Valorization of jatropha fruit biomass for energy applications

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Thesis

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CHAPTER 1

Introduction

1. Biobased economy

1.1. Definition

The European Commission defines a biobased economy as an economy that integrates the full range of natural and renewable biological resources – land and sea resources, biodiversity and biological materials (plant, animal and microbial) – and the processing and consumption of these bio-resources. A biobased economy therefore uses biological materials as the building blocks for industrial processes instead of fossil fuels. The traditional 'fossil-based' economy is to a great extent powered by non-renewable resources that are being depleted. The technologies developed and implemented in the biobased economy use renewable bioresources, biological tools, eco-efficient processes that enable green-house gas (GHG) emission reduction to produce sustainable bioproducts for pharmaceuticals, chemicals, materials, transportation fuels, electricity and heat (Langeveld and Sanders, 2010; Mosier et al., 2005; OECD, 2001). The technologies involved might differ in character, but they facilitate a significant substitution of fossil energy carriers by biomass.

1.2. Approaches in sustainable use of biomass

The drivers in a biobased economy include the wish to limit dependency on fossil fuels and oil-exporting countries, the need to facilitate a diversification of energy sources, the wish to provide options for regional and rural development in both developed and developing countries, and the need to reduce greenhouse gas (GHG) emissions (Langeveld and Sanders, 2010). These will strongly increase demand for biomass resources. Biomass production on land occasionally faces a variety of potential conflicts, such as the competition with food and feed production and the associated land-use changes. To satisfy both human basic needs (for food and feed) and the expanded demand of biomass in the production of biobased materials, energy, and fuels, a more efficient and sustainable use of biomass is essential. Sustainable use of biomass can be performed through biorefinery. Biorefinery is defined as the sustainable processing of biomass into a spectrum of marketable products (chemicals, materials, feed and food) and energy (biofuels, heat and power) (van Ree and Annevelink, 2007).

1.3. Plant as the main source of biomass

Biomass has been chosen as the main source in developing energy, materials and chemicals because it can be sustainably produced. Biomass is unique, because it is the only natural resource that can satisfy both the need for the production of biofuel and for the manufacture of chemicals and materials. Biomass materials are derived mainly from a range of plants and may include cellulose, lignin, starch, sugar, vegetable oil, proteins, amino acids, etc. In order to realize a sustainable production of biomass, production systems must ensure conservation, regeneration, recycling, and substitution of the resources: fossil energy, nutrients, water, soil organic matter (SOM), and biodiversity (Ostergard et al., 2010).

The amount of biomass can be expressed as energy content (in exajoules (EJ); 10^{18} Joules), whether the biomass is used as bio-energy or as biofeedstock. This allows the substitution of fossil fuels with biomass across the various sectors of the economy to be quantified in a comparable manner.

1.4. Global trends in biobased economy

Our present economy, which is strongly dependent on fossil oil, is gradually changing into an economy based mainly on renewable resources. This process is a transition, literally defined as the process of changing from one state or condition to another. In biobased economy, however, the term is used in a more specific scope, referring to a process of broad societal change, leading to an entirely new way of fulfilling societal needs, such as the need for food, housing, transport and energy (Langeveld and Sanders, 2010). Definitely, governments should promote research and innovation to facilitate a structural transition from a fossil-based to a bio-based industry, which will also offer great opportunities for economic growth and employment. The global biobased economy must ensure the following priorities in research and innovations: global food security; sustainable agricultural production; healthy and safe food; industrial use of renewable resources; and biomass-based energy sources. Conflicts may arise owing to the different objectives of these priorities, therefore we need holistic approaches that take into account the ecological, economic and social concerns in equal measure and integrate them in sustainable solutions.

According to a comprehensive study by OECD, Biotechnology will play a significant role in economic activities worldwide (OECD, 2001). Biotechnology is defined as "the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services"(OECD, 2014).

1.5. Future challenges

Challenges for sustainable development in a biobased economy have been described in detail (Ostergard et al., 2010). With respect to biomass availability, we have to overcome challenges for agriculture and challenges for the biorefinery sector. In the agricultural sector the challenge is how to develop sustainable biomass production. To achieve this, we need production systems that apply a greater proportion of recycling, higher efficiency in the exploitation of limited natural resources, and increased use of renewable resources. In the biorefinery sector the challenges are logistical, including how to efficiently collect and concentrate the biomass, which is scattered in a wide plantation area or located far away from the biorefinery processing site, and also how to recycle the nutrient to maintain the land fertility.

2. Biorefinery

2.1. Definition

There are several definitions of biorefinery, depending on the type of activity and the stakeholders involved. The definitions within the framework of IEA Bioenergy Task 42 on Biorefineries: Biorefinery is the sustainable processing of biomass into a spectrum of marketable products (chemicals, materials, feed and food) and energy (biofuels, heat and power) (van Ree and Annevelink, 2007). Figure 1 shows the principle of the biorefinery.

This definition includes the keywords (van Ree and Annevelink, 2007):

- Biorefinery: concepts, facilities, processes, cluster of industries
- Sustainable: maximizing economics, minimizing environmental aspects, fossil fuel replacement, socio-economic aspects taken into account

- Processing: upstream processing, transformation, fractionation, thermo-chemical and/or biochemical conversion, extraction, separation, downstream processing
- Biomass: crops, organic residues, agroresidues, forest residues, wood, aquatic biomass
- Spectrum: more than one
- Marketable: a market (acceptable volumes & prices) already exists or is expected to become available in the near future
- Products: both intermediates and final products, i.e. food, feed, chemicals, and materials
- Energy: fuels, power, heat

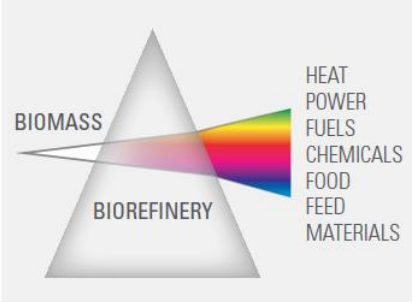

Figure 1. The principle of the biorefinery - IEA Bioenergy TASK 42 (Star-COLIBRI, 2011)

2.2. Biorefinery concept

The biorefinery concept was initiated by the idea to either further develop the existing food-based biorefineries such as sugar, starch, vegetable oil, and grain processing facilities, or to install completely new facilities, which could fractionate the whole biomass into its appropriate primary feedstock state, also known as precursors, and further refine it into sustainable bioproducts, such as chemicals, fuels, and heat and electricity (van Ree and Annevelink, 2007).

A schematic flow-chart of possible products made from biomass is shown in Figure 2 (van Ree and Annevelink, 2007). The main precursors given are carbohydrates, starch, hemicellulose, cellulose, lignin, lipids/oil, and protein. These precursors are converted to products called platforms, which are then transformed into so-called building

blocks, which can further be used to make secondary chemicals, intermediates and final products. These final products fullfil almost all our needs: industrial, transportation, textiles, safe food, environment, communication, housing, recreation and health.

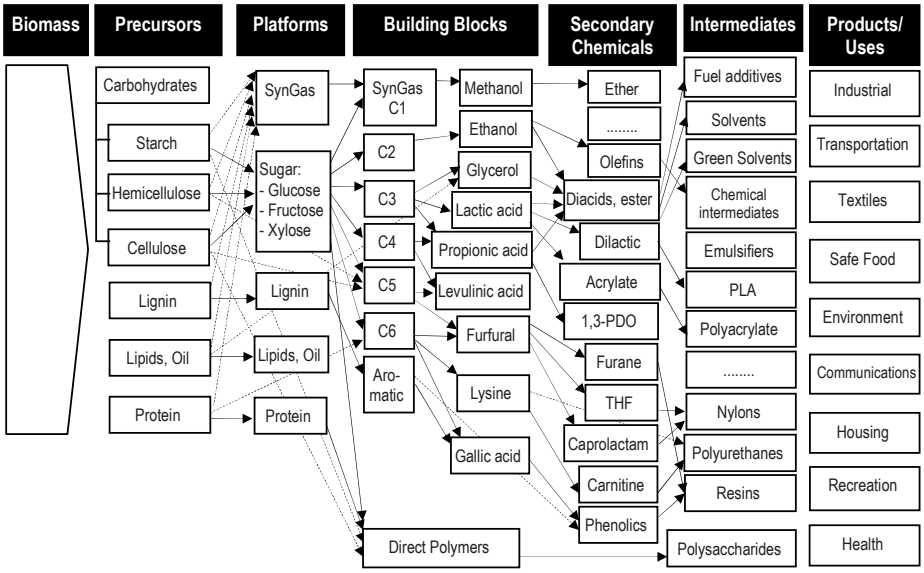

Figure 2. Possible products made from biomass (van Ree and Annevelink, 2007)

3. *Jatropha curcas,* L

During the past ten years *Jatropha curcas,* L (simply called "jatropha") has received a lot of attention because it has seeds with high oil content suitable for power generation or for the production of biodiesel. Jatropha has been introduced as one of the best candidates for future biodiesel production (Chen et al., 2008). Arguments for it are a high seed oil content (Misra and Murthy, 2011; Singh et al., 2008), the potential for high oil production levels per unit area in sub-humid tropical and subtropical climates, its drought-resistance and ability to grow well in marginal soils (Openshaw, 2000), though evidently this will reduce the oil productivity .

3.1. History of jatropha application

Historical records reveal that jatropha was used by Native American tribes in Central America and perhaps in North America as a traditional medicine (FACT-Foundation, 2010). Jatropha seeds were commercially traded in the Cape Verde Islands in 1836, and seeds were exported to Portugal and France and the oil used for street lights and soap production. Jatropha has been used for various other purposes as well. Figure 3 shows an overview of the several applications of jatropha and its products (Gubitz et al., 1999).

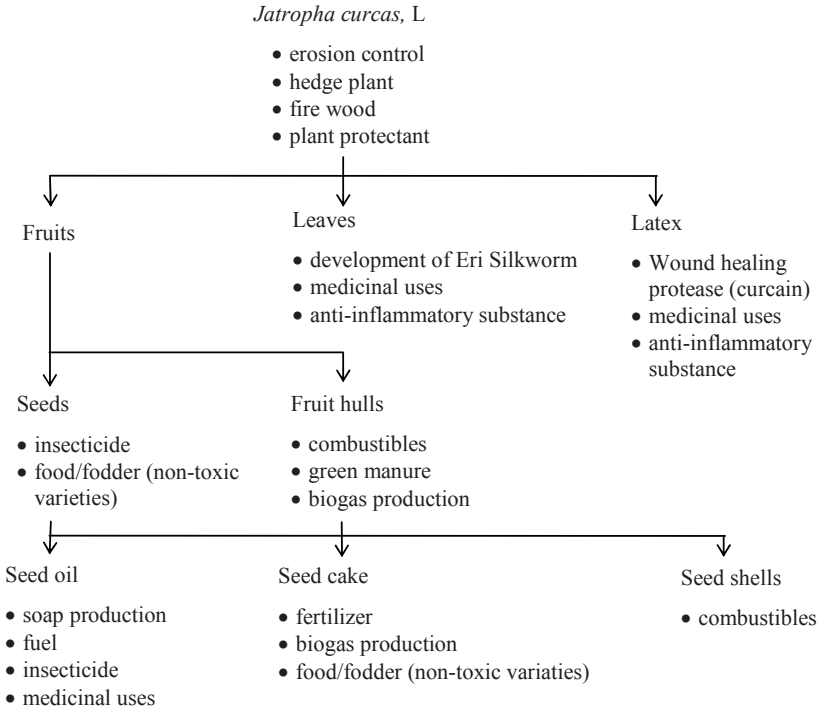

Figure 3. An overview of possible applications of jatropha (Gubitz et al., 1999)

3.2. Taxa and agronomy in brief

Jatropha curcas, L (purge nut, physic nut) is often simply called "jatropha" although there are approximately 170 species of jatropha. Taxonomically, jatropha belongs to the family of Euphorbiaceae. Being an attractive source of biodiesel in the research field, jatropha has so far not been commercially cultivated for industrial purposes due

to low seed productivity produced by the plant compared to other oilseeds plant. Therefore, improving the plant oil productivity is recently being the major research topic on agronomy aspects of this tree (Yue et al., 2013).

Jatropha is a perennial bush or small tree that can grow up to 6 m height; it can live more than 50 years (Achten et al., 2008). Numerous investigators have reported the ability of jatropha to grow in the soils with low nutrient content; however, our experiences reveal that intensive fertilization and sufficient watering are required to obtain high fruit production of jatropha growing in marginal soils. The fruits have an oval shape, of 35-45 mm length and each contains 3 black seeds (on average), with dimensions of 19-21 mm long and 8-13 mm wide (Figure 4). The branches contain a latex, which causes brown stains that are difficult to remove (Heller, 1996).

3.3. Plant parts and dry matter distribution

The dry matter distribution of jatropha is 25% in stem, 25% in leaf and 50% in fruit; the 50% DM fruit consists of 15% fuit hull and 35% seed; and the 35% DM seed consists of 23% kernel and 12% seed shell (Jongschaap et al., 2007b) (Figure 4).

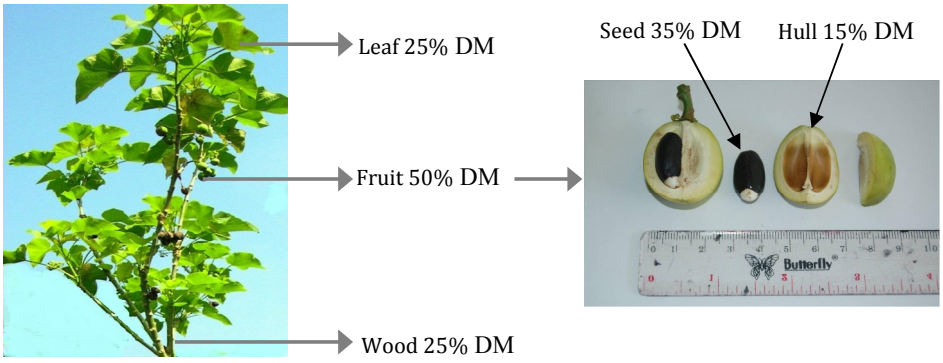

Figure 4. Dry matter distribution of jatropha

3.4. Fruit composition

On fruit weight basis, the fruit contains 23.5% oil, 13.0% protein and 30.0% carbohydrate, 12.6% lignin and 20.9% others (which may contain pectin, wax, tannin, ash, etc.). The carbohydrate fraction of the seed comprises mainly glucose, fructose,

saccharose, raffinose, stachyose, and galactose. The major fatty acids of Jatropha oil are oleic, linoleic, palmitic, and stearic acids (Martínez-Herrera et al., 2006). Arachidic and myristic acids are also reported. Phorbolesters are present in high concentrations in the kernels of toxic provenance. The protein contains essential amino acids and non-essential amino acids. Jatropha seed contains a heat stable 2S albumin (12 kDa) that has allergenic properties (Maciel et al., 2009). The glutelins, globulins, and albumins are the major of the total recovered protein found in jatropha kernel meals for both non-toxic genotypes from Mexico and a toxic genotype from Cape Verde, contributing 56.9, 27.4 and 10.8%, respectively, whereas prolamins and non-extracted residues were present in minor quantities (0.6 and 4.3%, respectively)

3.5. Toxicity of jatropha

Investigations have shown that the seed cake contains toxins and cannot be used as animal feed without further processing. Seeds contain the dangerous toxalbumin curcin, rendering them potentially fatally toxic (Devappa et al., 2010). Without being detoxified in a proper method, jatropha cake can not be used as food or feed owing to the presence of high levels of toxic and antinutritional factors such as trypsin inhibitor, phytic acid, saponins and glucosinolates (Devappa and Swamylingappa, 2008). Saponins were also present at a level of 2.6 - 3.4% (as diosgenin equivalent). Phorbolesters were present in kernels of the toxic variety (2.2 - 2.7 mg/g). Trypsin inhibitors and lectins are heat labile and can therefore be destroyed by heat treatment. Heat treatment for the Mexican variety and a combination of heat and chemical (NaOH and NaOCl) treatments or extraction with 80-90 % aqueous ethanol or methanol for toxic varieties hold promise for detoxification of jatropha meals (Makkar and Becker, 1997).

3.6. Current situation of jatropha in the world

By 2008, the global plantation of jatropha was estimated to be 900,000 ha, mostly concentrated in Asia (85%) and the rest in Africa (13%) and Latin America (2%); and in 2008 the plantation was projected to reach 12,800.000 ha worlwide by 2015 (GEXI, 2008). The current situation and trend reveal that this projection seems too optimistic.

In 2011, commercial airliners like Air Japan, Continental Airlines and Air New Zealand have done test flights of their planes using jatropha-based biofuel (Honeywell, 2011). Despite the advanced tests in use of jatropha-based biofuel, too many issues remain unanswered towards the commercial prospects of the jatropha-derived biofuel. Even though the spectacular seed yield of jatropha tree has been shown on many ocassions and in scientific fora, the factual yield is far behind expectations. Yields were inconsistent, and many farmers lacked of the basic agricultural practices to operate commercial-scale crops. Most of the jatropha plantations then shut down. Beside the knowledge gaps in the basic agricultural practices, the main drawbacks to mention are the long process of clone selection, breeding, and domestication of the most productive varieties of jatropha (Contran, 2013).

The previous arguments that jatropha could thrive and be productive, even with limited water availability and low fertility, has led to the failure of many projects implemented in arid areas. Some large scale jatropha plantations focusing on biodiesel production have provided intensive irrigation to obtain high yields, driving down the potential sustainable advantages of this tree (Contran, 2013). The regular post planting agronomic management seems to be well practiced at least in countries such as India and China (Gour, 2006). Unfortunately, it is still unclear if these agronomic practices can be successfully applied in other locations, such as arid and semi-arid areas of Africa. Furthermore, industrial processing of jatropha biomass is still in its infancy and a commodity market for jatropha oil and by-products does not exist yet. In contrast, the process technologies for jatropha oil, especially that linked to the biodiesel production at large scale, are improving rapidly (Achten et al., 2008; Contran, 2013; Islam et al., 2011; Tang et al., 2007).

