate itself can be converted to 2-KDG (2-keto-D-gluconate) by contacting gluconate
with GDH. Furthermore, 2-KDG can be converted to 2,5-DKG by contacting 2-KDG with 2-
KDGH. Gluconate can also be converted to 2-KDG by contacting gluconate with 2KR.
Glucose can also be converted to 1,3-propanediol by contacting glucose with E. coli. In
addition, glucose can be converted to succinic acid by contacting glucose with \textit{E. coli}.

Additional embodiments, as described herein are also provided by the present invention.

In some embodiments in which glucose is an intermediate, it is converted to ethanol by contacting glucose with an ethanologenic microorganism. In contacting the intermediate with an intermediate converting enzyme, it is contemplated that isolated and/purified enzymes are placed into contact with the intermediate. In yet another embodiment, the intermediate is contacted with bioconverting agents such as bacteria, fungi or other organism that takes in the intermediate and produces the desired end-product. In some embodiments, the organism is wild-type, while in other embodiments it is mutated.

Preferred examples of ethanologenic microorganisms include ethanologenic bacteria expressing alcohol dehydrogenase and pyruvate decarboxylase, such as can be obtained with or from \textit{Zymomonas mobilis} (See e.g., U.S. Pat. Nos. 5,028,539, 5,000,000, 5,424,202, 5,487,989, 5,482,846, 5,554,520, 5,514,583, and copending applications having U.S. Ser. No. 08/363,868 filed on Dec. 27, 1994, U.S. Ser. No. 08/475,925 filed on Jun. 7, 1995, and U.S. Ser. No. 08/218,914 filed on Mar. 28, 1994, each of which is incorporated herein by reference).

The sustainable reuse of palm oil mill effluent (POME) is taught in Wu, \textit{et al.} \textit{(Biotechnology Advances} 27 (2009) 40-52). The feedstock may be subjected to one or more processing steps selected from the group consisting of: milling, cooking and/or saccharification. A further surprising finding of the present invention is that the enzyme incubation preferably takes place without or with only minimal agitation.

Preferably the enzyme composition comprising cellulase for use in the methods and uses of the present invention is thermostable.

In a further embodiment, there is provided a use of at least one thermostable enzyme composition comprising cellulase in combination with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed with the enzyme composition comprises at most 40% water content w/w in the manufacture of palm oil for improving palm oil yield, for improving separation of palm oil from the sludge phase, for producing an aqueous phase with improved fermentable sugars, or for improving the speed of oil separation.

It may be possible to perform the enzyme incubation according to the present invention at any stage in the palm fruit processing plant following removal of palm fruitlet from the bunch. However, in practice the inventors have found that it may be undesirable to carry out this reaction in the digester. Current digester conditions are highly optimized to physically disintegrate the fruit so that the liquids can be pressed out. This takes place typically at 95 °C.
In one embodiment, the enzyme composition is incubated with the palm fruit or a portion thereof before the digestor, e.g. in a precooker. The temperature in the precooker may be lower than the temperature in the digestor, e.g. less than 95 °C. In one preferred embodiment the enzyme composition is incubated with palm fruit extract (e.g. pressed palm fruit liquid) either just before or during clarification (e.g. just before or in the clarifier).

The method of the present invention further comprises separating palm oil (crude palm oil) from sludge.

In some embodiments, the crude palm oil may be separated from the sludge by clarifying, decanting or a combination thereof.

In another embodiment, the crude palm oil may be separated from the sludge by centrifugation. In another embodiment, the palm fruit extract for use in the methods and/or uses of the present invention may be a pressed palm fruit liquid.

In one embodiment, the admixing of the at least one enzyme(s) and palm fruit or a portion thereof may occur during digestion of one or more palm fruit(s) or a portion thereof prior to pressing, preferably wherein the at least one enzyme(s) is added to the digestor.

In another embodiment, the at least one enzyme(s) may be admixed with a palm fruit extract after being pressed.

In some embodiments, the palm fruit extract is a pressed palm fruit extract and the admixing occurs prior to or during separation of crude palm oil from sludge.

In some embodiments, the admixture as defined in the method of the present invention may be incubated for about 2 hours.

The method may comprise applying the enzyme composition to the palm fruit or a portion thereof or a palm fruit extract by: (a) spraying; (b) admixing the enzyme composition with water added to the digestor (so long at the total water content does not exceed the maximum 40% w/w); (c) pouring the at least one enzyme into a vessel with the palm fruit or a portion thereof or palm fruit extract; or (d) combinations thereof.

In one embodiment, the enzyme or thermostable enzyme may be immobilized, e.g. to a surface or cross-linked. By way of example the enzyme or thermostable enzyme may be cross-linked using glutaraldehyde (Migneault et al., BioTechniques37: 790:802 (2004)).

In one embodiment, the separation of the crude oil from the sludge may be carried out by clarification. "Clarification", as used herein, means using gravity to allow the oil to settle out of the sludge. During clarification the oil becomes "clear". Clarification conventionally takes place between approximately 90 and 95 °C. Typically one would leave the admixture for 1-3 hours to allow for the clarification process to occur. However it has been found that enzyme treatment as taught in the present invention means it is possible to
conduct the clarification as a lower temperature, e.g. between 50 to 70°C. Without wishing to be bound by theory, the high conventional clarification temperatures were applied to secure low viscosity and good oil separation. The present methods facilitate low viscosity and good oil separation when the enzyme composition is used in an admixture with at most 40% water content. Advantageously, the present method provides admixing the enzyme composition with a palm fruit liquid (which comprises at most 40% water content) in the clarifier and incubating at a temperature of between 50 and 70 °C for 0.5 to 8 hours. Reducing the temperature in the clarifier can has significant advantages as the exposure of the oil to temperatures of 90 to 95 X can have a negative effect on the quality of the oil and/or cause oxidation of the oil.

Large settling tanks (so-called vertical clarifiers) may be used in which the crude oil settles out of the sludge. The oil may be skimmed off the sludge. With the sludge typically coming out of the bottom of a vertical clarifier (this sludge is typically called the "underflow"). "Sludge" as used herein is a heavy fraction of pressed palm fruit extract comprising oil, water and some organic material that has a tendency to settle out of pressed palm fruit extract. Sludge may be obtained by clarification, for example following separation as shown in Figure 1.

Separation of the oil may be into 2-phases or 3-phases. When a 2-phase system is used (e.g. separation into an oil phase and a sludge phase) the sludge phase is enriched in soluble fermentable sugars. When a 3-phase system is used (e.g. separation into an oil phase, an aqueous phase and a sludge phase) the aqueous phase is enriched in soluble fermentable sugars.

The phase enriched in soluble fermentable sugars may be used as a fermentation media, e.g. for the production of an alcohol (e.g. bioethanol) or other fermentation product as set forth herein. Mechanisms may be employed to improve recovery of residual oil from the settled sludge (e.g. post clarification). For example a centrifuge may be used to extract further oil from the sludge. Typically therefore the sludge may be sent to a centrifuge. Alternatively a decanter may be used in combination with clarification. For example, 3-phase centrifuges or decanters may be employed. By way of example only a 3-phase decanter is available as "Westfalia SEPARATOR® topd 3-Phase Decanter" may be used to separate the sludge from the clarification process into 3-phases: an oil-phase, solids, and virtually oil-free waste water. Alternatively, nozzle-type separates such as the one from GEA Westfalia may be used which separate the sludge from the clarification step into three phases: palm oil, solid concentrate and water.
The separation of the crude oil from the sludge may be carried out by decanting (e.g. without clarification). Decanters (3-phase) available as "Westfalia SEPARATOR® topd 3-Phase Decanter" can be used directly with enzyme treated pressed palm fruit extract without clarification (GEA Westfalia, Oelde, Germany). This may have the advantage of shorter processing time, smaller dimensions of the process lines. Also the risk of oxidation of the crude oil is less significant compared to the process using vertical clarifiers. Such a decanter separates the sludge into 3-phases: an oil-phase, a dry solids cake, and virtually oil-free waste water. Centrifugation may be used during or after the decanting process.

The method may further comprise purifiers downstream of the separation step (e.g. downstream of the clarification and/or decanting stage). The purifiers may remove even extremely small amounts of oil left in the effluent.

In another embodiment, the present methods may further comprise a desanding step - which step removes sand to avoid erosion problems caused thereby. The sand may be removed by any method known to one skilled in the art. As the skilled person will appreciate, a multicyclone system may be used to separate the sand. Typically the desanding step may occur prior to the centrifugation step.

The pressed palm fruit extract may be prepared by sterilizing the fresh palm fruit bunches, stripping the fruit from the sterilized bunches, optionally digestion of the fruit, and pressing. The digestion may take place in a digester. During the digestion, the palm fruit is disintegrated and oil released from the fiber. After digestion, the crude oil is separated from the fiber by pressing the digested fruits. The term "palm fruit extract", as used herein, may mean an extract obtained during sterilization of the fresh palm fruit bunches, stripping the fruit from the bunches, and optionally digestion of the fruit prior to pressing. The term "pressed palm fruit extract", as used herein, means an extract post-pressing. For example, pressed palm fruit extract may arise from the pressing step indicated in the flow diagram of Figure 1 and the pressed palm fruit extract or pressed oil may then be subject to further processing steps, e.g. screening, clarification, desanding, separation, purification, drying etc. The method may include removing large solid particulates/course fibres from the pressed palm fruit extract - e.g. this may be achieved by passing the pressed palm fruit extract through a screen which may be vibrating. This additional step may be carried out before clarification and/or decanting.

In one embodiment, the enzyme for use in the present invention is admixed with one or more palm fruit(s) or a portion thereof prior to pressing. Suitably the palm fruit(s) or portion thereof is/are admixed with the enzyme during a digestion step. In one embodiment, the palm fruit or portion thereof may be admixed with the enzyme in a digester. The enzyme may be added to palm fruit(s) prior to removal of the kernel (otherwise known as the nut).
another embodiment, the enzyme for use in the present invention may be admixed with digested palm fruit or portion thereof prior to pressing.

In one embodiment, the enzyme may be admixed with palm fruit or a portion thereof in an enzyme reaction tank. Suitably, such admixing may be prior to digestion of said palm fruit or portion thereof. Suitably the admixture may be incubated at a temperature and/or time that are optimised for digestion of said palm fruit or the portion thereof. It will be appreciated that the person skilled in the art will be capable of determining such a time empirically based on routine experimentation.

As used herein, the term "or a portion thereof in relation to the palm fruit(s) means the part of the palm fruits that are remaining post stripping the fruit from the bunches.

It is envisaged that in practicing the present invention the enzyme composition may be admixed with or applied to the palm fruit or portion thereof or palm fruit extract by any suitable means known in the art.

The aim when admixing the enzyme with the palm fruit or a portion thereof or palm fruit extract is to ensure good coverage of the enzyme on the palm fruit (or portion thereof or palm fruit extract). In one embodiment, the enzyme composition may be applied to the palm fruit or a portion thereof or palm fruit extract by spraying, admixing the at least one enzyme, pouring the at least one enzyme into a vessel with the palm fruit or portion thereof or palm fruit extract, or combinations thereof.

The term "admixed", as used herein, encompasses mixing, pouring, spraying, misting or other suitable means of contacting the enzyme(s) with the palm fruit or portion thereof or palm fruit extract.

Suitably, when the enzyme composition is admixed with or applied to the palm fruit or portion thereof or palm fruit extract this may occur behind a protective surface (e.g. a screen or a curtain). Advantageously, application behind a protective surface protects the individual(s) performing the application or admixing and minimises any negative effects to human health that could ensue by exposure (e.g. through inhalation) to the enzyme composition.

In one embodiment, the palm fruit or a portion thereof or the palm fruit extract may be one having a diglyceride content of about 3% w/w to about 8% w/w of total lipid content.

In another embodiment the enzyme for use in the present invention may be admixed with pressed palm fruit extract prior to separating the crude palm oil from the sludge (e.g. by clarification or decanting or centrifugation).

In one embodiment, the enzyme for use in the present invention may be admixed with pressed palm fruit extract during the separation step, e.g. when the crude palm oil is being separated from the sludge (e.g. by clarification or centrifugation). In particular, in one
embodiment the enzyme for use in the present invention may be admixed with pressed palm
fruit extract during clarification (e.g. in the clarifying tank).

In some embodiments, the enzyme may be added into the flow stream of pressed
palm liquid pumped into the clarifier (e.g. the clarifying tank).

One preferred mechanism for recovery of residual oil in the sludge is centrifugation.
The term "pressed palm fruit extract" is also known in the industry as "pressed liquid" or
"pressed oil". These terms are used interchangeably herein. For the avoidance of doubt, the
term pressed palm fruit extract is not a crude palm oil or semi-crude palm oil and has
typically not been water or enzymatically degummed. A crude palm oil is the resulting oil that
is purified and dried prior to shipment to refining/degumming plants.

Crude palm oil has three main components, which are a mixture of oil and water, oil-
in-water emulsions and water-in-oil emulsions. A crude palm oil is typically separated into
Stork & Co's Apparatenfabriek N.V. Amsterdam; Master Thesis: Separation Technique of Crude
Palm Oil at Clarification Area Via Optimum Parameters by Nurulhuda Binti Kasim, Universiti
Malaysia Pahang (April 2009)). An approximate average composition of screw pressed crude
palm oil might be 64% oil, 24% water and 12% non-oil solid (Maycock et al. (1987) Palm Oil
Factory Process Handbook Part 1, PORIM, Bangi). Without wishing to be bound by theory,
examination of sludge samples typically reveals the presence of oil droplets of sizes varying
from less than 1 µm. The difference in specific gravity between sludge and oil is practically
constant at 0.1 throughout the temperature range from 40 °C to 100 °C (Stork, 1960). The
largest solid impurity to be separated is the fibre and the smallest is the cellular debris.