3.7. Current situation of jatropha in Indonesia

In 2006, due to an energy crisis, Indonesia launched a new renewable energy program, which aimed to source 17 percent of its energy needs from renewable resources by 2025. The nation has been pushing the use of biofuels made from various biomass sources such as palm oil, sugar cane, cassava, and jatropha. Since then, the Indonesian national policy began introducing jatropha as the favored biofuel source. The ultimate goals were to improve economy, to expand job opportunities, to minimise poverty, and to reduce dependence on imported fossil fuels. Many

proponents believed that jatropha would be the best choice of future energy crop. However, no one would have been able to predict that the growth estimatation of the jatropha market in Indonesia would deminished so suddenly after only a few years. The predominant obstacle standing in the way of jatropha industry development is the low oil yield: farmers do not get sufficient income from jatropha cultivation. Moreover, up to now the government of Indonesia has been reluctant to fully support the biofuel industry. The reasons for this remain unclear.

The amount jatropha cultivation area in Indonesia shows a slightly increasing trend. As high as 68.200 hectare land (produced 7600 tonnes seeds) cultivated in 2007, increased to 69.200 hectare (7900 tonnes seeds) in 2008, and rose slightly to 69.300 hectare (8000 tonnes seeds) in 2009 (Syakir, 2010). Clearly, the seed production expresses low seed yields that lie between 111 to 116 kg seed per hectare per year, which is even much lower than the reported yields between 400 to 12,000 kg per hectare per year (Contran, 2013).

3.8. Application of jatropha

Whole plant use

Jatropha cultivation has a positive effect on the environment, such as improving soil condition, reducing soil erosion, and supporting marginal land reclamation and soil remediation (Openshaw, 2000). Traditionally, jatropha is used as a living fence to protect homestead, gardens and crop fields from livestock, to create fire barriers, and to prevent wind erosion.

Utilization of plant parts

Almost all parts of the plant (leaves, bark, roots, seeds, and latex), fresh or as a decoction, have pharmacological effects in human medication. The pharmacological effects include: purgative and laxative; anticancer, antimalaria, rheumatic and muscular pain (Thomas et al., 2008); antibacterial against *Staphylococcus aureus* and *Escherichia coli* (Ye et al., 2009); anticoagulant (Islam et al., 2011); mouth desinfectant, against skin diseases (Kumar and Sharma, 2008); eczema and rheumatic pains (Heller, 1996); and as contraceptive (Gubitz et al., 1999).

Jatropha oil

Jatropha oil is extracted from the seeds of *J. curcas,* L. Several techniques have been applied to extract oil from the jatropha seeds. The use of hexane recovers up to 98% oil from seeds (Singh et al., 2008; Winkler et al., 1997b); however strict regulations of hexane use due to its hazardous properties have driven research to discover safer and more economical extraction process alternatives. The other ways of oil extraction are hydraulic pressing and screw pressing (expeller) of dried seed, resulting in maximum 85% w/w oil recovered (Singh et al., 2008).

A continuous pressing has been widely applied in jatropha oil extraction. In some cylinder models, the nozzles are equipped with heaters. Care has to be taken since overheating can reduce the oil quality. A temperature of 60 °C has been regarded as a safe maximum. Above this temperature, excessive phosphorus may be released into the oil, leading to offspec oil, and in extreme cases the oil may be damaged (cracked) by very high temperatures (above 150 °C). After pressing, the oil contains 1-13% solids that can be separated from the oil by sedimentation, filtration or centrifugation, or a combination of these processes. The size of solid paticles must not exceed 5 µm. The cleaning process should follow shortly after the pressing process to avoid filtration problems when the oil is stored under unfavorable storage conditions. Some disadvantages of continuos expellers are:

- Oil from an expeller contains more impurities than oil from a batch press and must be filtered to obtain clean oil
- Maintenance costs are high and it requires skilled mechanics.

Another method of jatropha oil extraction is aqueous extraction process (AEP) that uses water as media to facilitate oil liberation from oilseeds. AEP offers many advantages compared to conventional extraction. For instance, it eliminates solvent consumption which may also reduce investment costs and energy requirement (Barrios et al., 1990a; Barrios et al., 1990b; Rosenthal et al., 1996). AEP also enables simultaneous separation of oil, protein and other interesting constituents from oilseeds. The oil obtained is also free of gums, thus eliminating de-gumming steps (Caragay, 1983). Water extraction only (AEP without enzymes) yielded 38 % of the total oil content of the seeds. AEP usually employs enzymes to disrupt oil barriers therefore increases overall oil yield. The use of enzymes mainly proteases in AEP of jatropha oil extraction had been reported with oil yield up to 86% w/w (Shah et al.,

2004; Shah et al., 2005). The cost of enzymes together with drying energy cost in the aqueous oil extraction process are the most decisive economic factors (Dominguez et al., 1994).

The use of whole-cell biocatalyst in place of purified enzyme in oil aqueous oil extraction is attractive in terms of process simplicity, which in turn may reduce production cost. Several investigations have reported the use of microbial cells such as bacteria, yeast and fungi as whole-cell biocatalysts in separation and bioconversion processes (Ban et al., 2001; Fujita et al., 2002; Konishi et al., 2005; Man et al., 1997; Narita et al., 2006). The use of whole cells has been reported to extract coconut oil with high yield (Man et al., 1997; Puertollano et al., 1970).

In all processes, about 50 % of the weight of the seeds remains as a press cake containing mainly protein and carbohydrates. Like any other vegetable oil with a comparable fatty acid composition, jatropha oil is liquid at room temperature. The oil has been used for many purposes, such as for making soap, and most importantly as biodiesel (Kumar and Sharma, 2008). Jatropha oil contains both saturated and unsaturated fatty acids. Jatropha oil is 80 percent unsaturated, with oleic and linoleic as the major fatty acids. The saturated fatty acids consist of 14-15% palmitic (16:0), 4-10% stearic (18:0), 0.3% arachidic (20:0), 0.2% behenic (22:0), and 0.1% myristic (14:0). The unsaturated fatty acids consist of 34-46% oleic (18:1) and 29-44% linoleic (18:2), and 0-0.3% linoleic (18:3) (Kpoviessi et al., 2004; Kumar and Sharma, 2005).

Oil conversion by trans-esterification has made jatropha oil an environmentally safe, cost-effective renewable source of biofuel and a promising substitute for diesel, kerosene and other fuels (FACT-Foundation, 2006). The trans-esterification process requires the addition of methanol or ethanol and caustic soda, adding additional cost to the final product. The resulting biodiesel can be used directly in any diesel engine without adaptations (except for pure rubber hoses which deteriorate after longer contact with pure biodiesel).

Jatropha protein

Extraction of jatropha protein from jatropha kernel and seed press cake with high protein recovery has been reported (Lestari et al., 2010). The solvent used was 0.055M NaOH. By using one-stage extraction, the protein yield from kernel (35.8%

protein content) and seed press cake (53.3% protein content) was 69.6 and 64.9%, respectively. The protein yield from seed press cake could be further increased to 71% by applying four stages of counter current extraction at solvent to solid ratio of 4 g/g. The best pH for protein precipitation found was between 4 to 5.5.

In relation to functional properties, jatropha seed protein has been reported to have best performance in alkaline region (pH higher than 9.0) due to its high solubility, high emulsifying, and high foaming properties (Lestari, 2012).

Jatropha seed protein has almost all essential amino acids in a higher content than those of the FAO reference protein (Makkar et al., 1997). The amino acid composition and the percentage of essential amino acids are comparable to those of other seeds and press cakes used as fodder.

Jatropha carbohydrates

The jatropha fractions with high carbohydrate content like stem (wood), dried fruit hulls, and seed shells can be burnt as fuel; however, jatropha wood is not popular as fuelwood because it is a light wood that burns too fast (Islam et al., 2011; Singh et al., 2008). Jatropha fruit hull has 33.8% cellulose, 9.7% hemicelluose, 11.9% lignin, 0.8% Na, 4.9% K, 0.7% K, 0.8% P is a good feedstock for biological conversion (fermentation) and for briquetting to be used as fuel for household and industries (Singh et al., 2008). Analysis shows that jatropha seed shells containing 4.0% ash, 71.0% volatile matter, and 25% fixed carbon with calorific value of 4044 kcal/kg is a suitable feedstock for laboratory model open core down draft gasifier in the production of CO, H_2, and CH_4 (Singh et al., 2008).

Other uses

The 37% tannin found in the jatropha bark give a dark blue dye; the latex of leaves also contains 10% tannin and can be used as marker; the oil can be used to make hard homemade soap (FACT-Foundation, 2006).

4. Lignocellulose conversion

Conversion of lignocellulosics to bioproducts such as ethanol or other fermentative products involves four major unit operations: pretreatment, hydrolysis, fermentation, and product separation/purification. Pretreatment is required to break macroscopic

structure of lignocellulose so that hydrolysis of the carbohydrate fraction to monomeric sugars occurs more rapidly and with greater yields. Hydrolysis aims at converting the smaller carbohydrate polymers into monomeric sugars. Enzymatic hydrolysis of cellulose provides opportunities to develop the technology with lower costs so that the ethanol produced is competitive when compared to other liquid fuels on a large scale (Wyman, 1999).

4.1. Pretreatment

Lignocellulosic biomass is a complex material made up of three major organic fractions: cellulose, hemicellulose, and lignin. It also contains ash and various extractives. Cellulose is a long polymer chain of glucose units that can be deploymerized by enzymes such as cellulase or by acids. However, hydrogen bonds that hold the long cellulose chains tightly together in a crystalline structure hampers its depolymerization into glucose. Hemicellulose is an amorphous polymer of mixed sugars, usually xylose, arabinose, galactose, mannose, and glucose, and smaller amounts of a few other compounds, such as acetic acid. Hemicellulose is more prone to enzyme attack to form their component sugars than cellulose. Lignin is a complex non sugar-based polymer with a phenol-propene backbone.

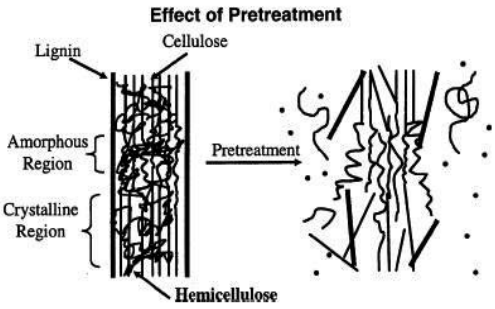

Figure 5. Schematic of goals of pretreatment on lignocellulosic material (Mosier et al., 2005)

Pretreatment is required to enhance the enzymatic digestibility of the lignocellulosic biomass in the conversion of carbohydrate into more simple sugars as represented in the schematic diagram of Figure 5. During pretreatment the lignin seal is broken and the crystalline structure of cellulose is dusrupted, making it more prone to enzymes (cellulases) attacks (Hendriks and Zeeman, 2009).

Biomass pretreatment can be classified in four groups: physical, physico-chemical, chemical, biological, and combination of these treatments (Hendriks and Zeeman,

2009; Sun and Cheng, 2002). Physical pretreatments include mechanical size reduction (chopping, pulverizing, and milling to reduce the cellulose crystallinity) and pyrolysis at temperatures greater than 300 °C. Physico-chemical pretreatments include steam explosion (autohydrolysis) and ammonia fiber explosion (AFEX). Chemical pretreatment includes ozonolysis, acid pretreatment, alkaline pretreatment, oxidative delignification, and organosolv pretreatment. Biological pretreatment employs microorganisms such as fungi to degrade lignin and hemicellulose in waste materials. The advantages of biological pretreatment are low energy requirement and mild environmental conditions. The low rate of hydrolysis is the main disadvantage of most biological pretreatments (Hendriks and Zeeman, 2009).

4.2. Biomass hydrolysis and ethanol process

Cellulose can be enzymatically or chemically hydrolyzed into glucose. Enzymatic hydrolysis uses cellulases whilst chemical hydrolysis usually employs sulfuric acid or other acids. Hemicellulases or acids hydrolyze the hemicellulose polymer to release sugars such as xylose. The six carbon sugars or hexoses (glucose, galactose, and mannose) are readily fermented to ethanol by many naturally occurring organisms such as *Saccharomyces cerevisiae*, but the pentoses xylose and arabinose (containing only five carbon atoms) are fermented to ethanol by only a few wild type strains, and usually at relatively low yields. Xylose and arabinose generally comprise a significant fraction of hardwoods, agricultural residues, and grasses and must be utilized to make the economics of biomass processing feasible (Lynd et al., 1999). Genetic modification of bacteria (Ingram et al., 1998) and yeast (Kuyper et al., 2005a; Kuyper et al., 2005b; van Maris et al., 2006) has produced strains capable of co-fermenting both pentoses and hexoses to ethanol and other value-added products at high yields.

In the ethanol process, the carbohydrate hydrolysis conducted separately from the fermentation step is known as separate hydrolysis and fermentation (SHF), while the hydrolysis carried out in the presence of the fermentative microorganism is known as simultaneous saccharification and fermentation (SSF). SSF is favorable because of lower process costs (Wright et al., 1988).

5. Research objectives

To develop sustainable technologies of jatropha oil extraction and jatropha biomass fractionations within a framework of bioconversions (enzymatic and microbial processings) that could suit the requirement for in situ (local) application, in this case Indonesia.

6. Research questions

The research questions are:

a) Which part of jatropha lignocellulosic biomass can be depolymerized to sugars and then valorized to chemicals/fuels, in particular to bioethanol?
b) Can a microbial method of jatropha oil extraction improve the oil yield in comparison to other known processes?
c) Can a microbial method of oil extraction preserve the original structure of jatropha protein?
d) Can Jatropha fruit by-products after valorization give better added-value products than just the seeds?

7. Outline of this thesis

After a general introduction in Chapter 1, Chapter 2 presents the investigation on the effect of dilute sulfuric acid pretreatment on enzymatic digestibility of jatropha seed shells, fruit hulls, and seed-cake. The goal is to estimate the feasibility of releasing monomeric sugars from several jatropha fractions.

Chapter 3 discusses the investigatation on the oil extraction from jatropha kernel assisted by a thermophilic bacterial strain namely *Bacillus licheniformis* strain BK23 isolated from paddy crab.

Chapter 4 discusses the investigation on the oil extraction from jatropha kernel assisted by a mesophilic bacterial strain namely *Bacillus pumilus* isolated from paddy crab.

Chapter 5 discusses optimization of the experimental variables (sulfuric acid concentration, time, and temperature) for jatropha fruit hull pretreatment and its hydrolysis into sugars. Simultaneous saccharification and fermentation (SSF) of jatropha fruit hull hydrolyzates by *Saccharomyces cerevisiae* for ethanol production is also discussed.

Chapter 6 provides general discussion and conclusion, focusing on the retrospectives of our research findings and the perspectives of the future utilization of jatropha, specifically in Indonesia.

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CHAPTER 2

The influence of dilute sulfuric acid pretreatment on enzymatic digestibility of jatropha seed shell, press cake and fruit hull

Abstract

Jatropha fruits consist of (in % *w/w* dry fruits) 50% seed kernels, 20% husks (seed shells) and 30% hulls (fruit exocarp). The fruit contains 23.5% (*w/w*) of vegetable oil. Fractionation of the fruit byproducts, mainly protein and carbohydrate, will increase the economy of jatropha processing factory. In this study, we investigated the effect of pretreatment using dilute sulfuric acid (0-500 mM) at 120 ^{o}C for 30 minutes on enzymatic digestibility of milled jatropha biomass: seed shell, seed-cake and fruit hull at 10% (*w/w*) solids loading by determining glucose and xylose release. The lignocellulose fractions of the jatropha seed shell and the jatropha seed cake were relatively recalcitrant to dilute sulfuric acid pretreatments suggesting that more intense pretreatment is necessary to disrupt lignin barriers sufficiently to improve enzymatic digestibility. However, dilute sulfuric acid pretreatment solubilized 65% of the available protein in the jatropha seed cake. The acidic jatropha seed cake hydrolyzate that is rich in amino acids may be further fractionated into single amino acids for the production of N-chemicals. After dilute acid pretreatment, the lignocellulose fraction of the jatropha fruit hull proved relatively more susceptible to hydrolysis by cellulases (GC220). As much as 70% glucose and 100% xylose was obtained from the jatropha fruit hull after a pretreatment with 500 mM sulfuric acid. A pretreatment at higher temperature may resolve the need of more diluted acid concentration and a shorter time to achieve comparable or higher sugar yields.

***Keywords**:*

Sulfuric acid, Pretreatment, Lignocellulose, Hemicellulose, *Jatropha curcas,* L, Biomass, Glucose, Xylose

1. Introduction

Jatropha curcas, L is a crop of which the fruits are used for oil production. The oil can be converted into biodiesel. The fruits consists (in *w/w*) of 50% seed kernel, 20% husk (seed shell) and 30% hull (fruit exocarp) (Jongschaap et al., 2007a). The overall composition of the total fruit is (on weight basis): 23.5% oil, 13.0% protein, 30.0% carbohydrate, 12.6% lignin and 20.9% other compounds.

To improve the economic value of the jatropha plant it is important not only to harvest the oil, but also to utilize the other fruit fractions. The side-stream from jatropha processing plant such as fruit hulls, seed shells, and the remaining de-oiled seed cake after pressing can be used for organic fertilization or can be directly burnt for more energy production (Jongschaap et al., 2007a). Alternatively, the seed cake and the fruit hulls can be anaerobically digested for biogas production. Direct burning causes the loss of most nutrients, e.g. the oxidation of organic nitrogen to NO_x, while digestion will secure application of most nutrients in the effluent as fertilizer.

Jatropha seed kernel contains approximately 25-30% protein (Gubitz et al., 1999; Openshaw, 2000). It however cannot be used directly as food or feed because it contains toxic or anti-nutritional components such as phorbol esters, trypsin inhibitor, lectin, and phytate (Devappa et al., 2010; Makkar et al., 2008; Rakshit et al., 2008). In view of its favorable amino acid composition for food and feed application, research focuses on the detoxification of jatropha protein concentrates (Devappa and Swamylingappa, 2008; King et al., 2009; Lestari et al., 2013; Makkar et al., 2008; Makkar and Becker, 2009). Non-food applications of the protein from the jatropha seed cake to be applied as binders/glues, emulsifiers, protein films and plastics have been extensively studied (Lestari et al., 2010; Moure et al., 2006; Scott et al., 2007).

Since jatropha lignocellulosic biomass is a source of hexose (C6) and pentose (C5) sugars, de-polymerized fruit carbohydrate can also serve as a source for the production of biofuels, chemicals and other economically valuable by-products (Gonzalez-Garcia et al., 2010; Wyman, 1994; Yang and Wyman, 2008). Unlike starch carbohydrates which are easier to be hydrolyzed into fermentable sugars, carbohydrate fractions in lignocelluloses are not readily attacked by enzymes and

therefore a pretreatment stage is necessary (Chang et al., 1997; Cheng et al., 2010; Kaar and Holtzapple, 2000; Kong et al., 1992).

Pretreatment is a costly processing step in the conversion of cellulosic biomass to fermentable sugars with costs as high as 0.08 USD/liter ethanol produced (Mosier et al., 2005). Highly efficient conversion of cellulosic biomass to fermentable sugars is therefore essential for making fermentative products commercially competitive in biotechnological processes (Sun and Cheng, 2002). Various methods of biomass pretreatment are available, including physical and chemical pretreatments or a combination of both followed by enzymatic hydrolysis.

Acid pretreatment has advantages compared to other methods, i.e. the solubilization of hemicellulose, and therefore setting the cellulases free from steric hindrance during cellulose hydrolyzation (Mosier et al., 2005). However, a risk of inhibitor formation during strong acid pretreatment is one of the disadvantages (Lawford and Rousseau, 2003; Mosier et al., 2002). Dilute acid pretreatment can overcome excessive inhibitor formation because secondary reactions to furfural and HMF can be reduced under optimal pretreatment conditions. Dilute acid pretreatment commonly uses sulfuric acid in the concentration of 50-300 mM at 100-200 ^{o}C to disrupt the lignin-carbohydrate matrix, and to facilitate enzymatic cellulose hydrolysis (Lawford and Rousseau, 2003; Lloyd and Wyman, 2005; Mosier et al., 2005; Zhu et al., 2009).

The thermo-chemical treatment of almost any protein-rich biomass with alkali or acid – at an appropriate temperature, biomass concentration, and time – cleaves peptides bonds to release soluble amino acids and small peptides/proteins (Coward-Kelly et al., 2006a; Coward-Kelly et al., 2006b; Kootstra et al., 2011). Moreover, the acid pretreatment of proteins promotes the destruction of some essential amino acids, such as tryptophan (Westall and Hesser, 1974).

Pretreatment and enzymatic conversion of crop residues (e.g. wheat straw, barley straw, corn cob, corn stover, bagasse, etc.) are widely studied. However, very limited studies have been conducted yet on jatropha biomass hydrolysis. The subject of this study is to investigate the effect of dilute sulfuric acid pretreatment on the enzymatic digestibility of polysaccharides and hydrolysis of protein of jatropha fruit hulls, seed

shells and seed cake. We study the influence of dilute sulfuric acid (0 to 500 mM) at 120 ^{o}C for 30 minutes on the following two factors:

1. Xylose and glucose released from the milled jatropha biomass (seed shells, seed cake and fruit hulls) just after pretreatment and after enzymatic hydrolysis by cellulases (GC220, Genencor) for 24 h and 72 h.
2. Soluble proteins / amino acids released from the milled jatropha seed cake due to dilute acid pretreatment.

2. Materials and Methods

2.1. Materials

Jatropha curcas, L fruits, ripened to a yellowish state, were harvested on November 2009 from a small jatropha plantation located in Serpong, Indonesia. The hulls of the fruits were removed, collected and dried at 60 ^{o}C for 48 h. The dried hulls were milled in a home blender (Philips HR 2071) and sieved through a strainer with 0.8 mm hole diameter. The dry matter of milled jatropha fruit hull was 95.3% (*w/w*).

Jatropha seed shells were obtained by removing the kernels from the seeds. The shells were milled in a Retsch mill (1 mm sieve). The dry matter of milled jatropha seed shell was 90.6% (*w/w*). The kernels obtained were milled in a home blender (Philips HR 2071) and sieved through a strainer with 0.8 mm hole diameter. The oil in milled kernel was extracted in a Soxhlet apparatus using n-hexane at 80 ^{o}C for 6 hours to obtain oil-free kernel.

Jatropha seed cake was the byproduct in the production of oil from jatropha seeds by screw-pressing. The Jatropha seed cake (provided on September 2008 by The Energy Center BPPT, Serpong, Indonesia) was extracted with hexane in a continuous extractor to remove the remaining oil (performed at Pilot Pflanzenöltechnologie Magdeburg e.V., Magdeburg, Germany). The de-oiled seed cake was milled in a Retsch mill (1 mm sieve). The dry matter of milled de-oiled jatropha seed cake was 92.2% (*w/w*).

The milled jatropha seed shell, oil-free kernel, jatropha seed cake, and jatropha fruit hull were kept in sealed plastic containers at 4 ^{o}C until used. All chemicals were of

analytical grade and used as received. GC220 is a cellulase enzyme mixture (batch 4900759148, 7608 IU/mL cellulase activity).

2.2. Sulfuric acid pretreatments

Five grams of the milled jatropha seed shell, jatropha seed cake, or jatropha fruit hull was mixed with 45.0 mL of sulfuric acid solution (50, 100, 150, 200, 300 or 500 mM) in a 250-mL Erlenmeyer flask. Demineralized water was used as a control. Every experiment was performed in duplicate. The mixtures were soaked for 20-24 hours at room temperature. After measuring the pH after soaking, the mixtures were autoclaved at 120 °C for 30 minutes using TOMY Autoclave SS-325. The time required to reach 120 °C was 12 minutes. After the holding time of 30 minutes, the mixtures in the autoclave chamber were let to cool down to 98 °C; the time required to reach this temperature was 10 minutes. The mixtures were quickly removed from the autoclave and quenched in ice water to cool to 25°C in less than 10 min. After the pretreatment, the pH was adjusted to 5.0 with 10 M NaOH, and a sodium azide solution (0.025% (*w*/*w*) final concentration; Merck KGaA, Darmstadt, Germany) was added. A 1-mL sample was taken for analysis.

2.3. Enzymatic hydrolysis

To start enzymatic hydrolysis, GC220 (0.4 g (*w*/*w*) dry matter hull) was added to a 250-mL flask containing the pH-adjusted (pH 5.0) pretreated biomass. The amount of GC220 used corresponded to 46 FPU/g original dry matter hull. The weight of the material plus the flask was determined, after which the flasks were closed with airtight plugs and placed in a water-bath incubator shaker (50 °C, 120 rpm, 3 cm reciprocal stroke). Samples of 1.0 mL were taken at t = 0, 24, and 72 h. After enzyme inactivation by heating at 90 °C for 10 min, samples were stored at -20 °C until analysis.

2.4. Protein extractions

Protein extraction was carried out by extracting 1 g of sample with 30 ml NaOH 0.055 M for 30 min in 50 ml capped centrifuge tubes (Lestari et al., 2010). The mixing was

conducted at room temperature by using a rotary mixer. Solid–liquid separation was conducted at 5000 *x* gravity for 15 min using a KUBOTA centrifuge.

2.5. SDS-PAGE analysis

After hot acid pretreatments of the jatropha seed cake, jatropha protein from both the aqueous phase and solid phase (pH of 5.0) was subjected to SDS-PAGE (Biorad Electrophoresis System). The protein in aqueous phase was directly subjected to SDS-PAGE analysis, while protein in the solid phase was first extracted with NaOH 0.055 M.

2.6. Analyses

The chemical composition of the jatropha biomass was analyzed in triplicate according to TAPPI methods (TAPPI, 2004a; TAPPI, 2004b; TAPPI, 2004c; TAPPI, 2004d; TAPPI, 2004e; TAPPI, 2004f), with minor modifications as described previously (Kootstra et al., 2009b). Glucose and xylose were measured using enzyme kit D-Glucose (K-GLUC 07/2008) and D-Xylose (K-XYLOSE 03/07), respectively, both from Megazyme (Ireland). Other hexose sugars (mannose, rhamnose and galactose) and pentose sugar (arabinose) were measured by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) as described earlier (Kootstra et al., 2009b). The monomeric sugar yield was calculated as follows:

$$Yield\,(\%) = \frac{S_L}{S_S} * 100\,(\%) \qquad (1)$$

where S_L is the amount of sugar (g) in the aqueous phase and S_S is the amount of sugar present in the sample of dry biomass (g monomeric sugar equivalents in polymeric sugar).

The solubilized protein after sulfuric acid pretreatment was calculated as follows:

$$Solubilized\ \ Protein\ (\%) = \frac{N_L}{N_S} * 100\,(\%) \qquad (2)$$

where N_L is the amount of protein (g) measured in the aqueous phase and N_S is the amount of total nitrogen present in the sample of dry biomass.

3. Results and Discussion

3.1. Raw material compositions

Table 1 shows the composition of jatropha seed shell, jatropha seed-cake, and jatropha fruit hull.

Table 1. Composition (% *w/w* of dry matter) of jatropha fruit fractions.

		Seed shell	Seed cake	Fruit hull
Extractives in	Ethanol/ toluene	2.7	4.3	4.1
	Ethanol	0.5	1.4	2.0
	Water	5.3	9.2	36.2
	Subtotal	**8.5**	**14.9**	**43.3**
Polysaccharides	Arabinose	0.7	1.3	0.8
	Xylose[a]	12.1	7.3	5.7
	Mannose	1.3	1.0	1.1
	Galactose	1.0	1.0	2.0
	Glucose[b]	28.9	22.6	20.4
	Rhamnose	0.3	0.2	0.8
	Subtotal	**44.3**	**33.4**	**30.8**
Uronic Acids		**0.8**	**0.7**	**3.2**
Lignin	AIL	43.7	28.3	11.7
	ASL	0.3	0.6	0.8
	Subtotal	**44.0**	**28.9**	**12.5**
Protein		**nd[c]**	**21.5**	**5.0**
Total		**97.6**	**98.7**	**94.8**

[a] The total xylose used calculate the xylose yield
[b] The total glucose used to calculate the glucose yield
[c] Not detected

The jatropha seed cake consists of the remaining material of the nut after pressing, so the seed shell (48% *w/w*) and remains of the kernel (52% *w/w*). The jatropha fruit hull contains the highest amount of extractives (43.3%) of which 36.2% are soluble in water. The extractives of the jatropha fruit hull are 3 and 5 times higher than those of the jatropha seed cake and the jatropha seed shell, respectively. The jatropha seed shell contains the highest percentage of polysaccharides (44.3%) followed by the jatropha seed cake (33.4%) and the jatropha fruit hull (30.8%). These polysaccharide

contents are lower than those reported for other lignocellulosic materials such as wheat straw (59%) and cardoon biomass (52%) (Ballesteros et al., 2008; Kootstra et al., 2009a). The total lignin value is the highest in the jatropha seed shell (44.0%), followed by the jatropha seed cake (28.9%) and the jatropha fruit hull (12.5%). The acid-soluble lignin (ASL) will solubilize during acid pretreatment, while the acid-insoluble lignin (AIL) remains in solid form. The ASL values of these jatropha byproducts account for less than 1.0%, implying that the majority of lignin from jatropha fruits is resistant to acid treatment. The jatropha seed cake has a considerable protein content (21.5%), all derived from the seed kernels. This protein content is more than four times higher than that of the jatropha fruit hull. No protein has been detected in the jatropha seed shell.

3.2. Pretreatment and enzymatic hydrolysis of jatropha seed shell

Determination of optimum pretreatment conditions for the industrial process should take into account the feasibility of the process, including equipment and process costs under local conditions. Pretreatment at high temperatures would require expensive pressurized equipment (Kaar and Holtzapple, 2000). For this reason, we identified 120 ^{o}C as a suitable temperature to be applied in Jatropha biomass pretreatment because the generated steam pressure, 1 atmosphere, can be easily maintained under local conditions. We tested pretreatment of the jatropha seed shell with sulfuric acid concentrations between 0 and 500 mM with a short incubation time of 30 min.

The curves of xylose and glucose yields as percentages of the total amount of respectively xylan and glucan present versus sulfuric acid concentration during pretreatment at different hydrolysis times are shown in Figure 1. The values of xylose and glucose yields at time zero (0h) depict the xylose and glucose liberation just after pretreatment of the Jatropha seed shell.

Samples that were pretreated in absence of sulfuric acid did not contain free xylose or glucose, indicating all sugars of the jatropha seed shell were polymeric. The addition of enzymes to the jatropha seed shell pretreated in absence of sulfuric acid resulted in the release of 10% of the available xylose and glucose. All hydrolyzable sugar was released after 24 hours of hydrolysis; a prolonged incubation of 72 hours did not result in further sugar release.

Dilute sulfuric acid pretreatment resulted in a maximum release of 75% and 20% of xylose and glucose respectively. Sulfuric acid concentrations higher than 150 mM had a strong hydrolytic effect on hemicellulose, also without addition of saccharolytic enzymes. This implies that at sulfuric acid concentrations higher than 150 mM, xylan solubilisation into soluble oligomers occured simultaneously with their complete depolymerization into monomer (xylose).

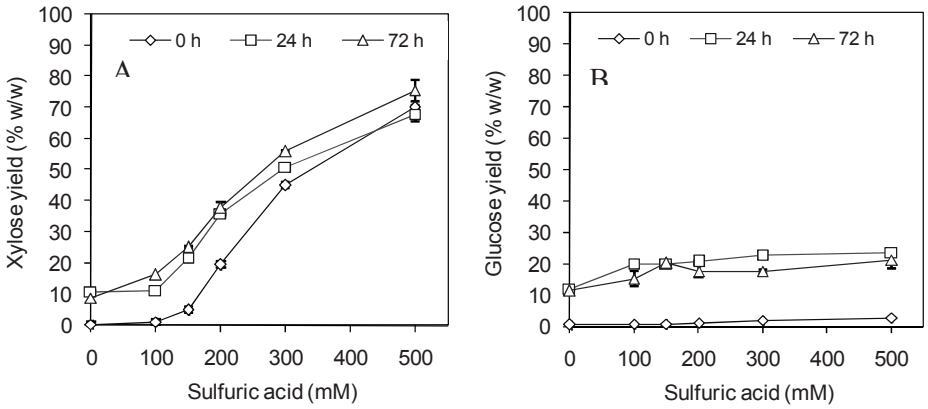

Figure 1. The trends of xylose yield (A) and glucose yield (B) of the sulfuric acid-pretreated jatropha seed shell during GC220 hydrolysis after 0, 24 and 72 h. Pretreatment conditions: 5.0 g milled jatropha seed shell, 45.0 mL sulfuric acid solution, 120 ^{o}C, 30 min. The milled jatropha seed shell (5.0 g; 4.53 g DM) contains = 1.31 g glucose and 0.55 g xylose.

Dilute sulfuric acid pretreatment had a slight positive effect on glucose release, but only 20% of total glucose could be released. It seems that the cellulose polymer of the jatropha seed shell was hardly cleaved by the hydrolytic enzymes. This is likely caused by steric hindrance of the cellulases by the lignin that was not affected by the pretreatment. Lignin acts as a physical barrier between cellulolytic enzymes and cellulose. Maximum conversion of cellulose into monomeric sugars occurs only if 50% or more lignin has been removed (Gould, 1984). Therefore, delignification of the jatropha seed shell with a proper method seems essential in achieving better conversion yield of glucose.

3.3. Pretreatment and enzymatic hydrolysis of jatropha seed cake

The jatropha seed cake was treated using the same conditions for pretreatment and hydrolysis as used for the jatropha seed shell. The curves of xylose and glucose yields versus sulfuric acid concentration at different hydrolysis times are shown in Figure 2.

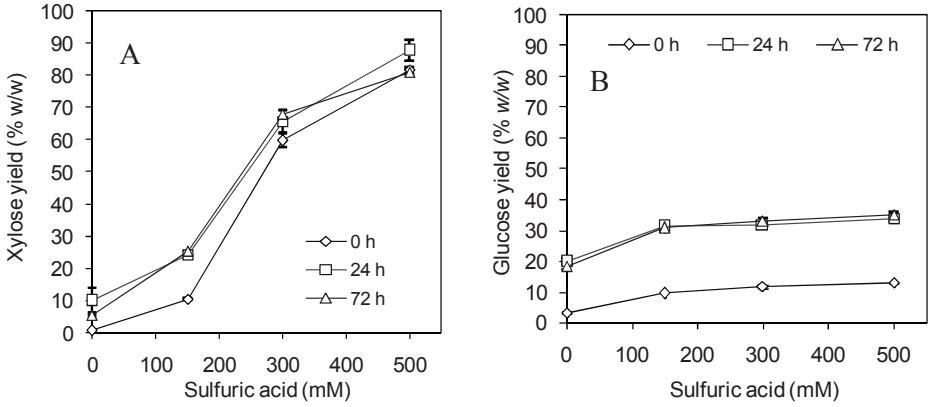

Figure 2. The trends of xylose yield (A) and glucose yield (B) of the sulfuric acid-pretreated jatropha seed cake during GC220 hydrolysis after 0, 24 and 72 h. Pretreatment conditions: 5.0 g milled jatropha seed cake, 45.0 mL sulfuric acid solution, 120 ^{o}C, 30 min. The milled jatropha seed cake (5.0 g; 4.61 g DM) contains = 1.04 g glucose and 0.34 g xylose.

The values of xylose and glucose yields at time zero (0h) depict the amount of glucose and xylose liberated just after pretreatment of the jatropha seed cake.

The release of xylose and glucose from pretreated the jatropha seed cake strongly resembles that of pretreated the jatropha seed shell, with slightly higher sugar release: 90% for xylose and 35% for glucose. The jatropha seed cake contains 48% seed shell and 52% seed kernel. Based on Table 1 it can be calculated that the kernel contributes 38% and the seed shells 62% of the total glucan present in the jatropha seed cake. If we assume that the release of glucose from the seed shell fraction in the jatropha seed cake is the same as for the jatropha seed shell (20%, see figure 1), this would mean that 60% of the glucan in the kernel fraction of the jatropha seed cake was hydrolyzed to glucose.