The pressed palm fruit extract is a composite containing: oil, water and non-oil solids
(comprising lignin, carbohydrates, proteins and inorganic solids). The mixture is an emulsion
of oil-in-water from which the oil is separated. The pressed palm fruit extract comprises
typically approximately 64% oil, 24% water, 12% non-oil solids (Maycock et al. (1987) supra)
(comprising lignin, carbohydrate, proteins and inorganic solids). It will be known to one skilled
in the art that the levels of each of these components in the pressed palm fruit extract may
vary depending on the starting palm fruits used, which vary according to location and year of
harvest as well as, for example, the amount of water in the fruit. Without wishing to be bound
by theory, the amount of water in the fruit may vary depending on the whether the climate
conditions are wet or dry during harvest, for example. However, a pressed palm fruit extract
for use in the present method typically has at least 50% oil. The water content of a pressed
palm fruit extract for use in the present method is below 40%.
Typically 7-10% w/w (e.g. 8.5% w/w) of the total non-oil solids in pressed palm fruit extract is protein. Therefore in one embodiment the present invention relates to a palm fruit extract (e.g. a pressed palm fruit extract) that comprises at least 7% w/w (suitably at least 8.4% w/w) total non-oil solids. In some embodiments, the present method comprises a palm fruit extract (e.g. a pressed palm fruit extract) that comprises at from at least about 6.5% to about 12% w/w total non-oil solids.

Approximately 0.5-2% w/w (typically approximately 1% w/w) of the total pressed palm extract is protein. In one embodiment, the present method is directed to a palm fruit extract (e.g. a pressed palm fruit extract that comprises at least 0.5%, preferably at least 1% w/w protein). For the avoidance of doubt, crude palm oil contains about 99% w/w oil because almost all of the non-oil solids have been removed. In one embodiment, the non-hydratable phosphorus content of the palm fruit extract (e.g. the pressed palm fruit extract) is less than 45 ppm, preferably less than 30 ppm calculated on the oil basis.

In some embodiments, the non-hydratable phosphorus content may be considered to be equivalent to non-hydratable phospholipids. Thus, in some embodiments, the non-hydratable phosphorus in non-hydratable phospholipids content of the palm fruit extract (e.g. the pressed palm fruit extract) may be less than 45 ppm, preferably less than 30 ppm. The incubation of the admixed at least one enzyme(s) and the at least one palm fruit or portion thereof or palm fruit extract in accordance with the present invention may be carried out at about 40 °C to about 100 °C.

Suitably, the incubation of the admixed at least one enzyme(s) and the palm fruit or portion thereof or palm fruit extract may be carried out at about 45 °C to about 95 °C, suitably at about 50 °C to about 95 °C; more suitably at about 50 °C to about 90 °C; and even more suitably at about 55 °C to about 85 °C. In one embodiment, the admixture may be incubated at above about 65 °C, preferably about 70 °C. In one embodiment, the admixture may be incubated between about 70 °C to about 90 °C; preferably at about 75 °C to about 80 °C; and even more suitably about 80 °C. In one preferred embodiment, the admixture may be incubated at above about 55 °C, preferably about 60 °C.

One skilled in the art will appreciate that the length of time necessary to achieve the effects will be somewhat dependent on the temperature at which the admixture of the enzyme composition and a palm fruit or a portion thereof or a palm fruit extract are incubated. The incubation of the admixed enzyme composition and the at least one palm fruit or portion thereof or palm fruit extract may be carried out for at least about 30 minutes to about 8 hours. Suitably, the admixture may be incubated for about 4 hours.

The water content (w/w) of the palm fruit or portion thereof or palm fruit liquid or sludge may be determined using the following method: by evaporation at 160 °C using a
ML50 Moisture analyzer (A&D Company Limited (Tokyo, Japan)) - in which the water readout is monitored until the change in weight is less than 0.1%/minute and the % water is read. Moisture can also be analyzed by Moisture Air-Oven Methods, AACC International Method 44-15.02.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Singleton, et al., Dictionary of Microbiology and Molecular Biology, 20 ED., John Wiley and Sons, New York (1994), and Hale & Marham, The Harper Collins Dictionary of Biology, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this disclosure. Another reference providing the skilled person with general information is Palm Oil Production, Processing, Characterization, and Uses; Volume 5 of AOCS Monograph Series on Oilseeds; edited by Oi-Ming Lai, Chin-Ping Tan and Casimir C. Akoh; and available from the publisher: Amer Oil Chemists Society, (2012). This disclosure is not limited by the exemplary methods and materials disclosed herein, and any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this disclosure. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, any nucleic acid sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

The headings provided herein are not limitations of the various aspects or embodiments of this disclosure which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

Amino acids are referred to herein using the name of the amino acid, the three letter abbreviation or the single letter abbreviation.

The term "protein", as used herein, includes proteins, polypeptides, and peptides.

As used herein, the term "amino acid sequence" is the sequence of amino acids as arrayed within a protein molecule. As such, the term may be used synonymously with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme". The terms "protein" and "polypeptide" are used interchangeably herein. In the present disclosure and claims, the conventional one-letter and three-letter codes for amino acid residues may be used. The 3-letter code for amino acids as defined in conformity with the IUPAC IUB Joint Commission on Biochemical
Nomenclature (JCBN). It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

Other definitions of terms may appear throughout the specification. Before the exemplary embodiments are described in more detail, it is to understand that this disclosure is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within this disclosure. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within this disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in this disclosure.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such candidate agents and equivalents thereof known to those skilled in the art, and so forth.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that such publications constitute prior art to the claims appended hereto.

ENZYME COMPOSITION

Suitably, the enzyme composition for use in the present invention comprises cellulase activity. The cellulase activity according to the present invention may be an endoglucanase (e.g. a \( \beta \)-glucanase) activity.

In one embodiment, the enzyme composition, in addition to comprising cellulase activity, comprises one or more of the following enzyme activities: mannanase activity, pectinase activity, and a combination thereof.

The enzyme composition for use in the present methods may be a crude or purified extract of a *Trichoderma reseei* fermentate.
In some embodiments, the enzyme composition may (in addition to having a cellulase (e.g. an endoglucanase (e.g. a β-glucanase)), activity further comprise one or more of the activities selected from the group consisting of: a mannanase activity, a pectinase activity, a xylanase activity, a glucuronidase activity, a galactanase activity, and combinations thereof.

Preferably the enzyme composition comprises endoglucanase (e.g. a β-glucanase) activity and mannanase activity. Suitably, the enzyme composition may comprise endoglucanase (e.g. a β-glucanase) activity and pectinase activity.

The terms "cellulases" or "cellulolytic enzymes", as used herein, are understood as comprising endo-glucanase (EC 3.2.1.4) activity. In one embodiment the enzyme composition used in accordance with the present invention is an endoglucanase (EC 3.2.1.4), e.g. an endoglucanase that cuts the cellulose chains at random. The cellulases may comprise a carbohydrate-binding module (CBM) which enhances the binding of the enzyme to a cellulose-containing fiber and increases the efficacy of the catalytic active part of the enzyme. A CBM is defined as contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity. For further information of CBMs see the CAZy (Carbohydrate Active Enzymes Database; Lombard V., et al. (2014), Nucleic Acids Res 42:D490-D495) or Tomme et al. (1995) in Enzymatic Degradation of Insoluble Polysaccharides (Saddler and Penner, eds.), Cellulose- binding domains: classification and properties, pp. 142-163, American Chemical Society, Washington. In a preferred embodiment the cellulases or cellulolytic enzymes may be a cellulolytic preparation as defined in U.S. provisional patent application no. 60/941,251. In some embodiments the enzyme composition is a cellulase enzyme, such as one derived from *Trichoderma reesei*. The cellulolytic activity may, in some embodiments, be derived from a fungal source, such as a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*; or a strain of the genus *Humicola*, such as a strain of *Humicola insolens*.

Endoglucanases (E.C. 3.2.1.4) catalyze endo-hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxy methyl cellulose and hydroxy ethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans and other plant material containing cellulosic parts. The authorized name is endo-1,4-beta-D-glucan 4-glucano hydrolase, but the abbreviated term endoglucanase is used in the present specification. Endoglucanase activity may be determined using carboxymethyl cellulose (CMC) hydrolysis according to the procedure of Ghose ((1987), Pure and Appl. Chem. 59: 257-268).

In some embodiments, endoglucanases may be derived from a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*; a strain of the genus *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of
Chrysosporium lucknowense. Suitably the cellulase for use in the present invention may be a Chrysosporium lucknowense cellulase available from Dyadic International USA Inc. (Jupiter, FL, USA), such as taught in US patent no. 7,892,812, the teachings of which are incorporated herein by reference.

In some embodiments, the enzyme composition for use in the methods and/or uses of the present invention may be the product of expression of one or more enzyme(s) in a suitable host cell (e.g. a fermentation product).

Suitably, the enzyme composition comprising any one or more of the activities indicated in the foregoing embodiment may be obtainable (e.g. obtained) from Trichoderma, preferably from Trichoderma reesei.

In one embodiment, a suitable enzyme composition for use in the methods and/or uses in accordance with the present invention may be or may comprise LAMINEX® BG2 (Available from DuPont Industrial Biosciences, Wilmington, DE ,USA). LAMINEX® BG2 is a commercial preparation of an enzyme complex hydrolyzing beta-glucans and related carbohydrates. It is produced by fermentation with a selected strain of Trichoderma reesei. LAMINEX® BG2 is composed of: water 59 - 71 % (w/w); cellulase 15 - 20 % (w/w); sorbitol 10.0 - 15.0 % (w/w); sodium chloride 4.0 - 5.0 % (w/w); and sodium benzoate 0.00 - 0.50 % (w/w).

In one embodiment the enzyme composition may be a fermentation of Penicillium funiculosum in combination with fermentation from Trichoderma, e.g. Trichoderma reesei.

In another embodiment, the cellulase according to the present invention may be LAMINEX® Super 3G (DuPont Industrial Biosciences). LAMINEX® Super 3G is a commercial preparation of an enzyme complex hydrolysing beta-glucans, pentosans and related carbohydrates. LAMINEX® Super 3G is produced by fermentation with selected strains of Trichoderma reesei and Penicillium funiculosum. LAMINEX® Super 3G is composed of: water 75.8 - 80.8 % (w/w); cellulase 15 - 20 % (w/w); sorbitol 6.50 % (w/w); sodium chloride 2.20 % (w/w); and sodium benzoate 0.50 % (w/w).

The enzyme composition comprising cellulase is preferably one which solubilises as much sediment as possible. The enzyme composition comprising cellulase is preferably one which comprises mannanase activity.

Without wishing to be bound by theory, the enzyme composition preferably comprises cellulase activity together with mannanase activity, in which case the enzyme composition comprises a minimum level of mannanase activity which when added to the substrate gives a mannanase concentration of at least 200 MVR/kg substrate. In some embodiments, the cellulase composition may comprise mannanase side activity. Where the mannanase activity is a side activity, it must represent at least 200 MVR/kg substrate. Alternatively, a separate
mannanase enzyme may be added to a cellulase composition to ensure at least 200 MVR/kg substrate mannanase activity is present in the substrate.

In one embodiment, the enzyme composition may comprise a xylanase. In one embodiment, the xylanase is of microbial origin, such as of fungal origin (e.g., Trichoderma, Meripilus, Humicola, Aspergillus, and Fusarium) or from a bacterium (e.g., Bacillus). In some embodiments, the xylanase is derived from a filamentous fungus, preferably derived from a strain of Aspergillus, such as Aspergillus aculeatus; or a strain of Humicola, preferably Humicola lanuginosa. The xylanase may preferably be an endo-1,4-beta-xylanase. Examples of commercial xylanases include GRINDAMYL™ POWERBAKE® 930 from Danisco A/S, Denmark or SHEARZYME™ and BIOFEED WHEAT™ from Novozymes A/S, Denmark.

In another embodiment, the enzyme composition may comprise a glucuronidase. The glucuronidase may be one or more selected from: a 1,2-alpha-glucuronidase (E.C. 3.2.1.131), an alpha-glucuronidase (E.C. 3.2.1.139), a beta-glucuronidase (E.C. 3.2.1.31), a glucuronosyl-disulfoglucosamine glucuronidase (E.C. 3.2.1.56) or any combination thereof.

As used herein, the term "beta-glucuronidase" is synonymous with "beta-glucuronidase glucuronohydrolase". In another embodiment, the glucuronidase may be a 1,2-alpha-glucuronidase (E.C. 3.2.1.131). In one embodiment, the glucuronidase may be an alpha-glucuronidase (E.C. 3.2.1.139). In another embodiment, the glucuronidase may be a beta-glucuronidase (E.C. 3.2.1.31). In another embodiment, the glucuronidase may be a glucuronosyl(disulfoglucosamine (E.C. 3.2.1.56).

In one embodiment, the enzyme composition may comprise a galactanase. The galactanase may be selected from an exo-galactanase (E.C. 3.2.1.23) or an endo-galactanase (E.C. 3.2.1.89) for example, an arabinogalactan endo-1,4-beta-galactosidase or a galactan endo-beta-1,3-galactanase (E.C. 3.2.1.181). Arabinogalactan endo-1,4-beta-galactosidase catalyzes the endohydrolysis of 1,4-D-galactosidic linkages in arabinogalactans.

The term "xylanase", as used herein, refers to an enzyme that is able to hydrolyze the beta-1,4 glycosyl bond in non-terminal beta-D-xylpyranosyl-1,4-beta-D-xylpyranosyl units of xylan or arabinoxylan. Other names include 1,4-beta-D-xylan xylanohydrolase, 1,4-beta-D-xylan xylanohydrolase, beta-1,4- xylan xylanohydrolase, (1-4)-beta-xylan 4-xylanohydrolase, endo-1,4-beta-xylanase, endo-(1-4)-beta-xylanase, endo-beta-1,4-xylanase, endo-1,4-beta-D-xylanase, endo-1,4-xylanase, xylanase, beta-1,4-xylanase, beta-xylanase, beta-D-xylanase. Xylanases can be derived from a variety of organisms, including plant, fungal (e.g. species of Aspergillus, Penicillium, Disporotrichum, Neurospora, Fusarium, Humicola, Trichoderma, Geosmithia, Talaromyces) or bacterial species (e.g. species of Bacillus,

In one aspect, the xylanase used in the methods described herein is an enzyme classified as EC 3.2.1.8. The official name is endo-1,4-beta-xylanase. The systematic name is 1,4-beta-D-xylan xylanohydrolase. Other names may be used, such as endo-(1-4)-beta-xylanase; (1-4)-beta-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; beta-1,4-xylanase; endo-1,4-xylanase; endo-beta-1,4-xylanase; endo-1,4-beta-D-xylanase; 1,4-beta-xylan xylanohydrolase; beta-xylanase; beta-1,4-xylan xylanohydrolase; endo-1,4-beta-xylanase; beta-D-xylanase. The reaction catalyzed is the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans.