To summarize our findings with respect to the jatropha seed shell and the jatropha seed cake hydrolysis, we conclude that hemicellulose depolymerization of the

jatropha seed shell is not the only factor in opening the barriers for cellulose hydrolysis. The lignin in seed shell which mostly remains intact following dilute sulfuric acid pretreatment has become a major barrier which in turn limits the accessibility of the enzymes to hydrolyze the cellulose.

The jatropha seed cake contains approximately 20% of protein. The utilization of this protein in technical applications to maximize the economic benefit requires that the protein remains largely intact. The protein solubilized from the jatropha seed cake just after pretreatment (120 °C, 30 minutes) given by different sulfuric acid concentration is shown in Figure 3.

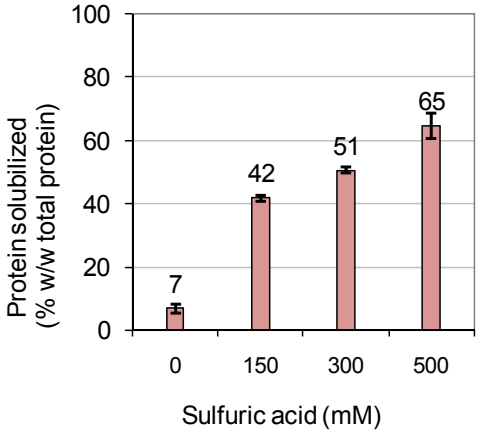

Figure 3. Protein of the jatropha seed cake solubilized after dilute acid pretreatment compared to water pretreatment at 120 °C for 30 min. Pretreatment conditions: 5.0 g jatropha seed cake, 45.0 mL sulfuric acid solution, 120 °C, 30 min. The milled jatropha seed cake (5.0 g; 4.61 g DM) contains 0.99 g protein.

In general, the amount of soluble protein increased significantly from the jatropha seed cake pretreated with sulfuric acid. Upon pretreatment with 500 mM sulfuric acid at 120 °C for 30 minutes, 65% soluble protein / peptides / amino acids (equivalent to 140 mg protein/g dry the jatropha seed cake) was released from the the jatropha seed cake sample, whereas only 7% soluble protein / amino acids (equivalent to 15 mg protein/g dry the jatropha seed cake) was released from the control sample (pretreated in absence of sulfuric acid).

SDS-PAGE analyses of the liquid phase and solid phase are shown in Figure 4 and 5. In general, the SDS-PAGE patterns of proteins in the jatropha seed cake (JSC) and the jatropha seed kernels (JSK) look almost the same, except that the proteins of 15 kDa from JSC are more visible than those from JSK, indicating difference sources of

jatropha seeds (used in this study) resulted in different concentration of the protein subunits.

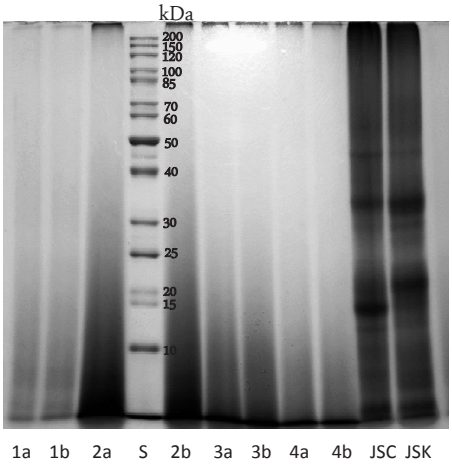

Figure 4. SDS-PAGE analysis of the protein in aqueous phase of the acid-pretreated jatropha seed cake. Bands of proteins from duplicate samples after pretratment with 0 mM (1a and 1b), 150 mM (2a and 2b), 300 mM (3a and 3b), and 500 mM (4a and 4b) of sulfuric acid solutions in comparison to NaOH-extracted jatropha protein from Jatropha seed cake (JSC) and from the oil-free jatropha seed kernels (JSK). S stands for protein molecular weight marker.

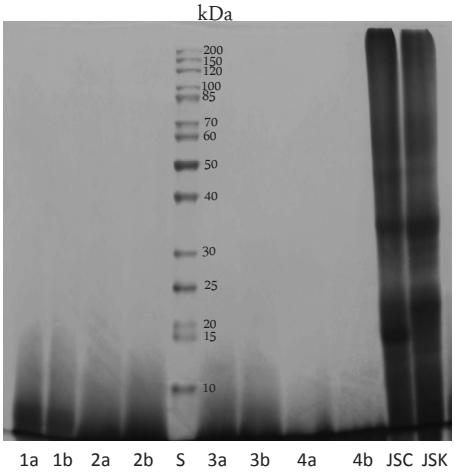

Figure 5. SDS-PAGE analysis of the NaOH-extracted protein from solid phase of acid-pretreated the jatropha seed cake. Bands of proteins from duplicated samples after prtreatment with 0 mM (1a and 1b), 150 mM (2a and 2b), 300 mM (3a and 3b), and 500 mM (4a and 4b) sulfuric acid solutions in comparison to NaOH-extracted jatropha protein from the jatropha seed cake (JSC) and from the oil-free jatropha kernels (JSK). S stands for protein molecular weight marker.

The protein bands of more than 200 kDa (located at the top of the gel) that were visible in the aqueous phase (Figure 4) were found totally invisible in the solid phase (Figure 5), indicating that these large proteins (higher than 200 kDa) were completely solubilized. These proteins seemed to be highly stable against the pretreatment conditions tested. In contrast to these large proteins, the smaller proteins (less than 200 kDa) were highly prone to degradation upon pretreatment, leading to their complete solubilisation.

As summary to SDS-PAGE analyses, pretreatments either with water only or with dilute sulfuric acid at 120 °C for 30 minutes resulted in a severe reduction of molecular weight of the less than 200 kDa proteins available in the the jatropha seed cake to below 10 kDa. Acid-hydrothermal treatment is known to cleave peptide bonds of protein into smaller, more soluble peptides and amino acids (AOAC, 1982).

3.4. Pretreatment and enzymatic hydrolysis of jatropha fruit hull

The jatropha fruit hull was pretreated in the same way as the jatropha seed shell and the jatropha seed cake.

Without enzymatic hydrolysis, the xylose yield which was initially low (approx 5%) but increased rapidly at sulfuric acid concentrations above 150 mM to achieve approx 80% at 500 mM sulfuric acid, indicating that susceptibility of the hemicellulose polymer against acid attack at 120 °C was more pronounced at sulfuric acid concentrations higher than 150 mM (Figure 6). For tougher biomass like corn stover, higher acid concentrations up to 1400 mM at higher pretreatment temperature (165-195 °C) with residence time of 3-12 minutes were needed to obtain more than 70% hemicellulose solubilization (Schell et al., 2003; Um et al., 2003). Hemicellulose solubilization of 96% from soybean hulls was achieved when the biomass was pretreated using 200 mM sulfuric acid at 140 °C for 30 minutes in combination with steam explosion (Corredor et al., 2008).

Without enzymatic hydrolysis the release of glucose from pretreated the jatropha fruit hull was low,- up to 5% glucose was released at 500 mM sulfuric acid - indicating that dilute sulfuric acid pretreatment conserved most of the cellulose in the solid form, an advantagous trait if the cellulose has to be separated from the hemicellulose for further hydrolysis.

After 24 h of enzymatic digestion of the jatropha fruit hull, pretreated in absence of sulfuric acid, the xylose yield and the glucose yield were increased by 15% and 40%, respectively, implying that heating contributed to the cleavage of long-chain carbohydrate polymers into smaller chains which facilitated further hydrolysis. It therefore can be concluded that hydrothermal treatment at 120 °C improved to some extent the enzymatic digestibility of the jatropha fruit hull carbohydrates. It was reported that heating lignocellulosic biomass in aqueous media at 150°C to 180°C is

required to solubilize parts of the biomass, mainly the hemicellulose and the lignin (Hendriks and Zeeman, 2009)

The addition of enzymes improved the xylose yield along the sulfuric acid range used for pretreatment with approximately 15% (Figure 6). Since the increase of the xylose yield is always the same, we may assume that the cellulases (GC220) liberate xylose from a different origin within hemicellulose structure of the jatropha fruit hull than the acid does. A complete hemicellulose solubilization (100%) was achieved at 500 mM sulfuric acid after 24 hours of enzymatic hydrolysis. Upon enzymatic hydrolysis for 24h, the glucose yield increased to 70% at 500 mM sulfuric acid. However, as much as 30% of the cellulose left as indigestible matter which requires a more severe petreatment condition to increase its enzymatic digestibility (Kabel et al., 2007).

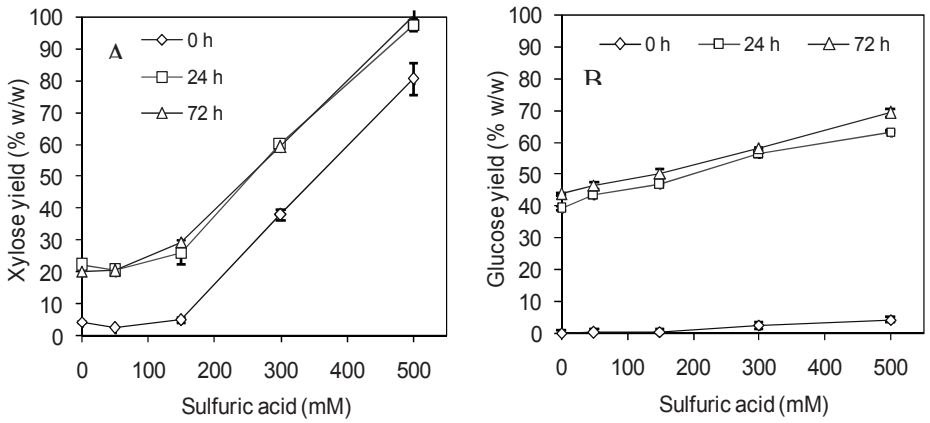

Figure 6. The trends of xylose yield (A) and glucose yield (B) of the sulfuric acid pretreated jatropha fruit hull during GC220 hydrolysis after 0, 24 and 72 h. Pretreatment conditions: 5.0 g milled jatropha fruit hull, 45.0 mL sulfuric acid solution, 120 ^{o}C, 30 min. The milled jatropha fruit hull (5.0 g: 4.77 g DM) contains = 1.13 g glucose and 0.48 g xylose.

To summarize our findings with respect to the jatropha fruit hull hydrolysis, pretreatment converted 100% xylan and 70% glucan available in the jatropha fruit hull into their monomeric sugars. The use of high sulfuric acid for industrial process would lead to large amounts of gypsum produced with respect to the use of as cheapest neutralizing agent, which can negatively affect the downstream process, and also results in a low-value-by-product stream (Kootstra et al., 2009a; Yang and Wyman, 2008). To reduce sulfuric acid concentration and time of pretreatment in the

jatropha fruit hull pretreatment, conducting pretreatment at a temperature higher than 120 °C is necessary, although robust design of equipments in industrial process, as a consequence, will be required to resist high pressures and corrosive chemicals (Yang and Wyman, 2008).

4. Conclusions

The lignocellulose fractions of the jatropha seed shell or the jatropha seed cake are relatively recalcitrant to dilute acid pretreatments. Delignification with a suitable method to disrupt lignin barriers seems essential to improve enzymatic digestibility of the jatropha seed shell or the jatropha seed cake for the release of more sugars. The lignocellulose fraction of the jatropha fruit hull proves relatively more susceptible to hydrolysis by cellulase following pretreatment with sulfuric acid than that of the jatropha seed shell or the jatropha seed cake. Therefore, the susceptibility of jatropha biomass studied against acid pretreatment and enzyme hydrolysis has a direct correlation with the total lignin content, in which more sugars are released at lower lignin content. The total lignin value is the highest in the jatropha seed shell (44.0%), followed by the jatropha seed cake (28.9%) and the jatropha fruit hull (12.5%).

As much as 70% glucan and 100% xylan conversions were obtained from the jatropha fruit hull after a pretreatment with 500 mM sulfuric acid at 120 °C for 30 minutes. Pretreatments at higher temperatures (more than 120 °C) may reduce sulfuric acid concentration and time of pretreatment to obtain a comparable or higher sugar yields from the the jatropha fruit hull. Therefore, optimisation of pretreatment parameters such as time and temperature of pretreatments as well as dilute sulfuric acid concentration is a challenge for the upcoming investigation with the aim to improve process economy and to limit the use sulfuric acid.

Dilute acid pretreatment solubilized more than 65% the available protein in the jatropha seed cake. The acidic the jatropha seed cake hydrolyzate rich in amino acids may be further fractionated into single amino acids for the production of N-chemicals. Because the jatropha seed cake contains antinutritional factors that are highly toxic to animal, the toxicity and the nutrition level of the resulting acidic the jatropha seed cake hydrolyzate needs to be thoroughly studied before application as animal feed is feasible.

Acknowledgements

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CHAPTER 3

Coconut oil extraction by the traditional Java method: An investigation of its potential application in aqueous jatropha oil extraction

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Abstract

A traditional Java method of coconut oil extraction assisted by paddy crabs was investigated to find out if crabs or crab-derived components can be used to extract oil from *Jatropha curcas*, L seed kernels. Using the traditional Java method the addition of crab paste liberated 54% *w/w* oil from grated coconut meat. Oil extraction using crab paste carried out under controlled temperatures and in the presence of antibiotics showed that enzymes from crab played a dominant role in liberating oil from grated coconut meat and aqueous jatropha kernel slurries when incubated at 30^{o}C or 37^{o}C. However, at higher temperature (50^{o}C), thermophilic bacterial strains present inside crabs played a significant role in the extraction of oil from both oilseeds tested. A thermophilic bacterial strain isolated from crab paste and identified based on 16s rRNA sequence as *Bacillus licheniformis* strain BK23, when added as starter culture, was able to liberate 60% *w/w* oil from aqueous jatropha kernel slurry after 24h at 50^{o}C. Further studies of BK23 and extraction process optimization are the challenges to improve jatropha oil extraction yield and process economy.

Keywords:
Java method, Coconut oil, *Cocos nucifera*, Jatropha oil, Paddy crabs, Thermophilic bacteria, *Jatropha curcas*, L, PCR, 16s rRNA, *Bacillus licheniformis*, Extraction economics

1. Introduction

Coconut is an established oil source for food and chemical applications due to its abundant availability in most tropical regions like Indonesia. Coconut is a traditional source of lauric oil used in a number of industrial products with well- developed processing technologies on large scale (TanoDebrah and Ohta, 1997). *Jatropha curcas*, L seeds are known as a potential oil source, which is, however, unsuitable for use in food due to anti-nutritional factors (Gubitz et al., 1999; Makkar et al., 1997). The use of jatropha oil as biodiesel has been described (Foidl et al., 1996; Makkar et al., 1997). The sustainable industrial production of biofuels from jatropha requires the development of efficient production and conversion processes, e.g. for the release of oil from the seeds.

Processes for removing oil from oilseeds can be categorized into solvent extraction, wet, and dry processing methods. Even though solvent extraction with hexane is the most efficient process, environmental and safety issues have been the major drawback of prolonged hexane use for vegetable oil extraction due to its toxic properties (Man et al., 1997; Rosenthal et al., 1996). In many developing countries where coconut is easily grown and chiefly produced, traditional wet methods of oil extraction are still practiced despite the availability of more efficient and modern processes (Man et al., 1996). One of the traditional wet methods practiced over the years in Java involves heating coconut milk to the boiling point in order to vaporize the water phase, leaving the oil above the residue to be collected. Generally, the traditional wet method results in low oil recovery (only 30-40% *w/w*) with inferior quality due to high moisture content and short shelf life (Hagenmai.R et al., 1973; Man et al., 1996). In some rural areas of eastern Java in Indonesia, indigenous people have practiced traditional coconut oil extraction for a long time using mashed wild paddy crabs collected from bunds. In the extraction process, crushed crab is added to grated coconut meat, wrapped tightly with a banana leaf or a plastic bag, and incubated overnight after which the oil is squeezed out. The process is a cold process (no fire wood required) and only mild heating under sunlight is applied to reduce the water content before squeezing. The oil yield of this process is 50-65% *w/w*, about 1.7 times more efficient than the traditional wet method (Haryoto, 1983).

The mechanism of oil liberation from grated coconut meat by crab paste is not well understood. Protocols have been described for coconut oil extraction on 100 kg scale of coconut meat, including a method for the propagation of microbial starter cultures from the mashed crab and grated coconut mixture in order to overcome the limited availability of crab (Haryoto, 1983). This indicates that oil release is a microbial process, but no scientific investigations have studied the microbial strains involved. Up to now this method has only been applied for the release of oil from coconut, its applicability for the recovery of oil from biofuel crops has not been assessed so far.

In the present report, coconut oil separation assisted by homogenized paddy crab was investigated and compared with literature values for conventional processes. The same method was applied to jatropha kernel slurries in order to assess its potential to liberate oil from this biofuel crop. Antibiotics were used in some treatments to distinguish between ongoing microbial degradation versus enzymatic effects. Bacterial strains able to release oil from both oil crops were isolated from the paddy crab. The best strain was identified on basis of its 16s rRNA sequence. The cost production of jatropha oil extraction employing isolated bacterial strain is also assessed and compared to that of mechanical oil extraction process (motor press).

2. Materials and Methods

2.1. Materials

Mature coconuts (from *Cocos nucifera,* L) were bought from a traditional market. Paddy crabs were collected on January 5th 2008 from bunds of a paddy field located in Pamulang, Indonesia (geocoordinates 6°20'52"S, 106°42'20"E). *Jatropha curcas*, L seeds were harvested on July 28th 2007 from jatropha plants more than 16 months old, grown in Serpong, Indonesia (geocoordinates 6°21'31"S, 106°40'33"E), kept dried at 4 °C until used. All chemical reagents, unless otherwise specified, were of analytical grade.

2.2. Traditional Java method of coconut oil extraction

Two crabs, equivalent to about 15 g, were cleaned in tap water and crushed with 30 ml demineralized water in a home blender and mixed with 150 g grated coconut meat.

Chloramphenicol and tetracycline each were added in a concentration of 1 g/kg coconut meat for antibiotics-treated samples. The mixture was tightly wrapped in a banana leaf and left stationary overnight (20-24h) at room temperature (28-32^{o}C). The samples were tested in duplicate for these combinations: (1) coconut only as control; (2) coconut and crab; (3) coconut, crab, chloramphenicol, and tetracycline. After incubation, the mixture was dried under sunlight for one day. The mixture was then squeezed using a dry screen cloth to remove the oil. The amount of oil obtained was directly weighed.

2.3. Crab paste preparation

Crabs of medium-to-big size (250 g in total) were cleaned with running tap water for 10 min after which they were soaked twice, first with diluted commercial NaClO solution at a concentration of 0.86mM (the CAS number 7681-52-9), followed by second soaking in 70% ethanol, for 5 min each. Crabs were rinsed and cleaned in sterile water several times, then homogenized aseptically for 5 min using a home blender (Philips) with 250 g sterile water. Total crab paste weight obtained was 500 g. Final crab concentration in the paste was 50% *w/w*.

2.4. Jatropha seed kernels slurry preparation

Jatropha seeds were cracked using a press beam and the husks (outer layer) were carefully removed. The kernels were pulverized and sieved through a strainer with 0.8 mm hole diameter to obtain a powder of homogenous particle size. To prepare the oilseed slurry, 150 g kernel powder was blended with 750 g demineralized water for 5 min using a home blender. Weight ratio of solid material to water in the slurry was 1:5. Under constant stirring to keep the slurry homogenous, 30 g of kernel slurry (equivalent to 5 g kernel) was weighed out and used for the oil extraction.

2.5. Coconut oil extraction assisted by crab in controlled conditions

To extract coconut oil, 20 g wet grated coconut meat was mixed with 8 g of crab paste. Chloramphenicol and tetracycline were added in concentrations of 1 g/kg coconut for antibiotic-treated samples. The mixture was incubated for 48 h at various temperatures (30, 37, or 50^{o}C) without stirring or shaking. The samples were

incubated in duplicate for the combinations: (1) coconut only as control; (2) coconut and crab; (3) coconut, crab, chloramphenicol, and tetracycline. After incubation, the content was transferred into 50 ml Falcon centrifuge tubes and 20 ml of water added. The tubes were reciprocally shaken at high speed (4 Hz) for 30 min to detach free oil from solid into liquid phase and then centrifuged at 500× gravity for 15 min. The amount of oil obtained was determined gravimetrically.

2.6. Jatropha oil extraction assisted by crabs

To extract jatropha oil, 2.0 g of crab paste was mixed with 30.0 g kernel slurry, shaken at 120 rpm for 24h at 37 or 50 ^{o}C. The samples were incubated in duplicate for the combinations: (1) kernel only as control; (2) kernel and crab; (3) kernel, crab, chloramphenicol, and tetracycline. After incubation, the mixture was centrifuged at 7400× gravity for 15 min. The extracted oil was assayed gravimetrically.

2.7. Crab bacteria isolation and selection

Grated coconut meat (5.0 g wet weight) was autoclaved at 121^{o}C for 15 min. After cooling to room temperature, 2 g crab paste was mixed with the autoclaved coconut and incubated at 50 ^{o}C for 48h. After incubation, one loop of the coconut-crab mixture was streaked out aseptically on nutrient agar (NA) media plates (Oxoid) and incubated at 50 ^{o}C for 24 h. Well separated colonies were picked out and sub-cultured on an NA medium slant in a tube of 15 cm length and 1.5 cm diameter, incubated at 50 ^{o}C for 24 h. The strains isolated were maintained on NA medium slants.

The isolated bacterium strains were selected for the best strain to extract oil from jatropha kernels with the following procedure: cells from a 24-h old slant culture were suspended in 2 ml sterile water and used to inoculate 30.0 g jatropha kernel slurry in a 100 ml shake flask, then incubated at 50 ^{o}C for 24 h. After incubation, the mixture was centrifuged at 7400× gravity for 15 min. The extracted oil was assayed gravimetrically.

2.8. Jatropha oil extraction assisted by starter culture of isolated bacterium

To prepare a bacterial starter culture, 50 ml germination medium (nutrient broth (NB), Oxoid) in a 250 Erlenmeyer flask was autoclaved at 121^{o}C for 15 min. Bacterial

cells from the slant were suspended in 2 ml sterile water, transferred into 50 ml NB medium and shaken at 120 rpm and 50 °C for 24 h. To extract the oil, 30.0 g jatropha kernel slurry was inoculated with 2.5 ml of the bacterial culture. The mixture was shaken at 120 rpm for 24 h (50 °C). After incubation, the slurry was centrifuged at 7400× gravity for 15 min. The extracted oil was assayed gravimetrically.

2.9. Gravimetric analysis of extracted oils

Petroleum ether (PE 40 – 60 °C) was used to dilute the free oil after microbial extraction to prevent oil loss due to small sample used (Lamsal and Johnson, 2007; Winkler et al., 1997a). The free oil on the surface of the liquid in the centrifuge tube was diluted with 3 ml petroleum ether without shaking (to prevent oil extraction from emulsion) over a minimum of 6 h. The top organic phase was carefully removed, and another 2 ml PE was used to rinse the top layer. The organic phases were collected in a pre-weighed dish and dried at 105 °C for 1.5-3 h until constant weight was reached.

2.10. Assay of total oil content, oil yield, and oil quality

Total oil in oilseeds was determined by Soxhlet method (AOAC, 1984). The total oil content was 0.35 kg/kg coconut meat and 0.47 kg/kg jatropha seed kernels. The total oil content was taken as 100% recovery of oil while calculating the oil yield. The free fatty acid (FFA) and moisture content was assayed by titration method and Karl Fischer method, respectively (AOAC, 2002b). Oxidative stability was assayed using 873-Biodiesel-Rancimat apparatus from Metrohm.

2.11. Identification of isolated bacterium

Genomic DNA was isolated by treating the bacterial cells using a FastPrep@DNA kit (MP Biomedicals, USA). The 16s rRNA sequence (ca. 1.1-kbp fragment) was amplified by PCR with the primers 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGATACCTTGTTACGA CTT-3'. The amplification was done by Thermalcycler Dice (Takara, Kyoto, Japan) with the following cycling parameters: 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C with final extension for 10 min. The PCR fragment was extracted, purified, and sequenced. Sequencing was performed with AB 3130xL Sequencer (Applied Biosystem) using ABI

BigDye v3.1 cycle sequencing kit with LA Taq (Takara) and appropriate primers. The rRNA sequence was blasted to NCBI database (http://www.ncbi.nlm.nih.gov/). The homology relationship was analyzed with ClustalX to create a dendrogram using the online analysis service available at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

2.12. Calculation of production cost

2.12.1. Aim of cost analysis

Cost analysis aims at comparing jatropha oil production cost between microbial process and other processes that have been commercially practiced (expeller extraction). To simplify cost analysis, the calculation is done on daily basis and focused on energy consumption, raw material cost, labor cost, and depreciation cost of general equipment involved in the process. Moreover, cost of energy, raw material, and labor are based on Indonesian price standards. Unit operations of a microbial assisted jatropha oil extraction factory with a capacity of 1000 kg/d of seeds is shown in Figure 1.

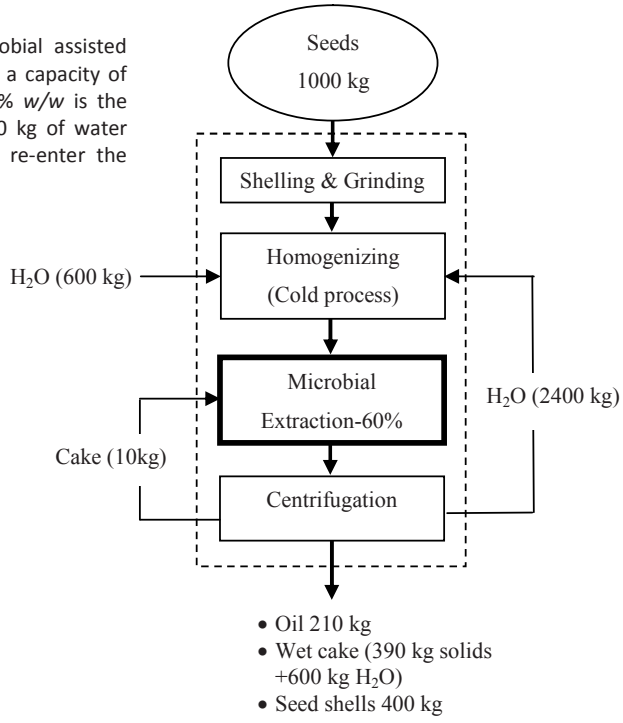

Figure 1. Unit operations of microbial assisted jatropha oil extraction factory with a capacity of 1000 kg/d of seeds. A yield of 60% *w/w* is the maximum oil extraction yield. 2400 kg of water (80%) is recycled and 10 kg cake re-enter the extraction reactor as inoculums.

2.12.2. Capital cost

Capital investment for a small jatropha oil factory (740 kg/d of seeds) using motor press, equipped with scales and utensils have been reported to be 5410 USD, linearly depreciated for 7 years (Openshaw, 2000). In a microbial extraction factory, the total price of main equipment is estimated to be 20400 USD (Appendix A1). The cost of installed equipment is estimated double of the total equipment price because additional budget is required for piping, control system, and installation, accounted for 40800 USD in total. Assuming that all equipment has the same lifetime of 7 years operation (250 d/yr or 1750 day in 7 years), the depreciation cost would be 23.3 USD/d. Taking into account maintenance cost (10% of capital investment per year equals to 16.3 USD/d) the capital cost becomes 39.6 USD/d without interest costs.

2.12.3. Energy cost

Electricity is the major energy source, except that required for water heater. The water heater providing 50 kg/h of 90 ^{o}C water is fueled by 400 kg/d of seed shells (LHV 17 MJ/kg) which are available as a byproduct on site. The industrial rate for electricity is 60 USD/MWh (Openshaw, 2000).

The energy components are calculated on the basis of 1000 kg of sun-dried jatropha seeds (10–12% moisture) that enters the process. Energy component from electric power is estimated based on equation of electric power consumption described earlier (Jekayinfa and Bamgboye, 2006):

Electrical energy consumed, E_p is expressed as:

$$E_p = \eta P t \ (kWh) \tag{1}$$

where P, rated power of motor, kW; t, hours of operation, h; η, power factor (assumed to be 0.8).

Main equipment, energy sources, and estimation of energy cost in a microbial jatropha oil processing plant are summarized in Appendix A2.

2.12.4. Raw materials and labor cost

Chemicals and water cost is estimated to be 5 USD/tonne of seeds, lower than their real cost for one-cycle process (about 20 USD/tonne of seeds), because 80% of water is recycled and 3% of protein cake re-enter the process as inoculums. Price of jatropha seed on factory site is 0.103 USD/kg (Openshaw, 2000). Oil container cost also included in the calculation. The labor cost is priced at 0.4 USD/person/h. The number of man-hour is shown Appendix A3.

3. Results and Discussion

3.1. Traditional Java method of coconut oil extraction

From Figure 2, without addition of crab paste (control) no free oil was obtained. Addition of crab paste alone liberated 54% coconut oil, agreeing well with that previously reported by Haryoto (Haryoto, 1983). Addition of crab paste in combination with antibiotics resulted in a small increase of the oil yield to 60%. In the antibiotic-treated samples, in fact, we could not discriminate crab enzymes from microbial enzymes that played the dominant role in oil liberation, because enzymes may come either from the crab muscle or from microorganisms living in crab's gastrointestinal tract before incubation.

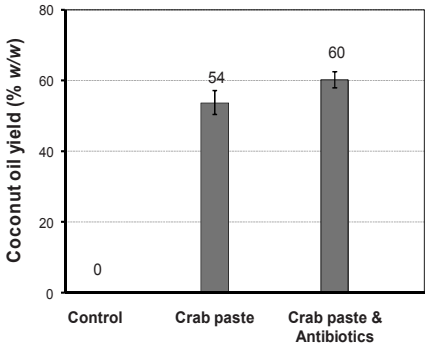

Figure 2. Coconut extraction yield assisted by crab paste according to traditional Java method.

3.2. Coconut oil extraction assisted by crab in controlled conditions

In this experiment, we studied the effect of incubation temperature on coconut oil yield. In Figure 3, the free oil in control samples decreased as temperature increased, ranging from 10% to 22%. Addition of crab paste doubled the oil yield from 22% to 42% at 30 ^{o}C and the yield remained relatively constant (45%) at 37 ^{o}C. The oil yield was significantly improved to 62% when the mixture was incubated at 50 ^{o}C. A different pattern of oil yield was observed if antibiotics were added, in which the

amount of oil extracted initially increased from 44% at 30°C to 53% at 37 °C, but then decreased to 34% at 50 °C.

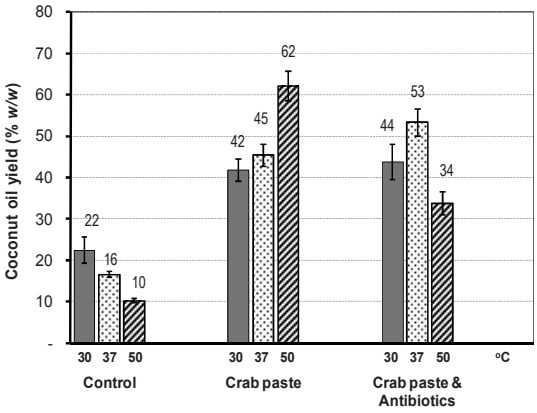

Figure 3. Coconut oil extraction yield assisted by crab paste at 30, 37 and 50 °C.

From these results we conclude that two mechanisms are involved in oil release by crab paste: an antibiotic insensitive mechanism – most likely enzymatic – dominating at lower temperatures and an antibiotic sensitive mechanism – likely thermophilic bacteria – active at higher temperatures. In the traditional Java method the role of bacterial activity in oil release could not be discriminated from the role of crab enzymes due to the lower incubation temperatures.

3.3. Application of crab paste to release oil from jatropha kernels

In Figure 4, the oil extracted in control samples was almost zero at 37 °C and only 9% at 50 °C. These results show that without crab paste addition, no or little oil liberated from the kernel slurry, even at higher temperatures (50 °C). The oil yields from this experiment were lower than those of aqueous jatropha oil extraction incubated overnight at 37, 40 and 50 °C, which was reported in the range of 17-21% (Shah et al., 2005).

Figure 4 also shows that addition of crab paste resulted in the release of approximately 30% jatropha oil at both temperatures tested. Inhibition of bacteria by antibiotics at 37 °C in kernel-crab mixture showed no effect on oil liberation; however,

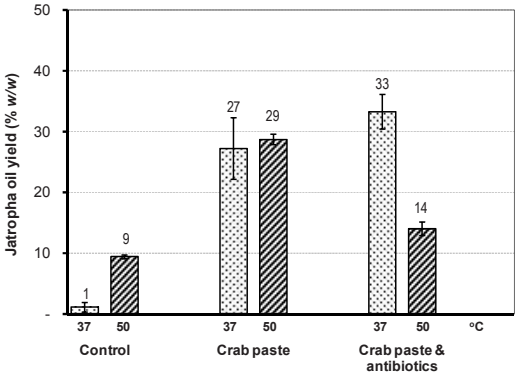

Figure 4. Jatropha oil extraction yield assisted by crab paste at 37 and 50 °C.

by increasing the temperature to 50 °C, the oil yield dropped to 14%. Furthermore, inhibition of bacteria that are resistant to high temperatures (50 °C) by addition of antibiotics caused a sharp fall of oil yield. These results strengthen our assumption that both crab enzymes and bacteria present in crab paste are involved in the liberation of oil from these oil seeds.

3.4. Isolation and selection of crab bacteria

We isolated 4 different rod-like thermophilic bacteria from crab at 50 °C: BK21, BK22, BK23, and BK24 (Note: BK stands for Bakteri Kepiting=crab bacteria). These strains were able to grow at room temperature (data not shown); indicating that the strains are facultative thermophiles.

Selection result of bacterial strains on jatropha kernel slurry is shown Figure 5. BK23 gave the best oil yield of 51%, 1.7 times higher than that obtained from incubation with crab paste at the same temperature (Figure 4). Selection results revealed that pure cells of the isolated strains generally gave higher jatropha oil yields compared to previous results of oil extraction using crab paste.

The presence of bacterial culture in oil separation would be a disadvantageous trait for the industrial application if the bacterium itself consumes the oil. We, therefore, examined if BK23 consumed coconut oil when incubated for 48 h at 50°C (Figure 6). The maximum coconut oil extracted by BK23 was found to be 59%, agreeing well with a similar treatment using crab paste. The total oil recovery – oil extracted plus oil left behind in the coconut material – was 100% indicating that BK23 does not degrade oil for its metabolic activities.

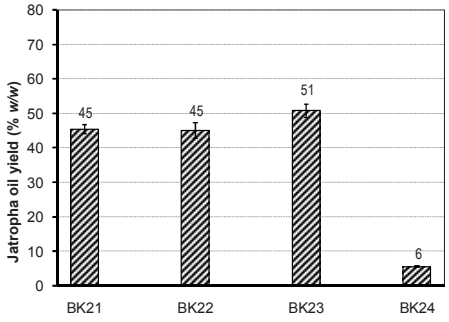

Figure 5. Jatropha oil extraction yield assisted by selected bacterial strains on jatropha kernel slurry to select best strain in oil liberation.

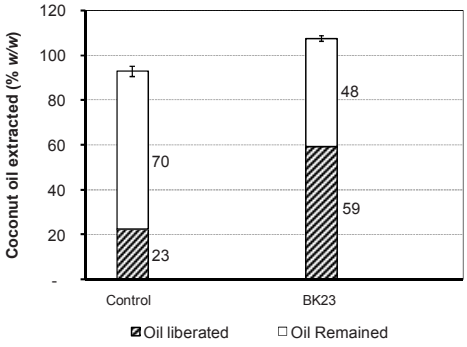

Figure 6. Coconut oil extraction yield assisted BK23 starter culture in comparison to control, incubated at 50 °C for 48 h.