In another embodiment, the mannanase for use in the present methods may be any commercially available mannanase. The mannanase may be an endo-1,4-p-D-mannanase (classified as E.C. 3.2.1.78) or a beta-mannosidase (classified as E.C. 3.2.1.25). In one embodiment, the mannanase is preferably an endomannanase, such as an endo-1,4-beta-D-mannanase. The classification for an endo-1,4-beta-D-mannanase (beta-mannanase) is E.C. 3.2.1.78. In another embodiment, the enzyme composition comprises a beta-mannanase (E.C. 3.2.1.78) from Bacillus. In one embodiment, the enzyme composition comprises a beta-mannanase (E.C. 3.2.1.78) from Bacillus lentus or Bacillus subtilis or Bacillus licheniformis. In a further embodiment, the enzyme composition may comprise a mannanase (e.g. E.C. 3.2.1.78) produced in Trichoderma, e.g. Trichoderma reesei. In another embodiment, the enzyme composition may comprise MANNASTAR® 375 (DuPont Industrial Biosciences).

In one embodiment, the enzyme composition comprises a beta-mannanase (E.C. 3.2.1.78) from Bacillus lentus, e.g. such as the commercial HEMICELL® and HEMICELL®-HT product from ChemGen Corp. (Gaithersburg, MD, USA; Elanco). In one embodiment, the enzyme composition comprises HEMICELL®-W (a commercial product sold by ChemGen Corp. comprising beta-Mannanase (EC 3.2.1.78) from Bacillus lentus and a xylanase (EC 3.2.1.8) from Trichoderma longibrachiatum). In one embodiment, the enzyme composition comprises a beta-mannanase (E.C. 3.2.1.78) from Bacillus licheniformis, such as the beta-Mannanase (EC 3.2.1.78) from Bacillus licheniformis sold in CTCZYME® - a product sold by CTC BIO Inc. (Gangwan-do, Korea). In one embodiment, the enzyme composition may comprise ZYMANASE® (a commercial product sold by ChemGen Corp. comprising a beta-Mannanase (EC 3.2.1.78) and a beta-glucanase. In one embodiment, the enzyme composition may comprise CTCZYME® - a product sold by CTC BIO Inc., and comprising a beta-Mannanase (EC 3.2.1.78) from Bacillus licheniformis (B. licheniformis gene expressed in B. subtilis). In one embodiment, the enzyme composition may comprise a mannanase taught in US Patent No. 7,846,705.
In another embodiment, the enzyme composition is suitably a thermostable enzyme composition. In one embodiment, one or more of the enzyme(s) used in the present methods and/or uses are thermostable enzyme(s). In one embodiment, the enzyme or thermostable enzyme is not a genetically modified enzyme.

The term "thermostability", as used herein, is the ability of an enzyme to resist irreversible inactivation (usually by denaturation) at a relatively high temperature. This means that the enzyme retains a specified amount of enzymatic activity after exposure to an identified temperature over a given period of time. There are many ways of measuring thermostability. By way of example, enzyme samples may be incubated without substrate for a defined period of time (e.g. 10 min or 1 to 30 min) at an elevated temperature compared to the temperature at which the enzyme is stable for a longer time (days). Following the incubation at elevated temperature the enzyme sample is assayed for residual activity at the permissive temperature of e.g. 30 °C (alternatively 25-50 °C or even up to 70 °C). Residual activity is calculated as relative to a sample of the enzyme that has not been incubated at the elevated temperature.

Thermostability can also be measured as enzyme inactivation as function of temperature. Enzyme samples are incubated without substrate for a defined period of time (e.g. 10 min or 1 to 30 min) at various temperatures and following incubation assayed for residual activity at the permissive temperature of e.g. 30 °C (alternatively 25-70 °C or even higher). Residual activity at each temperature is calculated as relative to a sample of the enzyme that has not been incubated at the elevated temperature. The resulting thermal denaturation profile (temperature versus residual activity) can be used to calculate the temperature at which 50% residual activity is obtained. This value is defined as the Tm value. Even further, thermostability can be measured as enzyme inactivation as function of time. Here enzyme samples are incubated without substrate at a defined elevated temperature (e.g. 76 °C) for various time periods (e.g. between 10 sec and 30 min) and following incubation assayed for residual activity at the permissive temperature of e.g. 30 °C (alternatively 25-70 °C or even higher). Residual activity at each temperature is calculated as relative to an enzyme sample that has not been incubated at the elevated temperature. The resulting inactivation profile (time versus residual activity) can be used to calculate the time at which 50 % residual activity is obtained. This is usually given as T1/2. These are examples of how to measure thermostability. Thermostability can also be measured by other methods. Preferably thermostability is assessed by use of the "Assay for measurement of thermostability" as taught herein. The thermostability of an enzyme for use in accordance
with the present invention may be determined using the "Assay for measurement of thermostability" (see below).

In contradistinction to thermostability, thermoactivity is enzyme activity as a function of temperature. To determine thermoactivity enzyme samples may be incubated (assayed) for the period of time defined by the assay at various temperatures in the presence of substrate. Enzyme activity is obtained during or immediately after incubation as defined by the assay (e.g. reading an OD-value which reflects the amount of formed reaction product). The temperature at which the highest activity is obtained is the temperature optimum of the enzyme at the given assay conditions. The activity obtained at each temperature can be calculated relative to the activity obtained at optimum temperature. This will provide a temperature profile for the enzyme at the given assay conditions.

Assay for measurement of thermostability

The thermal denaturation profiles of the enzyme is measured by diluting and pre-incubating the enzyme samples in 25 mM acetate buffer, pH 4.5 for 10 min at varying temperatures (60, 65, 70, 75, 80, 85 and 90°C, respectively) and subsequently measuring the residual activity of the enzyme when tested in the "Beta-Glucanase Activity Assay" described herein.

In the assay, activity measured without pre-incubation is set to 100 % and the residual activity of an enzyme at each temperature is calculated as relative to this. Tm value is calculated from the thermal denaturation profiles as the temperature at which 50 % residual activity is obtained.

In one embodiment, an enzyme is considered to be thermostable in accordance with the present method if it has a Tm value of more than 70°C, wherein the Tm value is the temperature at which 50% residual activity is obtained after 10 min incubation. This Tm value may be measured in accordance with the assay for measurement of thermostability as taught herein.

In one embodiment, an enzyme is considered to be thermostable in accordance with the present method if it has a Tm value of more than 75°C, wherein the Tm value is the temperature at which 50% residual activity is obtained after 10 min incubation. This Tm value may be measured in accordance with the assay for measurement of thermostability as taught herein.

In one embodiment, an enzyme is considered to be thermostable in accordance with the present method if it has a Tm value of more than 80°C, wherein the Tm value is the temperature at which 50% residual activity is obtained after 10 min incubation. This Tm value may be measured in accordance with the assay for measurement of thermostability as taught herein.
In certain embodiments, the thermostable enzymes described herein possessing residual activity post heat treatment may be used in subsequent processes (e.g., for use in a biorefinery application).

In one embodiment, the enzyme according to the present methods and/or uses is not CELLUCLAST® (by Novozymes, A/S).

In one embodiment, the enzyme according to the present methods and/or uses does not comprise pectinase activity.

In one embodiment, the enzyme or thermostable enzyme for use in the methods and/or uses described herein may be immobilized. The term "immobilized", as used herein, means that the enzyme or enzyme is fixed in position and its movement impeded but the activity of the enzyme or thermostable enzyme is not substantially altered by such immobilization. Suitably, an immobilized enzyme or thermostable enzyme may retain at least 50% of its activity when compared to a non-immobilized enzyme or thermostable enzyme. Suitably it may retain at least about 50%, 60%, 70%, 80%, 90% or 95% of its activity when compared to a non-immobilized enzyme or thermostable enzyme. An "immobilized" enzyme or thermostable enzyme may be fixed to a surface. This may be achieved by any known means within the art which do not substantially alter the activity of the enzyme or thermostable enzyme. Suitably, the enzyme or thermostable enzyme may be immobilized by cross-linking (e.g. cross-linking to a surface).

In one embodiment, the enzyme may be cross-linked with glutaraldehyde in order to improve the enzymes thermostability. For example, see the teachings of Schmid et al., Adv. Biochem Eng. 12, p 41,1 18 (1979) and European Patent No. EP0575323B1.

**ENZYME ACTIVITY ASSAYS**

**CELLULASE ACTIVITY ASSAY: BY THE CMC-DNS PROCEDURE:**

The assay of cellulase activity (e.g. endo-1,4^-glucanase activity) is based on the enzymatic hydrolysis of the 1,4-p-D-glucosidic bonds in carboxymethylcellulose (CMCellulose 4M, Megazyme Ltd) a β-1,4-glucan. The enzyme is diluted in ddH<sub>2</sub>O and 0.25 ml enzyme solution added to 1,75 ml substrate (1.5% CMC in 0.2M sodium acetate buffer, pH 5.0) at 50 °C. After 10 min of incubation a 2 ml 1% 3,5-Dinitrosalicylic acid (DNS) solution is added and the sample is placed in boiling water bath for 5 min. The products of the reaction (β-1,4 glucan oligosaccharides) are determined colorimetrically at 540 nm by measuring the resulting increase in reducing groups reacting with the DNS. Enzyme activity is calculated from the relationship between the concentration of reducing groups, as glucose equivalents, and absorbance using a glucose standard in the range 0.125-0.5 mg/mL. One
unit of cellulase activity is defined as the amount of enzyme which produces 1 μmole glucose equivalents per minute under assay conditions.

In one embodiment, the cellulase in accordance with the present method is a cellulase which reduces the amount of dry sediment (dry matter) by at least 20% when 25000 CMC-DNS/kg substrate is added to a palm fruit, a portion thereof or a palm fruit extract and incubated for 1 hr at 50°C and the sludge dry matter is analysed by the following method: After incubation the sample was placed in at water bath at 95 degrees for 10 minutes to stop the enzyme reaction, and transferred to a tarred 50-mL centrifuge tube. The sample was centrifuged at 4180 rcf and 60 °C for 10 minutes. The upper oil layer was removed, and remaining water phase was discharged. Water (30 mL) at 50 °C was added to each tube. The sample was centrifuged at 4180 rcf (relative centrifugal force) and 60 °C for 10 minutes. The water phase was removed and the side of the tube was wiped with a tissue to remove residual oil on the inside of the tube. The wet sediment was scaled, frozen and freeze dried. Weight of the dry sediment was determined after freeze drying.

**PECTINASE AND MANNANASE ACTIVITY (PVR U/G AND MVR U/G) ASSAYS MEASURED BY VISCOSITY REDUCTION PROCEDURE:**

An enzyme sample (25, 50, 75 or 100 μL) diluted in ddH₂O is added to hydrocolloid solution (0.5% GRINDSTED® GUAR 250 (DuPont Nutrition Biosciences) pH 6.7 or 1.4% GRINDSTED® Pectin SY200 (DuPont Nutrition Biosciences) pH 4 in Citric acid-Sodium phosphate buffer) and incubated 19 hours at 40 °C. The hydrocolloid is cleaved by the enzyme to oligosaccharides, thereby creating a drop in viscosity of the solution. Following this, samples are tempered for 20 minutes on ice before measuring viscosity at 0 °C using a Viscoman pipette (Gilson, Inc., USA). The viscosity reduction is calculated as the viscosity of a sample with addition of enzyme relative to viscosity of sample without enzyme. The viscosity reduction is plotted against LN (μl dosage in substrate) and should be linear within relative viscosity of 0.1 to 0.85. Activity of the sample in U/g is calculated using the regression line. Pectinase viscosity reduction (PVR) and Mannanase viscosity reduction (MVR) units are defined as the amount of enzyme that will degrade the hydrocolloid substrate solution to a 50% (0.5) viscosity reduction in 19 hours of incubation at 40 °C.

Where the enzyme composition comprises mannanase, preferably the enzyme composition comprises a minimum level of mannanase activity which when added to the substrate will give a mannanase concentration of at least about 200 MVR/kg substrate.

Where the enzyme composition comprises pectinase, preferably the enzyme composition comprises a minimum level of pectinase activity which when added to the substrate will give a mannanase concentration of at least about 9 PVR/kg substrate.
In one embodiment, the enzyme composition comprises low or no protease. In other words preferably there is no or only very low levels of protease activity in the reaction admixture during incubation.

**PROTEASE ACTIVITY (PU) ASSAY - WITH SULFANILAMIDE-AZOCASEIN**

The azocasein assay is based on hydrolyses of the azocasein which releases the azo dyed peptide in the supernatant where it is detected at 450 nm. These peptides cannot precipitate by the addition of acid, as against non hydrolysed azocasein, which precipitates. Substrate: 0.25% Azocasein (Sigma A2765; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50 mM sodium-citrate buffer pH 6.

Procedure: 100 pL enzyme solution is incubated with 250 pL substrate for 30 minutes at 40 °C.

50 pL 2M Trichloracetic acid is added, and the sample is centrifuged at 10000 rcf for 5 minutes. Supernatant (195 pL) is transferred to a microtiter filterplate (0.2 µm PVDF Hydrophilic membrane) and 85 µL 1M NaOH is added. The sample is filtered into another microtiter plate by centrifugation at 2400 rcf for 2 minutes. OD 450 nm of the filtrated sample is read.

The activity of the enzyme sample is measured based on a calibration curve obtained by analyzing different dilutions of a commercial protease, PROTEX® 14L (Standardized to 150 PU/mL) and construction of a calibration curve of OD₄₅₀ as a function of PU/g.

In one embodiment, the enzyme composition preferably has not more than about 0.1 PU/mL. In another embodiment, the enzyme composition preferably has not more than about 0.04 PU/mL.

**XYLANASE ACTIVITY ASSAY**

To 1.0 mL aliquots of assay buffer (0.1 M NaAc, pH 5.0) is added 25 pL, 50 pL, 75 pL and 100 pL of enzyme solution and the mixtures are equilibrated at 40.0 °C for 5 minutes. One XylaZyme tablet (Megazyme International cat no. T-XYZ100, Wicklow, Ireland) (e.g. containing AZCL-arabinoxylan (wheat)) is added to each test tube and the test tubes must not be stirred. After exactly 10 minutes incubation 10 mL stop solution (1% (w/v) Tris(hydroxymethyl)- aminomethane) is added. The test tubes are stirred and the solutions are filtered through Whatman No. 1 filter paper.

The absorbance at 590 nm of standard and test samples is measured against a blank sample without enzyme. The concentrations of standard and sample enzymes are adjusted so that the optical densities (OD) at 590 nm are within the range 0.2-1.1.

The Standard enzyme is xylanase from Aspergillus niger, Megazyme cat. no. E-XYAN4.
The OD₅₉₀'s for standard and test enzymes are plotted against the volumes of enzyme solution added. The best curve fit is found using linear regression. The volumes of standard (Vₛₜ) and test enzymes (Vₜ) corresponding to an OD₅₉₀ of 0.7 are calculated.

\[ XU/g = \frac{ACT_{st} \cdot V_{st} \cdot D_{st} \cdot A_{st}}{V_t \cdot D_{st} \cdot A_{t}} \]

where

- \( ACT_{st} \) = activity of standard enzyme preparation, XU/g
- \( V_{st} \) = volume of standard enzyme read on x-axis, µL
- \( D_{st} \) = dilution of test sample, mL
- \( A_{st} \) = amount of standard enzyme, g
- \( A_{t} \) = amount of test sample in g
- \( V_{t} \) = volume of test sample read on x-axis, µL

An enzyme is a xylanase for use in the present methods if in the Xylanase Activity Assay herein it has at least 100 Units/mL.

**DOSAGE**

The one or more enzyme(s) for use in the methods and/or uses may be dosed at predetermined amounts when treating the palm fruit or a portion thereof or palm fruit extract. Suitably, the one or more enzyme(s) for use in the methods and/or uses may be dosed at an amount which reduces the viscosity in palm fruit extract by at least 10%. In one embodiment, the one or more enzyme(s) may be dosed at 0.5 to 500 mg/kg palm fruit or extract. In another embodiment, the one or more enzyme(s) may be dosed at 1 to 200 mg/kg palm fruit or extract. In still another embodiment, the one or more enzyme(s) may be dosed at 1 to 100 mg/kg palm fruit or extract. In still other embodiments, the one or more enzyme(s) may be dosed at 1 to 50 mg/kg palm fruit or extract. In a yet further embodiment, the one or more enzyme(s) may be dosed at 1 to 10 mg/kg palm fruit or extract.

**HOST CELL**

The host organism can be a prokaryotic or a eukaryotic organism.

The at least one enzyme may be obtainable (e.g. obtained) from any source. The at least one enzyme may be a recombinant enzyme, for example an enzyme that is heterologous to the cell in which it is expressed. In other embodiments the enzyme may be native to the cell in which it is expressed.
In one embodiment, the one or more enzyme(s) is not obtainable (e.g. obtained) from a Trichoderma (e.g. Trichoderma reesei) host cell. Alternative host cells may be fungi, yeasts or plants for example. The host cell may be any Bacillus cell. Preferably, said Bacillus host cell being from one of the following species: Bacillus licheniformis; B. subtilis; B. alkalophilus; B. amyloliquefaciens; B. circulans; B. clausii; B. coagulans; B. firmus; B. lautus; B. lentus; B. megaterium; B. pumilus or B. stearothermophilus.

Suitably, the host cell may a fungal host cell. Suitably the host cell may be any a Trichoderma, Meripilus, Humicola, Aspergillus, Fusarium or Chrysosporium host cell.

Suitably, the host cell may be a protease deficient or protease minus strain and/or an a-amylase deficient or a-amylase minus strain.

The term "heterologous", as used herein, means a sequence derived from a separate genetic source or species. A heterologous sequence is a non-host sequence, a modified sequence, a sequence from a different host cell strain, or a homologous sequence from a different chromosomal location of the host cell. A "homologous", as used herein, sequence is a sequence that is found in the same genetic source or species i.e. it is naturally occurring in the relevant species of host cell.

REGULATORY SEQUENCES

In some applications, an enzyme for use in the methods and/or uses taught herein may be obtained by operably linking a nucleotide sequence encoding same to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell (such as a B. licheniformis cell). By way of example, a vector comprising the nucleotide sequence is operably linked to such a regulatory sequence, i.e. the vector is an expression vector, may be used.

The term "operably linked", as used herein, refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. The term "regulatory sequences", as used herein, includes promoters and enhancers and other expression regulation signals.

The term "promoter", as used herein, is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme having the specific properties as defined herein may also be achieved by the selection of regulatory regions, e.g. promoter, secretion leader and terminator regions that are not regulatory
regions for the nucleotide sequence encoding the enzyme in nature. Suitably, the nucleotide sequence may be operably linked to at least a promoter.

**PROMOTER**

The promoter sequence may be heterologous or homologous to the sequence encoding any one of the enzymes for use in the methods or uses taught herein. The promoter sequence may be any promoter sequence capable of directing expression of an enzyme in the host cell of choice.

Suitably, the promoter sequence may be homologous to a *Bacillus* species, for example *B. licheniformis*. Preferably, the promoter sequence is homologous to the host cell of choice.

In another embodiment, the promoter may be homologous to a *Geosmithia* species, for example *Geosmithia emersonii*.

Suitably, the promoter sequence may be homologous to the host cell. “Homologous to the host cell”, as used herein, means originating within the host organism; i.e. a promoter sequence which is found naturally in the host organism. Suitably, the promoter sequence may be selected from the group consisting of a nucleotide sequence encoding: an α-amylase promoter, a protease promoter, a subtilisin promoter, a glutamic acid-specific protease promoter and a levansucrase promoter.

Suitably, the promoter sequence may be a nucleotide sequence encoding: the LAT (e.g. the alpha-amylase promoter from *B. licheniformis*, also known as AmyL), AprL (e.g. subtilisin Carlsberg promoter), EndoGluC (e.g. the glutamic-acid specific promoter from *B. licheniformis*), AmyQ (e.g. the alpha amylase promoter from *B. amylophilicus*), and SacB (e.g. the *B. subtilis* levansucrase promoter).

Other examples of promoters suitable for directing the transcription of a nucleic acid sequence may include: the promoter of the *Bacillus lentus* alkaline protease gene (aprH); the promoter of the *Bacillus subtilis* alpha-amylase gene (amyE); the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene (amyM); the promoter of the *Bacillus licheniformis* penicillinase gene (penP); the promoters of the *Bacillus subtilis* xylA and xylB genes; and/or the promoter of the *Bacillus thuringiensis* subsp. *tenebrionis* CryIII A gene.

**SIGNAL PEPTIDE**

The enzyme produced by a host cell by expression of the nucleotide sequence encoding the enzyme may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. A signal sequence may be used to direct secretion of the coding sequences through a particular cell membrane. The signal sequences may be natural or foreign to the coding sequence of the enzymes. For instance, the signal peptide coding
sequence may be obtained from an amylase or protease gene from a *Bacillus* species, preferably from *Bacillus licheniformis*.

Suitable signal peptide coding sequences may be obtained from one or more of the following genes: maltogenic a-amylase gene, subtilisin gene, beta-lactamase gene, neutral protease gene, and/or prsA gene.

In some embodiments, a nucleotide sequence encoding a signal peptide may be operably linked to a nucleotide sequence encoding any one of the enzymes disclosed herein. In another embodiment, the enzyme may be expressed in a host cell as defined herein as a fusion protein.

**EXPRESSION VECTOR**

The term "expression vector", as used herein, means a construct capable of *in vivo* or *in vitro* expression. Preferably, the expression vector is incorporated in the genome of the organism, such as a *B. licheniformis* host. The term "incorporated", as used herein, preferably covers stable incorporation into the genome.

The nucleotide sequence encoding an enzyme as defined herein may be present in a vector, in which the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism (such as *B. licheniformis*), i.e. the vector is an expression vector.

The vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide having cellulase activity as defined herein. The choice of vector, e.g. plasmid, cosmid, virus or phage vector, genomic insert, will often depend on the host cell into which it is to be introduced. The present methods may use other forms of expression vectors which serve equivalent functions and which are, or become, known in the art. Once transformed into the host cell of choice, the vector may replicate and function independently of the host cell's genome, or may integrate into the genome itself. The vectors may contain one or more selectable marker genes - such as a gene which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Alternatively, the selection may be accomplished by co-transformation (as described in WO 91/17243). Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.
ISOLATED

In one aspect, the enzyme is a recovered/isolated enzyme. Thus, the enzyme produced may be in an isolated form.

PURIFIED

In one aspect, the enzyme may be in a purified form. The term "purified", as used herein, means that the sequence is in a relatively pure state - e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

ADVANTAGES

The methods and/or uses described herein have many advantages over methods and/or uses taught in the prior art. The enzyme composition(s) for use in the present methods is/are advantageously able to decrease oil in sediment, decrease viscosity of the oil obtained from the palm fruit or portion thereof or palm fruit extract (e.g. pressed palm fruit extract), improve palm oil yields, and/or increase fermentable sugars in the aqueous phase. The methods and uses taught herein make it possible to isolate a heavy sediment phase. The thus isolated heavy phase has a low water binding capacity making it easy to dry. This has advantages with regard to downstream processing of this waste-stream. For example, it can be burnt to generate steam for the plant. Advantageously, the method and/or uses taught herein also result in increased palm oil yields during palm oil processing. In particular, a lot of the oil which is held in the sediment can be advantageously released by use of the present methods. In a preferred aspect, the enzyme treatment is of the pressed palm oil liquid and occurs in or just before the clarifier. This has additional advantageous properties, for example one or more of the following:

- less dilution with water needed prior to or in the clarifier, thus helping reduce palm oil mill effluent (POME) and reduce water consumption.
- reduces the biochemical oxygen demand (BOD) in waste.
- clarifier operation possible at a lower temperature (55-65°C compared to 80-90°C previously) with associated energy savings and better oxidative stability due to exposure of oil to lower temperatures.

A further advantage taught herein is the aqueous phase of the centrifuged enzyme treated pressed palm oil liquid or palm fruit is enriched in fermentable sugars in comparison to waste water from palm oil mills not utilizing the enzymatic treatment of the present
methods. Thus this renders the aqueous phase suitable as a fermentation medium (e.g. together with empty fruit bunches), e.g. for the production of bioethanol. Thus again increasing the economy of the process.

In some embodiments, the water in the aqueous phase may be recycled back into the process before the clarification tank. Thus, one advantage of using the present method is to reduce the amount of POME in the processing of palm fruit.

The present method taught herein further relates to use of a thermostable enzyme, e.g. a thermostable enzyme in palm fruit processing. The thermostable enzyme is able to function at the high temperatures used in palm fruit processing. This allows the enzymatic solution to be directly applied in current processes with minimal investment in additional or new equipment (for example, a low temperature incubation tank) or disruption of existing technologies. In particular, the use of thermostable enzymes has the advantage that the palm fruit/palm fruit extract does not need to be cooled post-high temperature processing (e.g. sterilisation of the fruit bunch for instance) in order for the enzymes to function. The thermostable enzyme can be used directly in high-temperature environments, e.g. in the digester. This has the advantage that cooling (and often additional reheating thereafter) can be avoided in the process. Cooling (and additional reheating) is generally undesirable as it leads to a less efficient process and/or additional energy consumption. For example, such cooling may be mediated by addition of chilled water. This can lead to downstream purification problems because more water must be separated from the oil, and this excess water must be treated as industrial effluent ("POME" = palm oil mill effluent, Industrial Processes & The Environment (Handbook No.3) Crude Palm Oil Industry: Department of Environment, Ministry of Science, Technology, and the Environment, Malaysia, (1999), Mohd. Ishak Thani, Rahani Hussin, Wan Ramlah Bt. Wan Ibrahim, and Mohd Sanusi Sulaiman (editorial staff)). In addition cooling and reheating steps can actually prolong the overall time that pressed palm oil extract is exposed to higher temperatures, which can have an adverse effect on oil quality due to increased rates of free fatty acid formation at higher temperatures. Suitably the crude palm oil produced in accordance with the present methods, particularly when a thermostable enzyme is used, may have a low concentration of free fatty acids. In addition or alternatively the use of cooling and reheating (e.g. oscillating temperatures) may also result in degraded (e.g. oxidised) crude palm oil. This may be avoided by using the present methods.

The method and/or uses taught herein have one or more further advantages over prior art processes including:

- Enhanced oil yield from the sludge post clarification and/or decanting;
Reduced sludge formation - which improves the throughput of the plant and enhances the oil extraction rate;

Improved separation of the crude palm oil from the sludge;

The ability to run the clarifier or decanter at a lower temperature thus giving reduced energy consumption for the process;

Provision of a more environmentally friendly process (e.g. due to increased yields fewer hectares of land are required for plantations);

Reduced loss of oil to the fibre extract during processing; and/or

Reduced viscosity of the pressed palm fruit extract, thus reducing the requirement for water to be added. Thus the present methods can lead to a process which consumes less water.

The use of the enzymes in accordance with the present methods has surprisingly been found to be a very effective and efficient, as well as environmentally friendly, way of significantly improving crude palm oil yields from palm fruit or a portion thereof or palm fruit extracts (such as pressed palm fruit extracts).

A further advantage is that oil obtainable (e.g. obtained) from the present methods and uses separates more easily and faster from the pressed palm liquid thus reducing the amount of oil in the waste stream. This advantageously allows production capacity to be increased. Another advantage is that the sludge contains less oil so that the remaining oil can be more easily separated in the separator.