3.5. Microbial assisted jatropha oil extraction and the quality of oil

Jatropha oil liberation from kernel slurry by starter culture of BK23 at 37 °C and 50 °C is shown in Table 1. The oil yields correspond well with those obtained with crab paste: BK23 liberated more oil at 50 °C (60%) than at 37 °C (47%).

Oil quality analysis data (Table 2) show that, in general, microbial assisted oil extraction gave better oil quality compared to screw-press, disregarding seed sources. However, the free fatty acid (FFA) value of oil from microbial process is about 4 times higher than that of German fuel standard DIN V 51605 for pure plant oil (rapeseed oil).

Table 1. Jatropha oil extraction yield assisted by BK23 starter culture.

Temperature	Oil extracted			
	g	%	Yield (% *w/w*)	Mean yield (% *w/w*)
37 °C	1.106	22.1	47.1	47
	1.133	22.7	48.2	
50 °C	1.417	28.3	60.3	60
	1.416	28.3	60.3	

Table 2. The quality of oil from microbial process compared to that of screw-pressed. IP (induction period) is the time at which the oil starts to be oxidized at 110 ^{o}C by O_2 from purified air.

	IP (h)		Water (ppm)		FFA (%)		Remarks
	Sample	Std[a]	Sample	Std[a]	Sample	Std[a]	
Microbial extracted oil	5.6	5.0	718	750	4.4	1	Indonesian seeds (6 months)
Screw-pressed oil	15.7	5.0	1550	750	13.3	1	Indian seeds (6 months)

[a] German fuel standard DIN V 51605 for pure plant oil (rapeseed oil). Std = standard value.

3.6. Identification of strain BK23

The similarity rank analysis based on the 16s rRNA sequence showed that the strain BK23 is closely related to members of the genus *Bacillus* (Figure 7). Based on these data, we propose the assignment of this isolate as ***Bacillus licheniformis* strain BK23**. The nucleotide sequence of 16S rRNA from *Bacillus licheniformis* BK23 isolated from paddy crab is available in the GenBank nucleotide sequence database under accession number FJ775733.

It is well known that microorganisms from the genus Bacillus in general and *B. licheniformis* specifically are used for the industrial production of alkaline and heat stable proteases (Hubner et al., 1993; Lee and Chang, 1990; Mabrouk et al., 1999; Potumarthi et al., 2007; Potumarthi et al., 2008). It is, therefore, likely that the observed oil liberation is caused by excreted proteases. The oil yield at 50 ^{o}C obtained by the action of BK23 is in close agreement with the 64% yield obtained by using the alkaline component of Protizyme, a commercial protease preparation (Shah et al., 2005; Winkler et al., 1997b).

3.7. Production cost and industrial applicability

It is evident that the traditional Java method in applying crab paste for the liberation of oil from oil crops cannot be applied on a large industrial scale due to the limited availability of paddy crabs. This study clearly shows, however, that oil release can be catalyzed by bacterial strains isolated from crab paste. The most efficient strain, BK23,

was identified as *Bacillus licheniformis.* Incubation with strain BK23 liberated oil from grated coconut and jatropha kernels without degrading it.

A summary of the jatropha oil production cost estimation is presented in Table 3. It is clearly shown that production cost of oil from microbial process (0.83 USD/kg of oil) is about 40% higher than the cost of oil from motor screw-pressed (0.60 USD/kg of oil, re-calculated based on published data and jatropha oil density of 933 kg/m^3) (Openshaw, 2000; Pramanik, 2003). Unlike motor-press that uses whole seeds as processing material in a simpler process and less capital investment, microbial process utilizes seed kernels, and the involvement of more unit operations makes capital and energy costs much higher, thereby increasing oil production cost. However, in a microbial process, subsequent separation step such as protein purification will be much simpler and this will improve the profitability of the process. Furthermore, operation at somewhat larger scale will further reduce cost price as well.

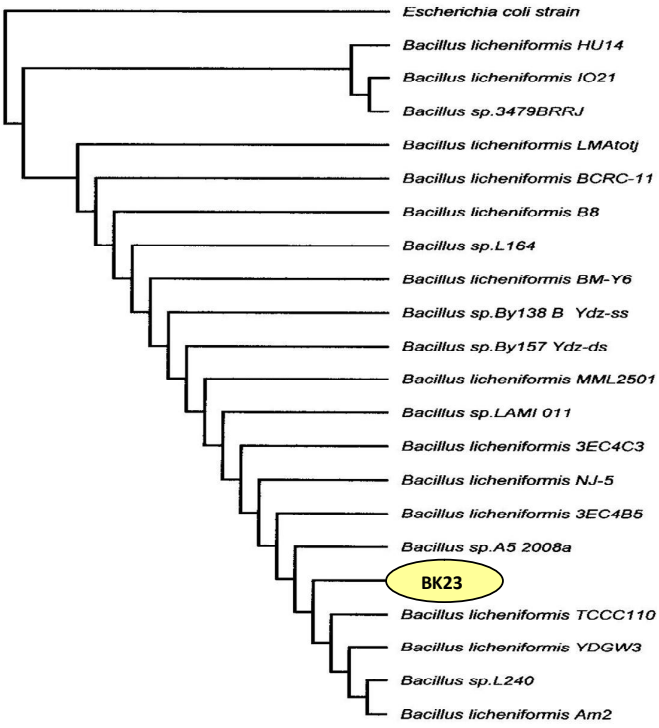

Figure 7. A phylogenetic dendrogram of the 16s rRNA sequences of BK23 and bacteria most closely related to it.

Table 3. Production cost of Jatropha oil by microbial assisted extraction process compared to motor press cost data (Openshaw, 2000)

	Cost components	This paper	Openshaw, 2000
Energy:			
1	Total energy consumed (kWh/d)	179.2	
2	Energy price (USD/kWh)	0.06	
3	Total energy cost (USD/d)	10.8	13.8
Labor:			
4	Total man-hour required	24	
5	Price (USD/man-hour)	0.6	
6	Total man-hour cost (USD/d)	14.4	3.0
Raw Materials:			
7	Jatropha seed (kg/d)	1000	737
8	Price of seeds (USD/kg)	0.103	0.103
9	Seed cost (USD/d)	103.0	75.9
10	Oil container (USD/d)	2,3	1.8
11	Chemicals and H_2O (USD/d)	5	0
12	Total raw materials cost (USD/d)	110.3	77.7
Capital Cost:			
13	Capital cost (USD/day)	39.6	8
Production cost of oil:			
14	Total cost (USD/d) (3+6+12+13)	175.0	102.7
15	Total oil produced (kg/d)	210	172
16	Production cost of oil (USD/kg)	0.83	0.60

The process as used in this study is not optimized yet and can be further improved. Options for further improvement to obtain higher oil yield, and thus lowering oil production cost concurrently, are the isolation of more efficient strain and improving the extraction parameters (kernel particle size reduction, pre-heating treatment, pH optimization, etc). Also the effect of the process on toxicity of oil and side streams should be assessed.

B. licheniformis is a species well-known for the industrial production of proteases. It is also likely that in this application, enzymes like proteases produced by the bacteria are responsible for oil release. The question arises whether bacterial starter cultures with *in situ* produced enzymes can compete with the application of off-site produced enzyme cocktails.

4. Conclusions

Two mechanisms play a role in the release of oil from grated coconut by crab paste during the traditional Java method: an activity dominating at lower temperatures which is most likely caused by enzymes and a bacterial associated activity more active at higher temperatures. The Java method could also be applied for the release of oil from the seed of the oil crop jatropha. A facultative thermophilic bacterium strain was isolated from paddy crab, identified as *Bacillus licheniformis* strain BK23. It is able to liberate 60% oil from aqueous jatropha seed kernel slurry. Further studies of BK23 as well as extraction process optimization are the challenges to improve aqueous Jatropha oil extraction yield and process economy.

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Appendix A1. The price of main equipment of microbial assisted jatropha oil extraction factory (1000 kg/d of seeds). SS=stainless-steel.

No	Unit Operation and Equipment	Function	Capacity	Power (kW)	Price (USD)
1	DESHELLING AND GRINDING				
	Decorticator	Removing shells	1000 kg/h	3	1500
	Desintegrator	Pulverizing	1000 kg/h	5	700
2	HOMOGENIZING				
	High shear water & powder mixer	Particle reduction	2000 kg/h of slurry	20	4000
	Centrifugal transfer pump	Material transfer	2000 kg/h of slurry	2	700
3	MICROBIAL EXTRACTION				
	Reactor unit (SS)	Microbial oil extraction	5 m^3	5.5	7500
	Wood-fired water heater	Heating the reactor	50 kg/h hot water	-	1000
	Air blower	Reactor aeration	60 m^3 /h	2	300
	Centrifugal transfer pump	Material transfer	2000 kg/h of slurry	2	700
4	CENTRIFUGATION				
	Centrifugal separator	Oil separation	4000 kg/h of slurry	10	4000
				Total	20400

Appendix A2. Estimation of energy consumption of the equipment calculated according Eq. (1). Thermal energy for water heater is derived from seed-shells burning and thus is excluded in the calculation.

Equipment	Qty	Power (kW)	Operation period (h)	Energy consumed (kWh)
Decorticator	1	3	2	4.8
Desintegrator	1	5	2	8
High shear water & powder mixer	1	20	2	32
Centrifugal transfer pump	2	2	2	6.4
Reactor unit (SS)	1	5	20	80
Air blower	1	2	20	32
Centrifugal separator	1	10	2	16
			Total	179.2

Appendix A3. Man-hour involved in oil extraction factory of 1000 kg/d of seeds

No	Unit Operation	Equipment	Number of person involved	Time required to accomplish (h)	Man-hour
1	Deshelling & Grinding	Decorticator	1	2	2
		Desintegrator	1	2	2
2	Homogenizing	High shear mixer	1	2	2
		Transfer pump	1	2	2
3	Microbial extraction	Reactor	1	6	6
		Water heater	1	4	4
		Transfer pump	1	2	2
4	Centrifugation	Centrifugal separator	1	4	4
				Total	24

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CHAPTER 4

Enhancing jatropha oil extraction yield from the kernels assisted by a xylan-degrading bacterium to preserve protein structure

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Abstract

We investigated the use of bacterial cells isolated from paddy crab for the extraction of oil from jatropha seed kernels in aqueous media while simultaneously preserving the protein structures of this protein-rich endosperm. A bacterial strain – which was marked as MB4 and identified by means of 16S rDNA sequencing and physiological characterization as either *Bacillus pumilus* or *Bacillus altitudinis* – enhanced the extraction yield of jatropha oil. The incubation of an MB4 starter culture with preheated kernel slurry in aqueous media with the initial pH of 5.5 at 37 ^{o}C for 6 h liberated 73% *w/w* of the jatropha oil. Since MB4 produces xylanases, it is suggested that strain MB4 facilitates oil liberation via degradation of hemicelluloses which form the oil-containing cell wall structure of the kernel. After MB4 assisted oil extraction, SDS-PAGE analysis showed that the majority of jatropha proteins were preserved in the solid phase of the extraction residues. The advantages offered by this process are: protein in the residue can be further processed for other applications, no purified enzyme preparation is needed and the resulting oil can be used for biodiesel production.

***Keywords**:*

Jatropha curcas, L, Aqueous oil extraction, *Bacillus pumilus*, *Bacillus altitudinis*, Protease, Xylanase

1. Introduction

Jatropha curcas, L is a well-known plant for the high fat and protein content of its seed ranging between 45 to 55% *w/w* and 20 to 30% *w/w* of the kernels, respectively (Gubitz et al., 1999; Lestari et al., 2010). This oil is economically attractive due to its potential application in biodiesel (Lin et al., 2003; Martinez-Herrera et al., 2006). In addition, the kernel contains 28% protein, which has been extensively studied for food and non-food application (Gubitz et al., 1999; Lestari et al., 2010; Lin et al., 2003; Martinez-Herrera et al., 2006). Lestari extracted more than 80% of the protein from the kernels and addressed some potential applications of the isolated protein in various fields such as adhesives, coatings, and chemicals (Lestari et al., 2010). Therefore, with respect to the overall economy of jatropha cultivation, it is interesting to find a commercial use for both oil and protein. Protein recovery from the kernel requires aqueous extraction; hence, it is interesting to see if aqueous extraction can also be used for the release of oil.

The common method of oil production from oilseeds as feedstock for biodiesel involves pressing of seeds and oil purification (degumming, deacidification, dewaxing, dephosphorization, dehydration, etc.). These processes, together with esterification/transesterification, contribute to over 70% of the total biodiesel production costs (Shuit et al., 2010; Zeng et al., 2009).

Aqueous oil extraction (AOE) uses water as medium to facilitate oil liberation from oilseeds. AOE eliminates organic solvent consumption and so improves process economy (Barrios et al., 1990a; Rosenthal et al., 1996). AOE also enables several purification steps such as degumming, deacidification, dewaxing, and dephosphorization to be carried out simultaneously within the extraction step (Caragay, 1983), resulting in a more efficient process.

We demonstrated earlier that thermophilic strains isolated from the gut of paddy crabs, one of which was identified as *Bacillus licheniformis*, enhanced oil liberation up to 60% from aqueous jatropha kernel, most likely via protein degradation (Marasabessy et al., 2010), which would be disadvantageous for protein recovery. In the present report, we confirmed that these thermophilic bacteria degraded extracted jatropha protein. We also examined if preheating the kernels degraded the proteins in

comparison to non-heated kernels by using SDS-PAGE analysis. Next, isolation and selection of mesophilic bacteria from the gut of paddy crabs were performed based on their ability to liberate oil from jatropha preheated kernel slurry. The aim was to obtain other microorganisms able to liberate oil without affecting the protein structures. The best strain was used for aqueous oil extraction from jatropha kernel. The molecular weight distribution of protein in the residue (water phase and solid phase) after microbial treatment was also investigated to examine protein integrity. The quality of recovered oil was analyzed and compared with those of standard values of feedstock for biodiesel.

2. Materials and Methods

2.1. Materials

Jatropha curcas, L seeds were harvested from the jatropha planted in Serpong, Indonesia (geocoordinates 6°21'31"S, 106°40'33"E). Kernels were obtained after removal of the shells. The sun-dried kernels were stored at 4 °C until used. Paddy crabs were collected from bunds of a paddy field located in Pamulang, Indonesia (geocoordinates 6°20'52"S, 106°42'20"E). All chemical reagents, unless otherwise specified, were of analytical grade.

2.2. Jatropha kernels slurry preparation

The kernels (500 g) were autoclaved at 121 °C for 15 min and then dried at 60 °C overnight. The kernels were milled and sieved through a strainer with 1.0 mm pore diameter. To prepare the preheated kernel slurry, 25 g milled kernel was homogenized with 125 g purified water (milli-Q) for 5 min using a Waring Blender. The weight ratio of solid material to water in the slurry was 1:5. Under constant stirring – to keep the slurry homogenous – 12 g of kernel slurry (equivalent to 2 g kernel) was used for the extraction of oil.

2.3. Protein extractions from kernels

Protein extraction was carried out by extracting 1 g of sample with 30 ml solvent for 30 min in 50 ml capped centrifuge tubes. The mixing was conducted at room

temperature by using a rotary mixer. The extracting solvents were water, NaCl 1.0 M, and NaOH 0.055 M as described previously (Lestari et al., 2010). Solid–liquid separation was conducted at 4,000×*g* for 15 min by using a SORVALL6+ centrifuge.

2.4. Evaluation of protein degradation by paddy crab bacteria

A mixture of 15 g/L Agar–agar (Merck) and 10 g/L of jatropha seed protein having a purity of ca. 83% *w/w* or 10.0 g/L of casein (Merck) in water was boiled to dissolve agar. After autoclaving (121 ^{o}C, 15 min), 15 ml protein–agar solution was aseptically poured in a sterile petri dish and brought to solidify overnight. The wells in protein–agar media were made by using a sterile rubber cork having a diameter of 9 mm. Two milliliters of a 24-h old bacterial starter culture was centrifuged at 20,000 rpm for 5 min. The supernatant was filtered through a 0.22-μm bacterial filter (Millipore), after which 50 μl of the filtrate (bacterial crude extract) was pipetted into the well. The plates were placed at 4 ^{o}C overnight to let the extract absorb into the protein–agar media, followed by incubation at 37 ^{o}C for 6 h and at 45 ^{o}C for 6 h. Clear zones surrounding the well indicating protein solubilization (degradation) by bacterial proteases were observed. A thermostable bacterial neutral protease from *Bacillus thermoproteolyticus* (Protex 14L, Genencor) at 200× dilution was used as the positive control, while the preheated bacterial crude extracts and preheated Protex 14L (100^{o}C, 10 min), respectively, were used as negative controls.

2.5. Jatropha oil extraction by paddy crab paste

The crab paste was prepared as described previously (Marasabessy et al., 2010). To extract oil, 2.0 g of crab paste was mixed with 30.0 g kernel slurry and incubated in a orbital shaker at 37 ^{o}C, 150 rpm for 24 h. Antibiotics were applied in some samples as described previously and the extracted oil was assayed gravimetrically (Marasabessy et al., 2010).

2.6. Isolation of mesophilic bacteria from paddy crabs

For the isolation of bacteria, one loop of crab paste was streaked out aseptically on a nutrient agar (NA) medium plate (Merck) and incubated at 37 ^{o}C for 24 h. Well

separated colonies were picked up, subcultured, and maintained on NA slants (37 °C for 24 h).