In addition, conventional industry practice for the production of crude palm oil requires very large volumes of water. Some of this is converted in to steam for the initial treatment step. A large amount of water is also added to the pressed fresh palm fruit (typically a 15-50% water addition, giving a standard dilution of approximately 40% oil) to decrease the viscosity of the liquid, to assist in the separation of the crude palm oil from the heavier sludge fraction. The present methods reduce the volume of water required for the process and lowering the environmental impact of the process.

DESCRIPTION OF PREFERRED EMBODIMENTS

In a first embodiment, a method of improving palm oil yields is provided, which method comprises:

a) admixing an enzyme composition comprising cellulase activity with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed comprises at most 40% water content w/w;
b) incubating the admixture for between 0.5 to 8 hours (preferably 4-8 hours) at a
temperature of between 50 to 95°C; and

c) separating the oil from other components of the admixture.

In a second embodiment, a use of an enzyme composition is provided comprising
cellulase activity in combination with a palm fruit or a portion thereof or a palm fruit liquid,
wherein said palm fruit or portion thereof or palm fruit liquid when admixed with the enzyme
composition comprises at most 40% water content w/w in the manufacture of palm oil for
improving palm oil yield, for improving separation of palm oil from the sludge phase, for
producing an aqueous phase with improved fermentable sugars, or for improving the speed
of oil separation.

In a third embodiment, a method is provided according to the first preferred
embodiment or second preferred embodiment wherein said palm fruit or portion thereof or
palm fruit liquid when admixed comprises at most 38% water content w/w, suitably at most
35% water content w/w, suitably at most 30% water content w/w.

In a fourth embodiment, a method is provided according to any one of the previous
preferred embodiments wherein the palm fruit liquid is a pressed palm fruit liquid.

In a fifth embodiment, a method is provided according to any one of the preceding
preferred embodiments wherein the oil is separated by gravity (e.g. by clarification).

In a sixth embodiment, a method is provided according to any one of the preceding
preferred embodiments wherein the oil is separated by centrifugation either instead of
gravitation separation or in addition to separation by gravity.

In a seventh embodiment, a method is provided according to the sixth preferred
embodiment wherein the separation (e.g. centrifugation) results in a triple phase composition
comprising an oil layer, an aqueous layer and a substantially solid sediment (or sludge) layer.

In an eighth embodiment, a method is provided according to the seventh preferred
embodiment wherein the aqueous phase is enriched in soluble fermentable sugars.

In a ninth embodiment, a method is provided according to any one of the preceding
preferred embodiments wherein said enzyme composition in addition to comprising cellulase
activity comprises one or more of the following enzyme activities: mannanase and pectinase.

In a tenth embodiment, a method is provided according to any one of the preceding
preferred embodiments wherein said enzyme composition comprises low or no protease.

In an eleventh embodiment, a method is provided according to any one of the
preceding preferred embodiments wherein the enzyme composition is a crude or purified
extract of a *Trichoderma reesei* fermentate.
In a twelfth embodiment, a method is provided according to any one of the preceding preferred embodiments wherein there is no or only minimal agitation during the incubation stage.

In a thirteenth embodiment, a method is provided according to any one of the preceding preferred embodiments wherein the cellulase in one which reduces the amount of dry sediment (sludge dry matter) by at least 20% when 25000 CMC-DNS/kg substrate is added to a palm fruit, a portion thereof or a palm fruit extract and incubated for 1 hr at 50°C.

In a fourteenth embodiment, a method is provided according to any one of the ninth, tenth, eleventh, twelfth, and thirteenth preferred embodiments wherein when the enzyme composition comprises mannanase, the enzyme composition comprises a minimum level of mannanase activity which when added to the substrate give a mannanase concentration of at least 200 MVR/kg substrate.

In a fifteenth embodiment, a palm oil phase, a sludge phase or an aqueous phase obtainable by the method according to any one of the preceding preferred embodiments.

In a sixteenth embodiment, a use of an aqueous phase obtained by the method according to any one of the preceding preferred embodiments as a fermentation medium is also provided.

In a seventeenth embodiment, a method is provided for producing a fermentation product comprising fermenting a feedstock comprising said aqueous phase obtainable (preferably obtained) by the method according to any one of the preceding preferred embodiments and recovering said fermentation product.

In an eighteenth embodiment, a method is provided according to the seventeenth preferred embodiment wherein said feedstock further comprises empty palm fruit bunches or processed empty palm fruit bunches.

In a nineteenth embodiment, a method is provided according to the seventeenth or eighteenth preferred embodiments wherein said fermentation product is a compound selected from the group consisting of: an alcohol, an organic acid, an antibiotic, an antimicrobial, bioinsecticide, a solvent, and a polyhydroxyalkanoate.

In a twentieth embodiment, a method is provided according to any one of the seventeenth, eighteenth or nineteenth preferred embodiments wherein the feedstock is subjected to one or more processing steps selected from the group consisting of: milling, cooking and/or saccharification.

In a twenty-first embodiment, a method provided according to the nineteenth or twentieth preferred embodiments wherein the alcohol is a biofuel (e.g. an ethanol, a butanol or a combination thereof).
EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only and should not be considered to limit the scope of the claims. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

The meaning of abbreviations is as follows: "sec" or "s" means second(s), "ms" mean milliseconds, "min" means minute(s), "h" or "hr" or "hrs" means hour(s), "µl." means microliter(s), "mL" means milliliter(s), "L" means liter(s); "mL/min" is milliliters per minute; "µg/mL" is microgram(s) per milliliter(s); "LB" is Luria broth; "µm" is micrometers, "nm" is nanometers; "OD" is optical density; "IPTG" is isopropyl-β-D-thio-galactoside; "g" is gravitational force; "mM" is millimolar; "SDS-PAGE" is sodium dodecyl sulfate polyacrylamide; "mg/mL" is milligrams per milliliters.

General Methods


EXAMPLE 1

IMPROVED PROCESSES TO REDUCE THE WASTE OF PALM OIL MATERIALS

Pressed palm liquid

Enzymes

LAMINEX® 750, (DuPont Industrial Biosciences) A03193G190 batch 4861668300, 2037
CMC-DNS/g, 20 MVU/g. LAMINEX® 750 is a commercial preparation of an enzyme
composition comprising: water 85 - 90 % (w/w); endo-1,3(4)-beta-glucanase 10 - 15 % (w/w),
and sodium benzoate 0.25 % (w/w).

LAMINEX® BG2, batch no, 4902043555 (DuPont Industrial Biosciences), 10166 CMC-
DNS/g, 185 MVU/g. LAMINEX® BG2 is a commercial preparation of an enzyme complex
capable of hydrolyzing beta-glucans and related carbohydrates. It is produced by
fermentation with a selected strain of Trichoderma reesei. LAMINEX® BG2 comprises: water
59 - 71 % (w/w); cellulase 15 - 20 % (w/w); sorbitol 10.0 - 15.0 % (w/w); sodium chloride 4.0
- 5.0 % (w/w); and sodium benzoate 0.00 - 0.50 % (w/w).

LAMINEX® Super 3G (DuPont Industrial Biosciences). LAMINEX® Super 3G is a
commercial preparation of enzyme complex capable of hydrolyzing beta-glucans, pentosans
and related carbohydrates. LAMINEX® Super 3G is produced by fermentation with selected
strains of Trichoderma reesei and Penicillium funiculosum. LAMINEX® Super 3G comprises:
water 75.8 - 80.8 % (w/w); cellulase 15 - 20 % (w/w); sorbitol 6.50 % (w/w); sodium chloride
2.20 % (w/w); and sodium benzoate 0.50 % (w/w).

LAMINEX® C2K (DuPont Industrial Biosciences). LAMINEX® C2K is a commercial
preparation comprising a cellulase enzyme composition (EC 3.2.1.4) derived from Penicillium
funiculosum containing other minor enzyme activities. These include beta-glucanase (EC
3.2.1.6), xylanase (EC 3.2.1.8), and glucan endo-1,3-B-D-glucosidase (EC3.2.1.39).

Experimental

Pressed Palm Liquid and enzyme was incubated with magnetic stirring at 50 °C or 60 °C for
0.5 or 4 hours. After incubation the sample was placed in at waterbath at 95 degrees for 10
minutes to stop the enzyme reaction, and transferred to a tarred 50 mL centrifuge tube. The
upper oil layer was removed, and a sample of the water phase was taken out for glucose
analyses and remaining water phase was discharged. 30 mL water at 50 °C was added to
each tube. The sample was centrifuged at 4180 x g and 60 °C for 10 minutes.

The water phase was removed and the side of the tube was wiped with a tissue to remove
residual oil on the inside of the tube. The wet sediment was scaled, frozen and freeze dried.
Weight of the dry sediment was determined after freeze drying. The dry sediment was ground for 25 sec at 25000rpm in IKA Tube-mill. 150 mg ground sediment was scaled in a 15 centrifuge tube and added 7.5 ml heptane:isopropanol (IPA) 3:2. The mixture was heated to 40 °C for 10 minutes and mixed for 30 minutes at Stuart rotator SB2.

The mixture was centrifuged for 3 min at 1280 rcf. 1 mL of the supernatant was transferred to TLC vials.

The triglyceride in the sample was calculated based on a calibration curve for palm oil analyzed in the same way.

TLC:
HPTLC analysis of triglycerides.

1.0 µl of the sample was applied to the 10 x 20 cm Silica HPTLC plate. Standard solutions of 0.5% Palm Oil (0.2, 0.5, 0.8, 1.2, 1.8 and 2.5 respectively) were also applied to the HPTLC plate by an automatic TLC applicator. The plate was eluted (7cm) with Running buffer: Heptane: Methyl-Tert-Butyl-ether (MTBE): Acetic acid 70:30:1. After elution the plate was dried on a plate heater for 2 minutes at 160 °C, cooled, and dipped in 6% cupric acetate in 16% H subscript 3 PO subscript 4. The plate was additionally dried for 6 minutes at 160 °C. The developed spots were quantified using a TLC plate scanner and components quantified based on calibration curves of the palm oil standard.

HPLC analysis of glucose in water phase.

Glucose in the supernatant from the centrifuged sludge was analyzed by HPLC using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) equipped with a DGP-3600SD Dual-Gradient analytical pump, WPS-3000TSL thermostat autosampler, TCC-3000SD thermostat column oven, and a RI-101 refractive index detector (Shodex, JM Science). Chromeleon datasytem software (Version 6.80, DU10A Build 2826, 171948) was used for data acquisition and analysis. The column used was a RSO oligosaccharide column, Ag+ 4% cross-linked (Phenomenex, The Netherlands) equipped with an analytical guard column (Carbo-Ag + neutral, AJO-4491, Phenomenex, The Netherlands) at 70 °C. The column was eluted with double distilled water (filtered through a regenerated cellulose membrane of 0.45 µm and purged with helium gas) at a flow rate of 0.3 mL/min. Glucose was quantified by construction of a calibration curve from analysis of D-(+)-glucose (min 99.5%, Sigma no G8270-100G).
To determine the effect of enzyme dosage, reaction time and impact of agitation the enzymes were tested in the following recipes (Table 1).

Table 1. Dosage, reaction time and impact of agitation the enzymes recipes,

<table>
<thead>
<tr>
<th>Pressed liquid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palm 3</td>
<td>g</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>LAMINEX® BG2</td>
<td>mL</td>
<td>0</td>
<td>0.03</td>
<td>0.05</td>
<td>0.13</td>
<td>0.25</td>
<td>0.25</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMINEX® 750</td>
<td>mL</td>
<td></td>
<td>0.05</td>
<td>0.13</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>mL</td>
<td>0.5</td>
<td>0.48</td>
<td>0.45</td>
<td>0.38</td>
<td>0.25</td>
<td>0.45</td>
<td>0.38</td>
<td>0.25</td>
<td>0</td>
<td>0.25</td>
</tr>
</tbody>
</table>

| Enzyme dos. v/w | % | 0 | 0.05| 0.1| 0.25| 0.5 | 0.5 | 1 | 0.5 | 0 |
| Reaction time, Hr | hr | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Temperature °C   | 60| 60| 60| 60| 60| 60| 60| 60| 60| 60 |
| Agitation        | + | + | + | + | + | + | + | + | + | - |

The experiment was conducted according to the procedure mentioned above.

After separation the pressed liquid was separated into 3 phases as indicated in Figure 2.

The effect of the enzyme on pressed palm liquid sediment was analysed with results shown in Table 2.

Table 2. Effect of LAMINEX® BG2 and LAMINEX® 750 on oil in sediment, sediment dry matter, water in wet sediment and % glucose in the water phase.

<table>
<thead>
<tr>
<th></th>
<th>Dosage, % v/w</th>
<th>Agitation</th>
<th>% Oil in sediment, based on sample</th>
<th>% Oil in Sediment dry matter</th>
<th>% water in wet sediment</th>
<th>% glucose in water phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMINEX® BG2</td>
<td>0</td>
<td>+</td>
<td>1,16</td>
<td>3,55</td>
<td>79,6</td>
<td>0,375</td>
</tr>
<tr>
<td>LAMINEX® 750</td>
<td>0,05</td>
<td>+</td>
<td>0,34</td>
<td>1,97</td>
<td>73,9</td>
<td>0,847</td>
</tr>
</tbody>
</table>
The results in Table 2 confirmed that LAMINEX® BG2 has strong effect on reduction of oil in the sediment after centrifugation. This effect is probably linked to the effect of LAMINEX® BG2 on solubilisation of approximate 50% of the sediment dry matter. The results also confirm that LAMINEX® BG2 works at 60°C, and it is possible to reduce the enzyme dosage by extending the reaction time to 4 hours.

The experiment also confirms that LAMINEX® BG2 works on the same level without agitation as with agitation, which is important for use of the enzyme in a clarifier where oil is separated from the sludge by gravity and without any agitation. Water content in the wet sediment is reduced by the enzyme treatment. This indicate that the water holding capacity is
reduced because the carbohydrate polymer are degraded to smaller molecules, which has less water binding.

EXAMPLE 2

OTHER ENZYME PREPARATIONS LAMINEX® SUPER 3G AND LAMINEX® C2K TESTED IN PRESSED PALM LIQUID

Other enzyme preparations LAMINEX® Super 3G and LAMINEX® C2K were tested in Pressed Palm Liquid according the recipe in Table 3.

Table 3. Enzyme Preparation Recipes of LAMINEX® Super 3G and LAMINEX® C2K tested in Pressed Palm Liquid.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palm 3, Pressed Palm liquid</td>
<td>g</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>LAMINEX® Super 3G</td>
<td>mL</td>
<td>0</td>
<td>0.025</td>
<td>0.05</td>
<td>0.125</td>
<td>0.25</td>
<td>0.05</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>LAMINEX® C2K</td>
<td>mL</td>
<td>0.5</td>
<td>0.475</td>
<td>0.45</td>
<td>0.375</td>
<td>0.25</td>
<td>0.45</td>
<td>0.375</td>
<td>0.25</td>
</tr>
<tr>
<td>Water</td>
<td>mL</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

The experiment was conducted according to the procedure mentioned above.

The effect of the enzyme on pressed palm liquid was analysed with results shown in Table 4.

Table 4. Effect of LAMINEX® Super 3G and LAMINEX® C2K on oil in sediment, sediment dry matter, water in wet sediment and glucose in the water phase.

<table>
<thead>
<tr>
<th></th>
<th>Dosage, v/w</th>
<th>% Oil in sediment, based on sample</th>
<th>% water in wet sediment</th>
<th>% Sediment dry matter</th>
<th>% glucose in water phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMINEX® Super 3G</td>
<td>0</td>
<td>1.16</td>
<td>78.45</td>
<td>3.54</td>
<td>0.403</td>
</tr>
</tbody>
</table>
The results in Table 4 confirm that LAMINEX® Super 3G has almost the same effect on the oil in sediment and sediment dry matter as LAMINEX® BG2, but higher dosage is needed to obtain the same effect.

LAMINEX® C2K is much less efficient than LAMINEX® BG2 and LAMINEX® Super 3G and the results indicate that this enzyme is more similar to LAMINEX® 750 with regard to effect on pressed palm liquid.

The results from Table 2 and 4 are illustrated graphically in Figure 3, Figure 4, Figure 5 and Figure 6.

CONCLUSION

Four different enzyme preparations LAMINEX® BG2, LAMINEX® Super 3G, LAMINEX® 750 and LAMINEX® C2K were tested in pressed palm liquid. After enzyme treatment the pressed palm liquid was separated into an oil phase, a water phase and sediment. Residual oil in sediment was analysed, which showed that LAMINEX® BG2 and LAMINEX® Super 3G contributed to a strong decrease in residual oil in sediment, and also the amount of sediment
was reduced because of solubilisation of insoluble carbohydrates. This was confirmed by a significant increase in amount of glucose in the water phase. LAMINEX® 750 has very little effect on release of oil in sediment and only small amount of glucose was produced. LAMINEX® C2K had similar performance as LAMINEX® 750.

**EXAMPLE 3**

**EFFECT LAMINEX® BG2 AND WATER DILUTION ON VISCOSITY AND OIL SEPARATION OF PRESSED PALM LIQUID**

Pressed palm liquid obtained by pressing the digested palm fruitlets is a rather viscous liquid. It is therefore normal procedure to dilute the pressed palm liquid with water before going to the clarifier in order to reduce viscosity.

The purpose of this study was to examine the effect on viscosity of enzyme treatment with LAMINEX® BG2 as well as water dilution. The impact on oil separation of LAMINEX® BG2 and water dilution was also investigated.

The effect on solubilisation of insoluble dry matter and amount of bound oil in the sediment from pressed palm liquid was also tested.

In this experiment three different pressed palm liquid were tested in order to study variations in raw material with different water content.

**Material**

- PALM_2 Pressed Palm Liquid, 24.3% water
- PALM_1 Pressed Palm Liquid, 32% water
- PALM_8 Pressed Palm Liquid, 35.6% water

**Enzyme** LAMINEX® BG2, batch no. 4902043555 (DuPont Industrial Biosciences), 10166 CMC-DNS/g, 185 MVU/g

**Experimental:**

- 30 gram Pressed Palm Liquid or 30 g diluted Pressed Palm Liquid and enzyme was incubated with magnetic stirring at 50 °C for 1 hour in an Alum cup fitted for Rapid Visco Analyzer.

After incubation the viscosity of the sample was immediately measured using a Rapid Visco Analyzer using the following program, Agitation 500 rpm, temperature setting 60 °C for 5 minutes, increase temperature to 70 °C, keep 70 °C for 4 minutes, increase temperature to
80 °C and keep 80 °C for 4 minutes, increase the temperature to 90 °C and keep 90 °C for 4 minutes. Cool down to 50 °C for 2 minutes.

The viscosity was calculated as the average of measurements for 1 minute at each temperature.

The sample was then transferred to a 50-mL centrifuge tube and heated to 70 °C for 10 minutes, and then turned upside down 10 times. The oil separation of the sample was followed visually for 10 minutes. The oil separation for the sample was measured as the volume % of upper oil layer after 10 minutes.

The sample was then centrifuged at 4180 rcf for 10 minutes at 60 °C. The upper oil layer and water was removed.

30 mL water at 50 °C was added to each tube without mixing. The sample was centrifuged at 4180 rcf for 10 minutes at 60 °C.

The water phase was removed and the inside of the tube was wiped with a tissue to remove residual oil. Weight of the wet sediment was determined. The wet sediment was scaled, frozen and freeze dried.

Weight of the dry sediment was determined after freeze drying.

The dry sediment was grounded for 25 sec at 25000rpm in IKA Tube-mill.

120 mg grounded sediment was scaled in a 15 centrifuge tube and added 7,5 mL Heptane: Isopropanol 3:2. The mixture was heated to 40°C for 10 minutes and mixed for 45 minutes at Stuart rotator SB2. The mixture was centrifuged for 3 min at 1280 rcf. 1 mL of the supernatant was transferred to TLC vials. The triglyceride in the sample was calculated based on a calibration curve for palm oil analyzed in the same way.

HPTLC analysis of triglycerides.

1,0 μL of the sample was applied to the 10 x 20 cm Silica HPTLC plate.

Standard solutions of 0.5% Palm Oil (0.2, 0.5, 0.8, 1.2, 1.8 and 2.5 respectively) were also applied to the HPTLC plate by an automatic TLC applicator. The plate was eluted (7cm) with Running buffer: Heptane: MTBE: Acetic acid 70:30:1 . After elution the plate was dried on a plate heater for 2 minutes at 160 °C, cooled, and dipped in 6% cupric acetate in 16% H₃PO₄.
The plate was additionally dried for 6 minutes at 160 °C. The developed spots were quantified using a TLC plate scanner and components quantified based on calibration curves of the palm oil standard.

5 Results

EXAMPLE 3A
VISCOSITY OF PRESSED PALM LIQUID PALM_2 TESTED WITH AND WITHOUT ENZYME TREATMENT AND WITH DIFFERENT WATER DILUTION

Viscosity of Pressed palm liquid PALM_2 was tested with and without enzyme treatment and with different water dilution as shown in Table 5.

Table 5.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PALM_2, Pressed</td>
<td>g</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>24</td>
<td>21</td>
<td>21</td>
<td>18</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>palm liquid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>mL</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>9</td>
<td>12</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>LAMINEX® BG 2</td>
<td>mL</td>
<td>0,15</td>
<td>0,15</td>
<td>0,15</td>
<td>0,15</td>
<td>0,15</td>
<td>0,15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water added (%)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>30</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>water in sample</td>
<td>%</td>
<td>24,3</td>
<td>24,3</td>
<td>39,4</td>
<td>39,4</td>
<td>47,0</td>
<td>47,0</td>
<td>54,6</td>
<td>54,6</td>
<td>62,2</td>
</tr>
<tr>
<td>LAMINEX® BG 2</td>
<td>%</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reaction time</td>
<td>hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>React. Temperature</td>
<td>°C</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

The experiment was conducted according to the procedure mentioned under experimental of Example 3 and the results from viscosity measurement in shown in Table 6 and graphically illustrated in Figure 7 and Figure 8.

Table 6. Effect of LAMINEX® BG2 and water dilution on viscosity of pressed palm liquid (PALM_2) treated according to Table 5.
The effect of LAMINEX® BG2 and water dilution on sediment dry matter, wet sediment and oil in sediment are provided in Table 6a below:

Table 6a. Effect of LAMINEX® BG2 and water dilution on sediment dry matter, wet sediment and oil in sediment.

<table>
<thead>
<tr>
<th>% water added</th>
<th>LAMINEX® BG2</th>
<th>Drymatter</th>
<th>Wet Sediment</th>
<th>Oil in sediment based on sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>7,27</td>
<td>27,3</td>
<td>2,44</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2,98</td>
<td>8,09</td>
<td>0,44</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>7,03</td>
<td>31,9</td>
<td>3,33</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2,71</td>
<td>7,96</td>
<td>0,59</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>6,36</td>
<td>32,8</td>
<td>3,17</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2,49</td>
<td>7,52</td>
<td>0,60</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>5,52</td>
<td>31,0</td>
<td>2,35</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>2,63</td>
<td>7,50</td>
<td>0,78</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4,39</td>
<td>26,5</td>
<td>1,72</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2,39</td>
<td>7,41</td>
<td>0,68</td>
<td></td>
</tr>
</tbody>
</table>

The oil separation of the control samples and the enzyme treated samples were followed visually over 10 minutes, and it was observed that enzyme treatment had a strong improvement on oil separation (Figure 9) It is however also observed that by water dilution
the enzyme treated samples had much less oil separation. The control samples behaved differently as increased water dilution improved oil separation up to 55% water. At higher amount of water added the oil separation also decreased in the control sample.

EXAMPLE 3B

PRESSED PALM LIQUID PALM_1 CONTAINING 32% WATER TESTED WITH DIFFERENT WATER DILUTION

Another pressed palm liquid PALM_1 containing 32% water was tested according to the same procedure with different water dilution as shown in Table 7.

Table 7. Pressed palm liquid PALM_1 containing 32% water tested with different water dilution.

<table>
<thead>
<tr>
<th>Palm _1, Pressed palm liquid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>mL</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Water added</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Water % in sample</td>
<td>%</td>
<td>32.0</td>
<td>32.0</td>
<td>45.6</td>
<td>45.6</td>
<td>52.4</td>
<td>52.4</td>
<td>59.2</td>
<td>59.2</td>
<td>66.0</td>
</tr>
<tr>
<td>LAMINEX® BG 2</td>
<td>%</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Reaction time</td>
<td>hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>React. temperature</td>
<td>°C</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

The experiment was conducted according to the procedure mentioned under example 3 and the results from viscosity measurement and oil separation is shown in Table 8 and illustrated in Figure 10 and Figure 11.

Table 8. Effect of LAMINEX® BG2 and water dilution on viscosity and oil separation of pressed liquid treated according to Table 7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LAMINEX® BG2</th>
<th>Water</th>
<th>Visc 60°C</th>
<th>Visc 70°C</th>
<th>Visc 80°C</th>
<th>Visc 90°C</th>
<th>Oil separation after 10 min, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0,5</td>
<td>32</td>
<td>239</td>
<td>213</td>
<td>195</td>
<td>185</td>
<td>63,3</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>32</td>
<td>130</td>
<td>117</td>
<td>109</td>
<td>100</td>
<td>7,4</td>
</tr>
</tbody>
</table>

46
The effect of LAMINEX® BG2 and water dilution on sediment dry matter, wet sediment and oil in sediment is shown Table 8a below:

Table 8a. Effect of LAMINEX® BG2 and water dilution on sediment dry matter, wet sediment and oil in sediment.

<table>
<thead>
<tr>
<th>Water added</th>
<th>LAMINEX® BG2</th>
<th>Drymatter</th>
<th>Wet Sediment</th>
<th>Oil in sediment based on sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>8,53</td>
<td>33,11</td>
<td>1,65</td>
</tr>
<tr>
<td>0</td>
<td>0,5</td>
<td>3,46</td>
<td>10,54</td>
<td>0,31</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>8,07</td>
<td>37,02</td>
<td>2,64</td>
</tr>
<tr>
<td>20</td>
<td>0,5</td>
<td>3,06</td>
<td>10,03</td>
<td>0,38</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>6,99</td>
<td>36,51</td>
<td>2,19</td>
</tr>
<tr>
<td>30</td>
<td>0,5</td>
<td>2,97</td>
<td>9,83</td>
<td>0,45</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>5,72</td>
<td>33,17</td>
<td>1,50</td>
</tr>
<tr>
<td>40</td>
<td>0,5</td>
<td>3,01</td>
<td>10,3</td>
<td>0,51</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>4,91</td>
<td>31,84</td>
<td>1,06</td>
</tr>
<tr>
<td>50</td>
<td>0,5</td>
<td>2,72</td>
<td>9,92</td>
<td>0,46</td>
</tr>
</tbody>
</table>

The oil separation of the control samples and the enzyme treated samples were then followed visually over 10 minutes, and it was observed that enzyme treatment had a strong improvement on oil separation (Figure 12) it is however also observed that by water dilution the enzyme treated samples had much less oil separation. The control samples behaved differently as increased water dilution improved oil separation up to 58% water. At higher amount of water added the oil separation also decreased in the control samples.
EXAMPLE 3C
THIRD PRESSED PALM LIQUID PALM_8 CONTAINING 35% WATER TESTED WITH DIFFERENT WATER DILUTION

A third pressed palm liquid PALM_8 containing 35% water was tested with different water dilution as shown in Table 9.