2.7. Selection of mesophilic bacteria for jatropha oil extraction

Under constant stirring, 12 g of preheated kernel slurry (equivalent to 2 g kernel) was weighed out in a 100-ml flask. This was inoculated with 2 ml bacterial suspension, prepared by suspending cells of a bacterial culture grown on an NA agar slant (in a tube having 1.5 cm diameter and 12 cm length) with 2 ml sterile water. The mixture was incubated at 37 °C and 150 rpm for 24 h using an Innova 44 Incubator Shaker (New Brunswick), after which it was centrifuged at 7,400 ×*g* for 15 min. The extracted oil was assayed gravimetrically (Marasabessy et al., 2010). Control experiments were performed using exactly the same treatment, however without bacterial inoculation. A bacterial strain showing the best performance was identified by partial sequence of 16*S* rDNA as well as physiological tests conducted by DSMZ (Germany).

2.8. Microbial jatropha oil extraction

Bacterial starter culture was prepared as described previously (Marasabessy et al., 2010), except that the nutrient broth medium (NB, Merck) was initially supplemented with 1.0% *w/v* milled jatropha kernel before autoclaving. To extract the oil, 12.0 g of jatropha kernel slurry was inoculated with 1.0 ml of the bacterial starter culture. Antibiotics were applied in some samples as described previously (Marasabessy et al., 2010). The mixture was shaken at 150 rpm and 37 °C. After incubation, the slurry was centrifuged at 7,400 ×*g* for 15 min. The free oil on the surface of the liquid in the centrifuge tube was assayed gravimetrically as reported previously (Marasabessy et al., 2010).

2.9. Detection of xylanase and glucanase activity in bacterial crude extracts

For xylanase detection, 15 µl bacterial crude extract was pipetted into a well (5 mm diameter) in an agar plate containing 0.2% Remazol Brilliant Blue Xylan (RBB-Xylan, Sigma) (Strauss et al., 2001). For cellulose detection, 50 µl bacterial crude extract was pipetted into a well (9 mm diameter) in an agar plate containing 0.4% carboxymethyl cellulose (CMC). The plates were placed at 4 °C overnight to let the extract absorb into

the agar media, followed by incubation at 37 °C for 6 h (RBB-Xylan agar) and 48 h (CMC agar). The CMC agar plate was stained with 0.03% Congo Red, followed by destaining with 1 M HCl (Teather and Wood, 1982). The clear zones surrounding the well indicate the hydrolysis of xylan and cellulose.

2.10. SDS-PAGE analysis

Molecular weight distribution of proteins was analyzed by using SDS-PAGE (NuPage Electrophoresis System with NuPage Novex Bis-Tris Gels 10% from Invitrogen).

2.11. Assay of total oil content, oil yield, and oil quality

The total oil content of the oilseeds was determined by Soxhlet method (AOAC, 1984). The total oil content was 0.47 kg/kg jatropha kernels. This amount was taken as 100% recovery of oil in the calculations of jatropha oil yield in the extraction experiments. The free fatty acid and moisture content of the extracted oil was assayed by the titration method and the Karl Fischer method, respectively (AOAC, 2002a). The oxidative stability index (OSI) was assayed using 873 Biodiesel Rancimat apparatus from Metrohm.

3. Results

3.1. The effect of thermophilic crab bacteria on jatropha protein integrity

In our previous publication, some thermophilic bacteria (namely BK21, BK22, and *Bacillus licheniformis* strain BK23 isolated from paddy crabs) extracted up to 60% of the jatropha oil from non-heated kernels after 24 h incubation at 50 °C under non-optimized conditions (Marasabessy et al., 2010). From the protein–agar plate experiment (Figure 1), we found that those strains hydrolyzed both jatropha kernel protein and casein upon incubation at 50 °C. BK21 showed the highest protease activity and *Bacillus licheniformis* BK23 the lowest, as indicated by the size of the clearing zone diameter, for both types of protein. The bright clear zones proved that proteins available in the kernel were completely solubilized.

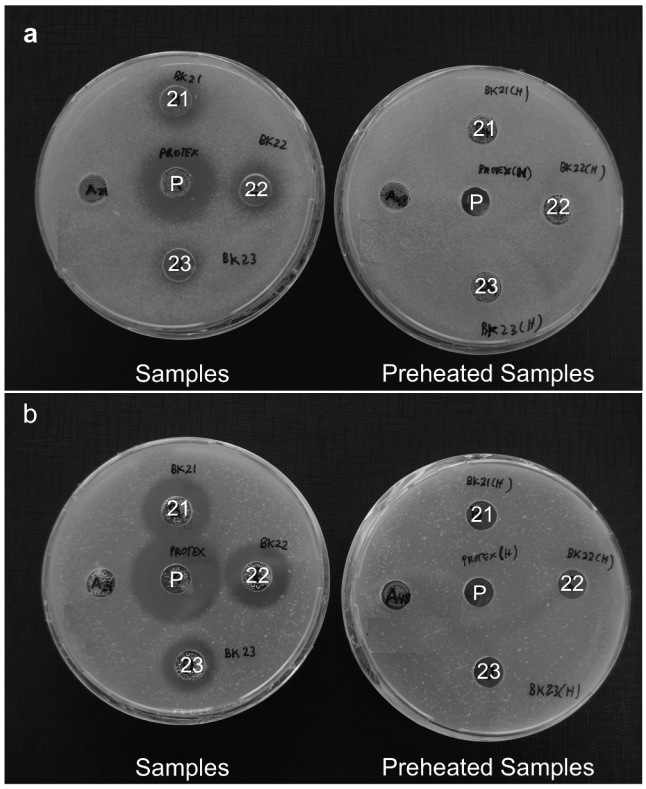

Figure 1. Protein degradation ability of thermophilic bacterial crude extract of BK21, BK22, and *Bacillus licheniformis* strain BK23 on (a) jatropha protein agar medium and (b) casein agar medium. These strains were isolated from paddy crabs (Marasabessy et al., 2010). A 200x dilution of Protex 14L from Genencor (P) was the positive control. The preheated samples (100 ^{o}C, 10 min) were the negative controls (showing no clear zone).

3.2. The effect of heat pretreatment on jatropha protein integrity

Because we wanted to study the effect of crab's gut bacteria working at lower temperature on the extraction yield of oil from jatropha kernels, internal factors within the kernels influencing oil liberation had to be minimized. Since the kernels contain microorganisms as well as seed enzymes which might interfere with the crab bacteria involved in oil liberation, we applied two different heat pretreatments on kernels, at 105 ^{o}C or 121 ^{o}C for 30 min, to deactivate enzymes and to kill microorganisms before being used for oil extraction. The proteins were extracted from the kernels and the extracts were subjected to SDS-PAGE analysis (Figure 2). The

solubility of proteins in water depends on various factors such as ionic strength and pH; therefore 1M NaCl and 0.055 M NaOH were also used as extractants besides water (Lestari et al., 2010). The protein pattern of the heat-treated kernels extracted with NaCl and NaOH was identical with that of the untreated kernels, showing that heat treatment at both 105 and 121 ^{o}C for 30 min did not affect the protein composition. Compared to the other samples, the untreated sample extracted with water is missing three small bands at molecular weights of approximately 20, 23, and 25 kDa, indicating that heat treatment increased the water solubility of the proteins.

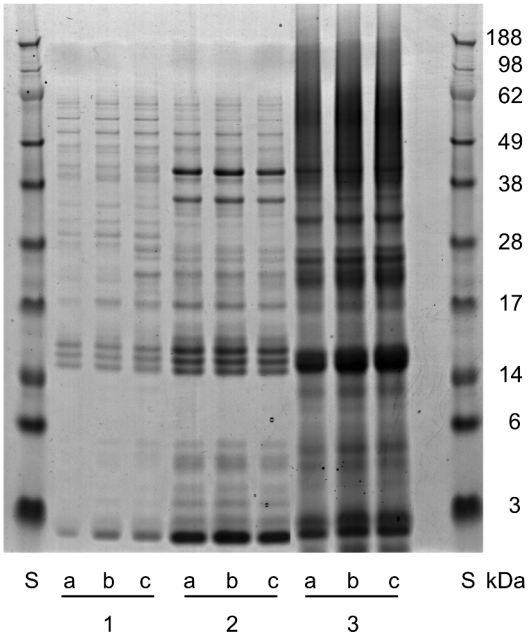

Figure 2. SDS-PAGE analysis of jatropha kernel proteins. Bands of proteins from jatropha kernel showing molecular weight distribution after protein extraction from jatropha kernel using (1) water, (2) 1.0 M NaCl, and (3) 0.055M NaOH with different pretreatment: (**a**) non-heated, (**b**) preheated at 105^{o}C for 30 min, and (**c**) 121^{o}C for 30 min.

Concluding, heat pretreatment did not have an effect on position and relative intensity of the different protein bands on the SDS-PAGE, indicating that no significant alteration of the chemical structures of the proteins occurred. We decided therefore to employ preheated kernels (by autoclaving at 121^{o}C for 15 min) for oil extraction in the subsequent experiments.

3.3. Effect of paddy crab paste on oil extraction

The presence of mesophilic bacteria in paddy crabs having a positive effect on jatropha oil liberation was determined by incubating 2 g paddy crab paste with 30 g preheated kernel slurry (containing 5 g kernel) at 37 ^{o}C and 150 rpm for 24 h with and without addition of antibiotics (Figure 3). Oil extraction in control experiments to which no crab paste was added resulted in 7% oil after 24 h. Addition of antibiotics to control samples also gave a low oil yield (4%). Incubation of preheated kernel slurry with crab paste and antibiotics significantly improved the oil yield to 62%. It is evident that paddy crab paste exhibits a strong effect towards oil liberation from jatropha kernel. Furthermore, it was shown that excluding antibiotics from the crab–kernel sample resulted in even higher oil liberation (70%). The significant yield improvement from 62% to 70% indicates that mesophilic bacteria derived from paddy crabs take part in the entire mechanism of jatropha oil liberation from preheated kernel. Based on these results, we decided to isolate mesophilic bacteria living in the gut of paddy crabs as our experimental strains for microbial jatropha oil extraction.

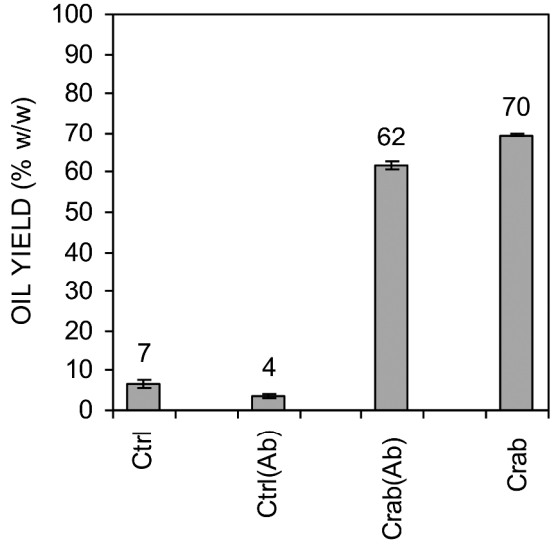

Figure 3. Jatropha oil extraction yield from preheated-kernel slurry incubated with paddy crab paste at 37 ^{o}C, 150 rpm for 24 h in comparison to control samples. Ctrl=control samples; Ab=antibiotics

3.4. Protease, xylanase, and glucanase activity of bacterial crude extract

We isolated 20 colonies of mesophilic crab bacteria, but we selected only 7 colonies for further testing, namely strains MB4, MB5, MB7, MB11, MB12, MB13, and MB20 based on differences in colony form and microscopic observation. The detection of protease activity in crude extract revealed that MB4 is the only strain exhibiting protease, with different strengths of activity against the two types of protein tested: casein and jatropha protein (Figure 4a, b). The MB4 protease showed strong activity against casein as shown by a bright clear zone in the casein layer due to casein degradation by hydrolysis (Figure 4a). However, the MB4 protease did not function with jatropha protein under the conditions tested, as shown by the absence of a clear zone formed in the jatropha protein layer (Figure 4b). Xylanase activity was found only in MB4 crude extract as shown by formation of a clear zone in RBB-xylan agar medium (Figure 4c). Congo Red staining in CMC agar medium showed a negative result for glucanase activity in the crude extract of all strains tested (Figure 4d). Figure 4d depicts that the enzyme GC220 (Genencor Inc, USA) lacked glucanase activity as no clear zone formed in CMC agar medium.

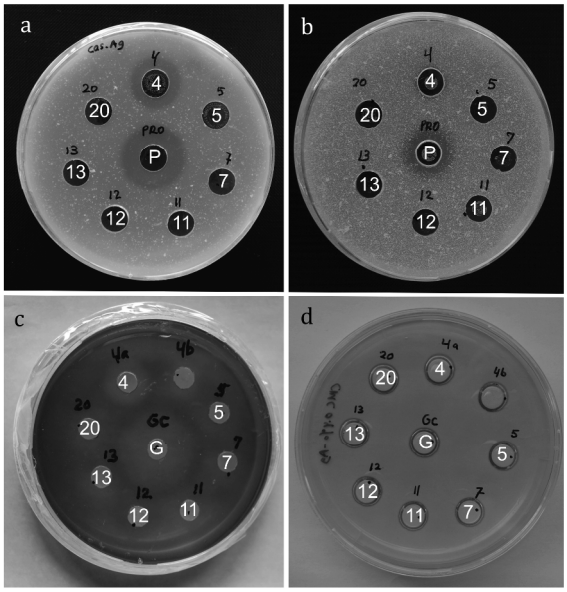

Figure 4. Protease, xylanase, and glucanase activity of crude extracts of isolated bacterial strains on (**a**) casein agar plate, (**b**) jatropha protein agar plate, (**c**) RBB-xylan agar plate, and (**d**) CMC agar plate. A 200x dilution of a protease: Protex 14L (*P*) or a cellulase: GC-220 (*G*) from Genencor was the positive control. *Numbers* on the plates denote the strain: 4, 5, 7, 11, 12, 13, and 20 for MB4, MB5, MB7, MB11, MB12, MB13, and MB20, respectively.

3.5. Selection of paddy crab bacterial strain for jatropha oil extraction

Figure 5 shows the amount of oil extracted from 12 g preheated jatropha kernel slurry (containing 2 g kernel), inoculated with the isolated bacterial strains directly prepared by suspending the NA culture slant with 2 ml water. We found that MB4 gave the highest jatropha oil yield (63%), a 15-fold increase compared to a control experiment containing antibiotics. MB4 was selected for further tests at different conditions of incubations.

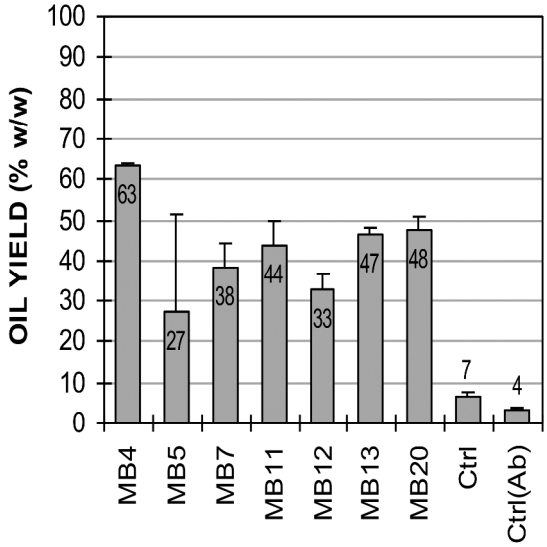

Figure 5. Jatropha oil extraction yield from preheated kernel slurry after inoculation with different mesophilic bacterial strains from paddy crabs (MB4, MB5, MB7, MB11, MB12, MB13 and MB20) and incubated at 37°C, 150 rpm for 24h.

3.6. Identification of strain MB4

The phenotypical characterization conducted by DSMZ (Germany) indicates that MB4 is a *Bacillus pumilus* strain (Table 1). The partial sequencing of 16S rDNA (data not shown) conducted also by DSMZ shows a similarity of 100% to *Bacillus altitudinis* and 99% to the type strain of *Bacillus pumilus*. The partial sequence has been submitted to the GenBank (accession number HQ860795). Considering the result of the partial sequencing, a clear identification to species level is not possible. Further examinations will be required to find out the novelty of the strain MB4. The strain MB4 has been deposited in the DSMZ collection as DSM 24473 *Bacillus* sp. BioMcc B-0081.

3.7. Microbial jatropha oil extraction: optimization

The optimization of microbially assisted oil extraction was conducted in three steps. In first instance, we incubated preheated jatropha kernel slurry with the starter culture of strain MB4 at 37 °C over 24 h in order to find the optimum incubation time (Figure 6a). Second, we studied the effect of initial pH (4.5, 5.5, 6.5, 7.5, and 8.5) of the kernel slurry on oil liberation by strain MB4; kernel slurry pH was adjusted to the desired value by using 4M sodium hydroxide or 4M sulfuric acid solutions before inoculation of bacteria starter cultures (Figure 6b). Third, we optimized incubation temperature (37, 45, 50, and 55 °C) for jatropha oil extraction (Figure 6c).

Table 1. Phenotypical characteristics of strain MB4.

Observed Item	Result
Shape of cells	Rods
Width (μm)	0.6-0.7
Length (μm)	2.0-3.0
Aminopeptidase Test	-
KOH Test	-
Catalase	+
Spores	Oval+
Sporangium swollen	-
Anaerobic growth	+
VP reaction	+
pH in VP	4.9
Growth temperature positive up to	50 °C
Growth in	
Medium pH 5.7	+
NaCl 2%	+
NaCl 7%	+
NaCl 10%	+
Lysozym 0.001%	+
Acid from:	
D-Glucose	+
D-Fructose	+
D-Xylose	+
D-Mannitol	+
L-Arabinose	+
Gas from D-Glucose	-
Hydrolysis of	
Starch	-
Gelatin	+
Casein	+
Tween 80	+
Esculine	+
Lechitinase	+
Tyrosin degradation	-
Indol reaction	-
Use of	
Citrate	+
Propionate	-
Phenylalanine deaminase	-
Nitrate reduction	-
Arginine dihydrolase	-

Figure 6a shows that the oil yield in the control samples (containing antibiotics) remained below 10% throughout incubation for 24 h. The addition of MB4 starter culture to preheated kernel slurry resulted in a sharply increased oil yield to about 60% (tenfold increase compared to the control experiment) only within 6–8 h, after which it remained constant until 24h. Based on these results, we decided to shorten the incubation time to 6 h in the subsequent experiments of microbial–assisted oil extraction.