Table 9. Third pressed palm liquid PALM_8 containing 35% water tested with different water dilution.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palm_8, Pressed</td>
<td>g</td>
<td>30</td>
<td>30</td>
<td>26</td>
<td>26</td>
<td>22,5</td>
<td>22,5</td>
<td>19,5</td>
<td>19,5</td>
<td>16</td>
</tr>
<tr>
<td>palm liquid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>mL</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>7,5</td>
<td>7,5</td>
<td>10,5</td>
<td>10,5</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMINEX® BG 2</td>
<td>mL</td>
<td>0,15</td>
<td>0,15</td>
<td></td>
<td></td>
<td>0,15</td>
<td>0,15</td>
<td></td>
<td></td>
<td>0,15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water added</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>30</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water in sample</td>
<td>%</td>
<td>35,0</td>
<td>35,0</td>
<td>43,7</td>
<td>43,7</td>
<td>51,3</td>
<td>51,3</td>
<td>57,8</td>
<td>57,8</td>
<td>65,3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMINEX® BG 2</td>
<td>%</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td>0,5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reaction time</td>
<td>hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>React. temperature</td>
<td>°C</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

The experiment was conducted according to the procedure mentioned under experimental and the results form viscosity measurement and oil separation is shown in Table 10 and illustrated in Figure 13 and Figure 14.
Table 10. Effect of LAMINEX® BG2 and water dilution on viscosity of pressed liquid treated according to Table 9

<table>
<thead>
<tr>
<th>Sample</th>
<th>LAMINEX® BG2</th>
<th>Water</th>
<th>Visc 60°C</th>
<th>Visc 70°C</th>
<th>Visc 80°C</th>
<th>Visc 90°C</th>
<th>Oil separation after 10 min., %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>35,0</td>
<td>271</td>
<td>244</td>
<td>231</td>
<td>205</td>
<td>33,3</td>
</tr>
<tr>
<td>2</td>
<td>0,5</td>
<td>35,0</td>
<td>136</td>
<td>125</td>
<td>117</td>
<td>103</td>
<td>66,7</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>43,7</td>
<td>178</td>
<td>161</td>
<td>147</td>
<td>132</td>
<td>50,0</td>
</tr>
<tr>
<td>4</td>
<td>0,5</td>
<td>43,7</td>
<td>153</td>
<td>141</td>
<td>127</td>
<td>116</td>
<td>56,7</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>51,3</td>
<td>169</td>
<td>151</td>
<td>143</td>
<td>131</td>
<td>43,3</td>
</tr>
<tr>
<td>6</td>
<td>0,5</td>
<td>51,3</td>
<td>99</td>
<td>98</td>
<td>97</td>
<td>89</td>
<td>10,0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>57,8</td>
<td>118</td>
<td>116</td>
<td>112</td>
<td>102</td>
<td>10,3</td>
</tr>
<tr>
<td>8</td>
<td>0,5</td>
<td>57,8</td>
<td>81</td>
<td>80</td>
<td>77</td>
<td>75</td>
<td>10,0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>65,3</td>
<td>85</td>
<td>85</td>
<td>78</td>
<td>74</td>
<td>17,2</td>
</tr>
<tr>
<td>10</td>
<td>0,5</td>
<td>65,3</td>
<td>63</td>
<td>66</td>
<td>64</td>
<td>59</td>
<td>13,3</td>
</tr>
</tbody>
</table>

The effect of LAMINEX® BG2 and water dilution on sediment dry matter, wet sediment and oil in sediment is show in Table 10a below:

Table 10a. Effect of LAMINEX® BG2 and water dilution on sediment dry matter, wet sediment and oil in sediment.

<table>
<thead>
<tr>
<th>Water added</th>
<th>LAMINEX® BG2</th>
<th>Drymatter</th>
<th>Wet Sediment</th>
<th>Oil in sediment based on sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>5,80</td>
<td>24,17</td>
<td>1,96</td>
<td></td>
</tr>
<tr>
<td>0,5</td>
<td>3,55</td>
<td>12,64</td>
<td>0,48</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>4,60</td>
<td>21,33</td>
<td>2,55</td>
<td></td>
</tr>
<tr>
<td>0,5</td>
<td>3,10</td>
<td>12,17</td>
<td>0,61</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>4,18</td>
<td>23,27</td>
<td>2,11</td>
<td></td>
</tr>
<tr>
<td>0,5</td>
<td>3,01</td>
<td>11,95</td>
<td>0,79</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>3,73</td>
<td>20,75</td>
<td>1,78</td>
<td></td>
</tr>
<tr>
<td>0,5</td>
<td>2,96</td>
<td>12,08</td>
<td>0,91</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4,09</td>
<td>21,06</td>
<td>1,67</td>
<td></td>
</tr>
<tr>
<td>0,5</td>
<td>2,91</td>
<td>11,73</td>
<td>0,77</td>
<td></td>
</tr>
</tbody>
</table>

The oil separation of the control samples and the enzyme treated samples were then followed visually over 10 minutes, and it was observed that enzyme treatment had a strong improvement on oil separation (Figure 15). Dilution with 20% water also gave good oil
separation of enzyme treated sample but less than undiluted sample. At higher water dilution the oil separation decreased for the enzyme treated samples. The control samples behaved differently as increased water dilution improved oil separation up to 51% water. At higher amount of water added, the oil separation also decreased in the control samples.

EXAMPLE 4
EFFECT OF MANNASTAR 375, PECTINASE FE, PROTEX 14L COMBINED WITH LAMINEX BG2 ON PRESSED PALM LIQUID

Effect of LAMINEX® BG2 combined with MULTIFECT® pectinase FE, MANNASTAR® 375 or PROTEX® 14L on pressed palm liquid was investigated by enzyme incubation for 4 hr at 50°C.

LAMINEX BG2 had a clear effect on solubilization of insoluble dry matter in pressed liquid, but also MANNASTAR® 375 and MULTIFECT® pectinase FE improved the solubilisation of insoluble dry matter.

The amount of oil left in the dry matter is reduced by the enzyme treatment and a very clear additive effect of MANNASTAR® and MULTIFECT® pectinase FE in combination with LAMINEX® BG2 is seen.

Protease - PROTEX® 14L had a negative effect on oil release form insoluble dry matter.

Studies have shown that LAMINEX® BG2 is able to solubilize 50% or more of the insoluble substrate in pressed palm liquid. Together with this solubilization it has been observed that amount of bound oil in the sediment is reduced by more than 60%. Studies have also shown that other cellulose degrading enzymes may not be as efficient as LAMINEX® BG2. In order to investigate this in further detail experiments with a combination of LAMINEX® BG2 and MANNASTAR® 375 was set up. MANNASTAR® 375 is a mannanase preparation obtained by fermentation of Trichoderma reesei (DuPont Industrial Biosciences). In this study combination of LAMINEX® BG2 with MULTIFECT® Pectinase FE was also investigated.

Material

Pressed Palm Liquid (PALM_2), May 2014. 24.3% water.

Enzyme

LAMINEX® BG2, batch no. 4902043555 (DuPont Industrial Biosciences), 10166 CMC-DNS/g, 185 MVU/g
Mannanase, MANNASTAR® 375 batch no. 4881561660. MANNASTAR® 375 is a commercial preparation comprising mannanase activity (Danisco USA).

Pectinase, MULTIFECT® pectinase FE batch no. 4861512246. MULTIFECT® Pectinase FE (DuPont Industrial Biosciences) is a commercial preparation of a pectinase complex derived from a strain of *Aspergillus niger*.

Protease, PROTEX® 14L, batch no. 4861905794. PROTEX® 14L is a commercial protease preparation comprising a protease from *Geobacillus* sp. (DuPont Industrial Biosciences).

Experimental:

Pressed Palm Liquid and enzyme was incubated with magnetic stirring at 50 °C 4 hours. After incubation the sample was transferred to a tarred 50 mL centrifuge tube and placed in a water bath at 95 degrees for 10 minutes to stop the enzyme reaction.

Picture of the samples were taken and the amount of oil phase was measured.

The sample was centrifuged at 4180 rcf and 60 °C for 10 minutes.

The upper oil layer was removed, and a sample of the water phase was taken out for glucose analyses and remaining water phase was discharged. 30 mL water at 50 °C was added to each tube without mixing. The sample was centrifuged at 4180 rcf and 60 °C for 10 minutes.

The water phase was removed and the inside of the tube was wiped with a tissue to remove residual oil and water. The wet sediment was scaled, frozen and freeze dried.

Weight of the dry sediment was determined after freeze drying.

The dry sediment was grounded for 25 sec at 25000 rpm in IKA Tube-mill. 120 mg grounded sediment was scaled in a 15 centrifuge tube and added 7,5 mL Heptane:Isopropanol 3:2. The mixture was heated to 40 °C for 10 minutes and mixed for 45 minutes at Stuart rotator SB2.

The mixture was centrifuged for 3 min at 1280 rcf. 1 mL of the supernatant was transferred to TLC vials, and triglycerides determined by TLC. The triglyceride in the sample was calculated based on a calibration curve for palm oil analyzed in the same way.

HPTLC analysis of triglycerides.
1.0 µl of the sample was applied to the 10 x 20 cm Silica HPTLC plate. Standard solutions of 0.5% Palm Oil (0.2, 0.5, 0.8, 1.2, 1.8 and 2.5 respectively) were also applied to the HPTLC plate by an automatic TLC applicator. The plate was eluted (7cm) with Running buffer: Heptane: MTBE: Acetic acid 70:30:1. After elution the plate was dried on a plate heater for 2 minutes at 160 °C, cooled, and dipped in 6% cupric acetate in 16% H₃PO₄. The plate was additionally dried for 6 minutes at 160 °C. The developed spots were quantified using a TLC plate scanner and components quantified based on calibration curves of the palm oil standard.

Results

LAMINEX® BG2 was tested in pressed palm liquid according to the recipe in table 11.

Table 11. Recipes of LAMINEX® BG2 tested in pressed palm liquid.

<table>
<thead>
<tr>
<th>Pressed Palm Liquid, (PALM_2)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMINEX® BG2</td>
<td>mL</td>
<td>0</td>
<td>0,05</td>
<td>0</td>
<td>0,05</td>
<td>0</td>
</tr>
<tr>
<td>MANNASTAR® 375 10% solution</td>
<td>mL</td>
<td>0</td>
<td>0</td>
<td>0,5</td>
<td>0,5</td>
<td>0</td>
</tr>
<tr>
<td>MULTIFECT® pectinase FE</td>
<td>mL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0,05</td>
<td>0,05</td>
</tr>
<tr>
<td>Water</td>
<td>mL</td>
<td>0,55</td>
<td>0,5</td>
<td>0,05</td>
<td>0</td>
<td>0,5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LAMINEX® BG2</th>
<th>%</th>
<th>0</th>
<th>0,1</th>
<th>0</th>
<th>0,1</th>
<th>0,1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MANNASTAR® 375</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>0,1</td>
<td>0,1</td>
<td>0</td>
</tr>
<tr>
<td>MULTIFECT® Pectinase FE</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0,1</td>
<td>0,1</td>
</tr>
<tr>
<td>reaction time</td>
<td>hr</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Temperature</td>
<td>°C</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Agitation</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The experiment was conducted according to the procedure mentioned above in Example 5.
After the enzyme reaction the samples were kept at ambient temperature for 10 minutes for the sludge to settle. As indicated in Figure 16 there is a significant effect of LAMINEX® BG2 on oil separation, but adding MANNASTAR® 375 or MULTIFECT® pectinase FE further improved the separation, and it can also be seen that the amount of entrapped oil in the sludge is less. Results from measurement of the volume of free oil and sludge are shown in Figure 17.

The samples were centrifuged and the sediment analyzed with results shown in Table 12.

Table 12. Effect of LAMINEX® BG2, MANNASTAR® 375, and MULTIFECT® pectinase FE on oil in sediment, water in wet sediment, sediment dry matter and glycose in water phase.

<table>
<thead>
<tr>
<th>LAMINEX® BG2</th>
<th>MANNASTAR® 375</th>
<th>MULTIFECT® pectinase FE</th>
<th>Oil in sediment, based on sample</th>
<th>Water in wet sediment</th>
<th>Sediment dry matter</th>
<th>% Glucose in water phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>%v/w</td>
<td>%v/w</td>
<td>%v/w</td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1,34</td>
<td>75,5</td>
<td>5,19</td>
<td>0,43</td>
</tr>
<tr>
<td>0,1</td>
<td>-</td>
<td>-</td>
<td>0,71</td>
<td>68,3</td>
<td>3,33</td>
<td>0,97</td>
</tr>
<tr>
<td>-</td>
<td>0,1</td>
<td>-</td>
<td>1,11</td>
<td>76,1</td>
<td>4,89</td>
<td>0,71</td>
</tr>
<tr>
<td>0,1</td>
<td>0,1</td>
<td>-</td>
<td>0,37</td>
<td>67,4</td>
<td>2,67</td>
<td>1,14</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0,1</td>
<td>1,20</td>
<td>73,3</td>
<td>4,62</td>
<td>1,30</td>
</tr>
<tr>
<td>0,1</td>
<td>-</td>
<td>0,1</td>
<td>0,57</td>
<td>63,1</td>
<td>2,68</td>
<td>2,30</td>
</tr>
</tbody>
</table>

The results in Table 12 confirm that LAMINEX® BG2 as well as MANNASTAR® 375 and MULTIFECT® pectinase FE has an effect on amount of dry matter as well as oil in sediment. LAMINEX® BG2 has the strongest effect on solubilization of dry matter, but it is seen that MANNASTAR® 375 and MULTIFECT® pectinase FE has a clear additive effect on top of LAMINEX® BG2. It is also observed that both MANNASTAR® 375 and MULTIFECT® pectinase FE have an effect on the amount of glucose in the water phase.

The results are illustrated in Figures 18 to 20.

The results showed that LAMINEX® BG2 had a strong impact on the solubilization of insoluble material in pressed palm liquid and also released significant amount of the bound oil in the sediment. It was however also observed that MANNASTAR® 375 and
MULTIFECT® pectinase FE had a positive effect alone and in combination with LAMINEX® BG2.

A Fungal protease PROTEX® 14L was also tested.

Table 13. Recipes with Fungal protease PROTEX® 14L

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressed liquid (PALM_2)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>LAMINEX® BG2</td>
<td>0</td>
<td>0,05</td>
<td>0</td>
<td>0,05</td>
</tr>
<tr>
<td>PROTEX® 14L</td>
<td>0,05</td>
<td>0,05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0,1</td>
<td>0,05</td>
<td>0,05</td>
<td>0</td>
</tr>
<tr>
<td>LAMINEX® BG2</td>
<td>0</td>
<td>0,1</td>
<td>0</td>
<td>0,1</td>
</tr>
<tr>
<td>PROTEX® 14L</td>
<td></td>
<td>0,1</td>
<td>0,1</td>
<td></td>
</tr>
</tbody>
</table>

The experiment was conducted according to the procedure mentioned under experimental with results listed in table 14

Table 14. Effect of PROTEX® 14L on pressed palm liquid.

<table>
<thead>
<tr>
<th>LAMINEX® BG2</th>
<th>PROTEX® 14L</th>
<th>Oil in sediment, based on sample</th>
<th>Drymatter</th>
<th>Water in wet sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>% v/w</td>
<td>% v/w</td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>1,43</td>
<td>6,49</td>
<td>73,9</td>
</tr>
<tr>
<td>0,1</td>
<td>-</td>
<td>0,97</td>
<td>4,63</td>
<td>70,4</td>
</tr>
<tr>
<td>0</td>
<td>0,1</td>
<td>1,59</td>
<td>6,47</td>
<td>75,0</td>
</tr>
<tr>
<td>0,1</td>
<td>0,1</td>
<td>0,96</td>
<td>4,54</td>
<td>71,1</td>
</tr>
</tbody>
</table>

The results in table 14 confirm that protease PROTEX® 14L has a negative effect on release of oil form the sediment of pressed palm liquid.

Conclusion

The Experiments conducted with enzyme treatment of pressed palm liquid showed that LAMINEX® BG2 have strong effect on solubilization of insoluble dry matter and release of
bound oil in the sediment. MANNASTAR® 375 also had a positive effect on release of oil from the sediment of pressed palm liquid. It was also observed that a protease PROTEX® 14L has a negative effect on release of oil from sediment, this might be explained by solubilization of protein and formation of peptides with emulsification properties.

**EXAMPLE 5**

**EFFECT OF LAMINEX® BG2 ON VISCOSITY OF PRESSED PALM LIQUID**

**Introduction**

Pressed palm liquid obtained by pressing the digested palm fruitlets is a rather viscous liquid. It is therefore normal procedure to dilute the pressed palm liquid with water before going to the clarifier in order to reduce viscosity.

The purpose of this study was to examine the effect on viscosity of enzyme treatment with LAMINEX® BG2 as well as water dilution. The effect on solubilisation of insoluble dry matter and amount of bound oil in the sediment from pressed palm liquid was also tested.

**Material**

PALM_8, Pressed Palm Liquid.

LAMINEX® BG2, batch no. 4902043555 (DuPont Industrial Biosciences), 10166 CMC-DNS/g, 185 MVU/g

**Experimental:**

30 gram Pressed Palm Liquid and enzyme was incubated with magnetic stirring at 50 °C for 4 hours in an Alum cup fitted for Rapid Visco Analyser After incubation the viscosity of the sample was immediately measure using a Rapid Visco Analyzer using the following program, Agitation 400 rpm, temperature setting 60 °C for 2 minutes Increase temperature to 80 °C, keep 80 °C for 3 minutes

The viscosity at 60 °C was calculated as the average of measurements between 60 and 120 seconds and viscosity at 80 °C was calculated as average measurements between 300 and 360 seconds.
The sample was then transferred to a 50-mL centrifuge tube and centrifuged at 4180 rcf for 10 minutes at 60 °C. The upper oil layer and water was removed.

30 mL water at 50 °C was added to each tube without mixing. The sample was centrifuged at 4180 rcf and 60 °C for 10 minutes.

The water phase was removed and the inside of the tube was wiped with a tissue to remove residual oil. Weight of the wet sediment was determined. The wet sediment was scaled, frozen and freeze dried.

Weight of the dry sediment was determined after freeze drying.

The dry sediment was grounded for 25 sec at 25000rpm in IKA Tube-mill. 120 mg grounded sediment was scaled in a 15 centrifuge tube and added 7,5 mL Heptane: Isopropanol 3:2. The mixture was heated to 40 °C for 10 minutes and mixed for 45 minutes at Stuart rotator SB2. The mixture was centrifuged for 3 min at 1280 rcf. 1 mL of the supernatant was transferred to TLC vials.

The triglyceride in the sample was calculated based on a calibration curve for palm oil analyzed in the same way.

HPTLC analysis of triglycerides.

The sample (1.0 µL) was applied to the 10 x 20 cm Silica HPTLC plate. Standard solutions of 0.5% Palm Oil (0.2, 0.5, 0.8, 1.2, 1.8 and 2.5 respectively) were also applied to the HPTLC plate by an automatic TLC applicator. The plate was eluted (7 cm) with Running buffer: Heptane: MTBE: Acetic acid 70:30:1. After elution the plate was dried on a plate heater for 2 minutes at 160 °C, cooled, and dipped in 6% cupric acetate in 16% H₃PO₄. The plate was additionally dried for 6 minutes at 160 °C. The developed spots were quantified using a TLC plate scanner and components quantified based on calibration curves of the palm oil standard.

Results

Effect of LAMINEX® BG2 and water dilution of pressed palm liquid was tested according to recipe shown in Table 15.

Table 15. Recipes of LAMINEX® B2 and water dilution of pressed palm liquid.
The experiment was conducted according to the procedure mentioned under Example 5 and the results are provided in Table 16.

Table 16. Results showing the effect of LAMINEX® BG2 and water dilution of pressed palm liquid.

<table>
<thead>
<tr>
<th>LAMINEX® BG2</th>
<th>Water added</th>
<th>Wet sediment</th>
<th>Dry sediment</th>
<th>Oil in dry matter based on sample</th>
<th>Viscosity, 60°C</th>
<th>Viscosity, 80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>cPoise</td>
<td>cPoise</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>22,9</td>
<td>5,79</td>
<td>1,79</td>
<td>282</td>
<td>223</td>
</tr>
<tr>
<td>0,05</td>
<td>0</td>
<td>14,3</td>
<td>3,97</td>
<td>0,48</td>
<td>117</td>
<td>80</td>
</tr>
<tr>
<td>0</td>
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<td>23,2</td>
<td>5,47</td>
<td>1,62</td>
<td>201</td>
<td>160</td>
</tr>
<tr>
<td>0,05</td>
<td>10</td>
<td>13,8</td>
<td>3,89</td>
<td>0,56</td>
<td>120</td>
<td>87</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>21,8</td>
<td>4,66</td>
<td>0,97</td>
<td>146</td>
<td>106</td>
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<tr>
<td>0,05</td>
<td>20</td>
<td>14,2</td>
<td>3,77</td>
<td>0,61</td>
<td>127</td>
<td>95</td>
</tr>
</tbody>
</table>

The results are also illustrated graphically in Figures 21 and 22.

The results illustrated in Figure 21 indicate that both LAMINEX® BG2 and water dilution has a strong effect on viscosity, but enzyme treatment without dilution decreases viscosity more than dilution with 20% water. It is however also observed that dilution of enzyme treated sample has a slightly negative effect on viscosity. Further it is observed that the lowest amount of oil in sediment is found in the enzyme treated sample without dilution. Based on these findings it is concluded that it is not preferable to dilute pressed palm liquid if it is treated with LAMINEX® BG2.
Various modifications and variations of the described methods will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.
CLAIMS

1. A method of improving palm oil yields, which method comprises:
   a. admixing an enzyme composition comprising cellulase activity with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed comprises at most 40% water content w/w;
   b. incubating the admixture for between 0.5 to 8 hours, preferably 4-8 hours, at a temperature of between 50 °C to 95 °C; and
   c. separating the oil from other components of the admixture.

2. Use of an enzyme composition comprising cellulase activity in combination with a palm fruit or a portion thereof or a palm fruit liquid, wherein said palm fruit or portion thereof or palm fruit liquid when admixed with the enzyme composition comprises at most 40% water content w/w in the manufacture of palm oil for improving palm oil yield, for improving separation of palm oil from the sludge phase, for producing an aqueous phase with improved fermentable sugars, or for improving the speed of oil separation.

3. A method according to claim 1 or use according to claim 2 wherein said palm fruit or portion thereof or palm fruit liquid when admixed comprises at most 38% water content w/w, suitably at most 35% water content w/w, suitably at most 30% water content w/w.

4. A method or use according to any one of claims 1 to 3 or use according to claim 2 or claim 3 wherein the palm fruit liquid is a pressed palm fruit liquid.

5. A method according to any one of the preceding claims wherein the oil is separated by gravity.

6. A method or use according to any one of the preceding claims wherein the oil is separated by centrifugation either instead of gravitation separation or in addition to separation by gravity.

7. A method or use according to claim 6 wherein the separation results in a triple phase composition comprising an oil layer, an aqueous layer and a substantially solid sediment layer.
8. A method or use according to claim 7 wherein the aqueous phase is enriched in soluble fermentable sugars.

9. The method or use according to any one of the preceding claims wherein said enzyme composition in addition to comprising cellulase activity comprises one or more of the following enzyme activities: mannanase and pectinase.

10. The method or use according to any one of the preceding claims wherein said enzyme composition comprises low or no protease.

11. The method or use according to any one of the preceding claims wherein the enzyme composition is a crude or purified extract of a *Trichoderma reesei* fermentate.

12. The method or use according to any one of the preceding claims wherein there is no or only minimal agitation during the incubation stage.

13. The method or use according to any one of the preceding claims wherein the cellulase in one which reduces the amount of dry sediment (sludge dry matter) by at least 20% when 25000 CMC-DNS/kg substrate is added to a palm fruit, a portion thereof or a palm fruit extract and incubated for 1 hr at 50°C.

14. The method or use according to any one of claims 9 to 13 wherein when the enzyme composition comprises mannanase, the enzyme composition comprises a minimum level of mannanase activity which when added to the substrate give a mannanase concentration of at least 200 MVR/kg substrate.

15. A palm oil phase, a sludge phase or an aqueous phase obtainable by the method according to any one of claims 1-14.

16. Use of an aqueous phase obtainable by the method according to any one of claims 1-14 as a fermentation medium.

17. A method for producing a fermentation product comprising fermenting a feedstock comprising said aqueous phase obtainable by the method according to any one of claims 1-14 and recovering said fermentation product.
18. A method according to claim 17 wherein said feedstock further comprises empty palm fruit bunches or processed empty palm fruit bunches.

19. A method according to claim 17 or claim 18 wherein said fermentation product is a compound selected from the group consisting of: an alcohol, an organic acid, an antibiotic, an antimicrobial, bioinsecticide, a solvent, and a polyhydroxyalkanoate.

20. A method according to any one of claims 17 to 19 wherein the feedstock is subjected to one or more processing steps selected from the group consisting of: milling, cooking and/or saccharification.

21. A method according claim 19 or claim 20 wherein the alcohol is a biofuel selected from ethanol, butanol or a combination thereof.
FRESH FRUIT BUNCHES (1000kg) → STEAM → STERILIZATION → CONDENSATE → STRIPPING → EMPTY BUNCHES (234kg)

FIBROUS MATERIALS → STEAM → DIGESTION → FRUITS (666kg)

WATER → PRESSING → PRESS CAKE

PRESSED OIL → PRESSING → SCREENING → OIL

CLEAR OIL (180kg) → CLARIFICATION → DESANDING → SEPARATION → SLUDGE (180kg)

PURIFICATION → DRYING → CRUDE PALM OIL (225kg)

NUT/FIBRE SEPARATION → FIBRE (180kg)

NUT DRYING → NUT CRACKING → CRACKED MIXED

DRY SHELL (total shell 73kg)

WET SHELL

WASTEWATER

KERNELS (67kg) → KERNEL DRYING → FUEL

FIG. 1
SUBSTITUTE SHEET (RULE 26)
**FIG. 3**

![Graph showing percentage of oil in sediment based on sample vs. percentage of enzyme.](image)

**FIG. 4**

![Graph showing percentage of sediment dry matter vs. percentage of enzyme.](image)
Effect of % water on viscosity of pressed palm liquid at different temperature

**FIG. 7**

Effect of % water on viscosity of enzyme treated pressed palm liquid at different temperature

**FIG. 8**
FIG. 9

Viscosity of pressed palm oil as a function of % water at different temperature

FIG. 10
FIG. 11

Viscosity of pressed palm oil treated with 0.5% Laminex BG2 as a function of % water

% water in pressed liquid

cPoise

FIG. 12

1 Control  2 ENZ  3 Control  4 ENZ  5 Control  6 ENZ  7 Control  8 ENZ  9 Control  10 ENZ
FIG. 13

FIG. 14
**FIG. 17**

Oil separation before centrifugation

**FIG. 18**

Oil in sediment, % based on sample
% water in wet sediment

% 80,0 75,0 70,0 65,0 60,0 55,0 50,0

- 75,5 76,1 73,3
- 68,3 67,4 63,1
- 0,1 0,1
- 0,1 0,1

FIG. 19

Sediment drymatter

% 6 5 4 3 2 1 0

- 5,19 4,89 4,62
- 3,33 2,67 2,68
- 0,1 0,1
- 0,1 0,1

FIG. 20
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

**INV. C11B/02 C11B13/00**

**ADD.**

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)

C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>page 2, line 26 - page 3, line 10 page 4, lines 1-15 page 5, lines 1-12 ; claim 3 ; example 1A</td>
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See patent family annex.

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**Date of the actual completion of the international search**

9 February 2016

**Date of mailing of the international search report**

18/02/2016

**Name and mailing address of the ISA/ **

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Fax. (+31-70) 340-3016

**Authorized officer**

Rinaldi, Francesco
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<td>WANG ZUNSHENG ET AL: &quot;Cel l u l ase production on and o i l palm empty frui t bunch sacchari fication on by a new i sol ate ofTri choderma koni ngi i D-64&quot;, PROCESS BIOCHEMISTRY, vol. 47, no. 11, 2012, pages 1564-1571, XP028960370, ISSN: 1359-5113, DOI: 10.1016/J.PROCBIO.2012.07.001, abstract Secti ons 1, 2.3 and 2.4</td>
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