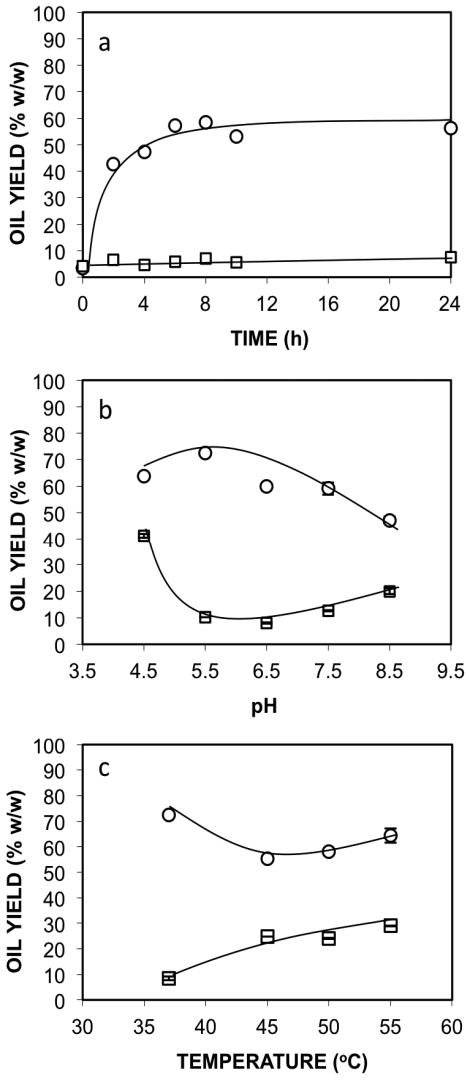

Figure 6. Incubation of preheated jatropha kernel slurry with MB4 (o) and without MB4 (□): (a) oil extraction yield after incubation (37 °C for 24h), the initial pH was not adjusted; (b) oil extraction yield after incubation at different initial pH (37 °C for 6h); and (c) oil extraction yield after incubation at pH 5.5 at different temperatures (37, 45, 50, and 55 °C for 6 h).

The oil yield of kernel slurry incubated with MB4 for 6 h at different pH values (4.5, 5.5, 6.5, 7.5, and 8.5) is shown in Figure 6b. The oil yield of MB4-treated sample increased from 65% at pH 4.5, to peak at 73% at pH 5.5, and then decreased to 50% at pH 8.5. Contrary to the curve trends obtained with MB4, the oil yield of control sample (containing antibiotics) decreased rapidly from 40% at pH 4.5 to 10% at pH 5.5, and then increased to 20% at pH 8.5. As conclusion, strain MB4 has an optimum initial pH of 5.5 at 37 °C. Based on these results, we therefore studied the effect of incubation temperature on oil liberation by MB4 at pH 5.5 for 6 h.

The oil yield from kernel slurry incubated with strain MB4 for 6 h at pH 5.5 and different temperatures is shown in Figure 6c. It is evident that the highest extraction yield of 73% was obtained at an incubation temperature of 37 °C. The oil yield of MB4-treated sample decreased from 73% to 60% as the temperature increased from 37 °C to 45 °C. The oil yield of MB4-treated sample slightly increased to 64% when the temperature increased from 45 °C to 55 °C. The oil yield of the control sample showed a slow increasing trend from 10% (37 °C), reaching a maximum oil yield of 30% only

at 55 °C. This slow increase can at least partially explain the increasing trend of oil liberation in the MB4-treated sample at temperature in the 45-55 °C range.

3.8. Evaluation of protein integrity after microbial oil extraction

We investigated the molecular weight distribution of protein in liquid phase (supernatant) and solid phase (cake) after MB4 oil extraction, in comparison to those extracted with 0.055M NaOH, by using SDS-PAGE analysis as shown in Figure 7. We did not recover protein in the interfacial phase for SDS-PAGE analysis because we observed a very low amount of solid in the interfacial phase (between oil–water) after centrifugation, indicating a lower amount of oil–water emulsion after MB4 treatment.

Figure 7 shows that almost all proteins in the range of 1.0 to 88.5 kDa available in 0.055M NaOH-extracted sample were also available in the solid phase, with the exception of one protein (88.5 kDa) that was missing in the solid phase. Three additonal proteins of 14.7, 27.4, and 44.9 kDa that were not available in NaOH-extracted sample were found in the solid phase as well as in the liquid phase. Six proteins of 1.7, 8.5, 9.4, 10.6, 11.3, and 32.1 kDa that were available in NaOH-extracted sample were not detected in the liquid phase. Furthermore, 13 additional proteins of 2.8, 11.9, 13.5, 14.7, 20.1, 25.1, 27.4, 29.2, 41.3, 44.9, 59.3, 100.1, and 130.4 that were not available in NaOH-extracted sample were found in the liquid phase.

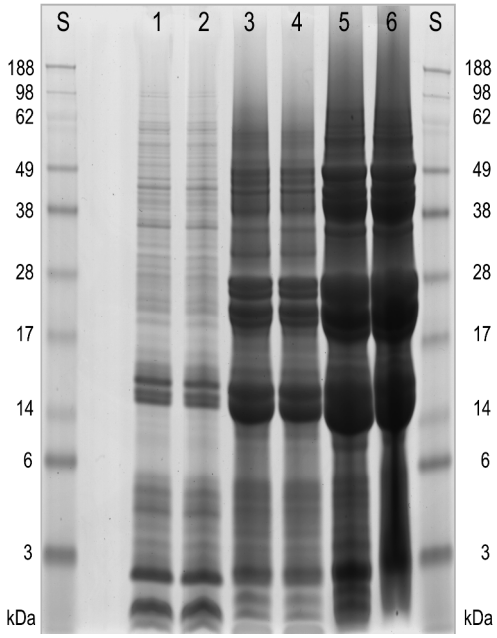

Figure 7. SDS-PAGE analysis of MB4 treated jatropha kernel proteins. Bands of proteins from duplicated samples of water phase (*1 and 2*), solid phase /cake (*3 and 4*) in comparison to jatropha protein (*5 and 6*). *S* stands for standard of protein marker.

3.9. The quality of oil after aqueous oil extraction

Oil quality data in Table 2 show that the oil obtained by MB4 extraction, in general, meet the German fuel standard DIN V 51605 for pure plant oil (rapeseed oil), except for the acid value (AV) which was found 8.6, or more than eight times higher than the standard value.

Table 2. The quality of oil extracted from Jatropha kernel using MB4 bacterial strain (AOE-MB4) compared to that extracted by expeller and the standard values.

Parameters	Methods and oil quality		
	AOE-MB4	Expeller	Standard value[a]
Seeds source	*J. curcas,* L	*J. curcas,* L	-
Feed type	Preheated kernels	Whole seeds	-
Expeller type	-	De-Smet UK	-
Conditions	6h, 150 rpm, 37^{o}C	25 rpm, 80-85 ^{o}C	-
OSI (h)	7,8 ± 0.06	10.7	Min 6
AV (mg KOH/g oil)	8.6 ± 0.20	10.3	Max 2
Water (ppm)	719 ± 32	1147	Max 750

OSI, Oxidative Stability Index; *AV*, Acid Value.
[a] German Fuel Standard DIN V 51605

4. Discussion

Jatropha seed kernels have a high fat and protein content ranging between 45–55% *w/w* and 20–30% *w/w*, respectively (Gubitz et al., 1999; Lestari et al., 2010). The oil is investigated for its suitability as a biofuel, whereas the protein has been extensively studied for food and non-food application (Gubitz et al., 1999; Lestari et al., 2010; Lin et al., 2003; Martinez-Herrera et al., 2006). Therefore, with respect to the overall economy of jatropha cultivation, it is important to find commercial outlets for both oil and protein.

In studying the effect of heat pretreatment on protein integrity, we found proteins resolution on electrophoresis gel gave identical band positions among non-heated, preheated at 105 ^{o}C for 30 min, and preheated at 121 ^{o}C for 30 min (Figure 2). This means that the structure of jatropha protein exhibits high thermal stability against thermal processing upon heating up to 121 ^{o}C for 30 min. Thermal properties of

proteins are important to study the changes during heat processing which, in turn, are useful in the processing designs for protein-based products (Horax et al., 2011).

Aqueous extraction is necessary for the recovery of the protein from the kernel, and in order to decrease process costs it is therefore interesting to liberate the oil from the seed in the same step. In protease-assisted aqueous oil extraction from oilseeds, oil-bound proteins are hydrolyzed into smaller fractions, thereby altering their structure and functionality (Moure et al., 2006). Similar studies in jatropha oil extraction reported previously did not highlight the importance of preserving protein structure during oil extraction process. If the protein structures are to be conserved to a large extent in the recovery of oil from oilseeds, the use of bacterial strains or enzymes liberating oil by other means than protein solubilisation is a reasonable choice.

Apart from proteases, a number of microbial enzymes have been studied to enhance oil extraction yields from oilseeds: amylase, glucanase, pectinase, cellulolytic, and hemicellulolytic enzymes (Dominguez et al., 1994). We were therefore interested to isolate and select other microbial strains from the crab's gut capable of assisting oil liberation without degrading protein.

The paste of paddy fields crabs are traditionally used for coconut oil extraction in Java. In a previous article, we have also applied paste crab to release oil from jatropha kernels (Marasabessy et al., 2010). Whereas we now were able to release 70% of the oil, we previously only liberated 30% of the oil. Even though the experimental conditions in using paddy crab paste as the research material between the present study and the previous study (Marasabessy et al., 2010) look similar, they are not entirely the same for two reasons. First, in our present study, preheated kernels were used as substrate instead of non-heated kernel used in the previous study. Preheating the kernels might have enhanced the dissolution of cell components which were previously bound the original structures of cells (Williams, 2005), allowing crab's enzymes or microbial enzymes to have access in breaking oil barriers, resulting in the release of more oil as compared to control experiments (Figure 3). Second, the different batch of crab paste used in the present study might have resulted in differences in oil liberation.

We found that MB4 starter culture was able to extract 73% oil from jatropha kernel slurry when incubated for 6 h at 37 °C and pH 5.5. This is in good agreement with the jatropha oil yield of 85.6% and 74% extracted by using protease of Alcalase (Novo Nordisk, Denmark) and Protizyme (Jaysons Agritech, India), respectively (Shah et al., 2005; Winkler et al., 1997b). The use of Viscozyme (Novo Nordisk, Denmark) as a hemicellulase/cellulase formula gave a comparable oil yield of 70% (Winkler et al., 1997b).

We have shown that protease from strain MB4 bears no activity against jatropha protein. Hence, by considering the optimal pH and temperature of MB4 (pH 5.5 and 37 °C, respectively) and also the presence of xylanase in the crude extract of MB4, it is most likely that the strain MB4 facilitates oil liberation at 37 °C via the degradation of hemicellulose that forms the oil-containing cell wall structure of the kernel (Rosenthal et al., 1996).

Bacterial identification results suggested the strain MB4 as *B. pumilus* or the closely related *B. altitudinis*. In case of *B. pumilus*, previous investigations have reported the potential application of *B. pumilus* as xylanase producer (Ahlawat et al., 2007; Battan et al., 2007; Kapoor and Kuhad, 2007; Kapoor et al., 2008; Nagar et al., 2010; Wang et al., 2010; Yasinok et al., 2010). In contrast, we found that only a few publications are available on the potential application of *B. altitudinis.*

After MB4-assisted oil extraction, the extracted oil has an AV below 14% (Table 2), which seems applicable for biodiesel production since a chemical pretreatment to reduce the acid value from 14% to 1% before transesterification of jatropha oil into biodiesel has been established recently, which results 99% yield of biodiesel (Tiwari et al., 2007).

Concluding, strain MB4 identified as *B. pumilus* or *B. altitudinis* isolated from paddy crab liberated 73% *w/w* of jatropha oil from preheated kernel in aqueous system after 6 h incubation at 37 °C. It is suggested that the strain MB4 facilitates oil liberation via degradation of hemicellulose. Incubation of jatropha kernel with strain MB4 preserves the jatropha protein structure to a large extent. MB4-assisted oil extraction has several advantages: (a) no purified cocktail enzyme preparation is required, (b)

protein integrity is mostly preserved, and (c) this method results in jatropha oil with a quality which is suitable for biodiesel production.

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CHAPTER 5

Dilute H_2SO_4-catalyzed hydrothermal pretreatment to enhance enzymatic digestibility of *Jatropha curcas* fruit hull for ethanol fermentation

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Abstract

Dilute sulfuric acid pre-treatment of jatropha fruit hull at high temperatures (140 to180 ^{o}C) performed in a 110-mL stainless steel reactor was investigated to enhance the enzymatic digestibility of its lignocellulosic components. Carbohydrates accounted for 43% of the dry matter of jatropha fruit hull biomass. The goal of the study was to optimize the pretreatment conditions (acid concentration, time, and temperature) in order to obtain the highest sugar yield after subsequent enzymatic hydrolysis. A Box-Behnken Design was applied to the experimental set up in order to reduce the number of experiments. The optimal pretreatment conditions are 30-min incubations at a temperature of 178 ^{o}C with a sulfuric acid concentration of 0.9% (*w/v*). Using these pretreatment conditions for a fruit solid loading of 9.52% followed by a 24-h enzymatic hydrolysis resulted in a liberation of 100% of all pentoses present (71% yield and 29% degradation to furfural) and 83% of the hexoses (78% yield and 5% degradation to 5-hydroxymethylfurfural). The simultaneous saccharification and fermentation experiment showed that acid-pretreated fruit hull can be used as a substrate for *Saccharomyces cerevisiae* to produce ethanol.

Keywords:

Box-Behnken, *Jatropha curcas*, L, Fruit hull, Hemicellulose, Cellulose, Pentose, Hexose, Ethanol

1. Background

Jatropha curcas, L has become widely known as a biofuel crop due to the high oil content of the oilseeds harvested from this plant (Gubitz et al., 1999). The biodiesel produced from jatropha oil via transesterification compares well to petrochemical diesel and meets the latest biodiesel standards (Tiwari et al., 2007). However, whereas palm oil is widely used as feedstock for biodiesel production in Indonesia, jatropha oil has not been traded at price levels that satisfactorily reward growers/farmers. Low oil productivity and high labor cost for fruit harvesting are the most important reasons. Many potential Indonesian farmers in the subsistence sector are now reluctant to invest time and money in planting jatropha since the profit from jatropha products is much less compared to other agricultural commodities. Hence, one of the strategies to improve the economics of this potentially profitable plant is to increase the value of the side streams of oil production (trimmed stems/branches, seed or kernel cake, seed shells, and fruit hulls).

Jatropha curcas, L bears fruit from the second year after crop establishment, but seed production becomes economically feasible from the fifth year onward (Foidl and Eder, 1997; Nallathambi Gunaseelan, 2009). In Indonesia, jatropha is planted at a 2×2-m distance, yielding 2,500 plants/ha. A productivity of 2 kg seeds/plant/year on a jatropha plantation in Indonesia has been reported (Purwaamijaya et al., 2007). One hectare of jatropha plant therefore would yield 5 tons of seed annually (containing 25% to 33% oil), with 1 ton per year of fruit hull biomass (dry weight) as side stream. Fruit hull is composed of lignocellulose (39.3% cellulose and 14.2% lignin) with 5.3 % protein (Nallathambi Gunaseelan, 2009), making this biomass a potential raw material for the production of bioethanol, enzymes, organic acids, and other fermentative products, or as animal feed (Gonzalez-Garcia et al., 2010; Wyman, 1994). The use of fruit hulls so far focuses on applications such as fertilizer and substrate for co-firing installations or for biogas production (Foidl and Eder, 1997; Gubitz et al., 1999; Nallathambi Gunaseelan, 2009; Openshaw, 2000). Another way to valorize the fruit hull side stream is to convert it to fermentation products such as ethanol and lactic acid. In that case, pretreatment is required to enhance the enzymatic digestibility of the lignocellulosic biomass. Pretreatment aims at a partial dislocation of the inter- and intra-fibrillic structure of lignocellulose with a reasonable energy input (Abatzoglou et

al., 1986). From an economic point of view, pretreatment must improve fermentable sugar yield, maintain carbohydrate content, minimize formation of degradation products that are inhibitory to subsequent fermentation processes, and be cost effective (Sun and Cheng, 2002). Various methods, from physical to chemical pretreatments or combinations, have been widely studied; one of which is hot dilute sulfuric acid pretreatment. Dilute acid pretreatment of lignocellulosic biomass conducted at high temperature (more than 160 ^{o}C) results in high xylan conversion yields and low levels of sugar degradation, thus resulting in an improved overall cellulose hydrolysis (Sun and Cheng, 2002). There are no reports on pretreatment of the fruit hull of jatropha yet.

The subject of this study is the pretreatment of the jatropha fruit hull by sulfuric acid at an elevated temperature, in order to improve the enzymatic digestibility of this lignocellulosic byproduct. We study the influence of varying sulfuric acid concentration, pretreatment time, and temperature on the following three factors:

1. Pentose and hexose degradations after fruit hull pretreatment, calculated from furfural and 5-HMF formed, respectively
2. Pentose and hexose yields after subsequent enzymatic hydrolysis of the pretreated fruit hull
3. Total sugar yield after subsequent enzymatic hydrolysis of the pretrerated fruit hull.

A Box-Behnken Design was applied to the experimental set up in order to reduce the number of experiments. The main objective is to obtain an optimum sulfuric acid concentration, time, and temperature of pretreatment that give a low level of sugar degradation and a high level of sugar yield in the subsequent enzymatic hydrolysis. Simultaneous saccharification and fermentation (SSF) of jatropha fruit hull hydrolysates by *Saccharomyces cerevisiae* for ethanol production is also studied.

2. Methods

2.1. Experimental design and setup

Design-Expert 8.0.3 software (Stat-Ease, Inc., MN, USA) was used for the experimental design, model fitting, and statistical data analysis. In order to reduce the number of experiments, a Box-Behnken Design (BBD) (Box and Behnken, 1960) was applied.

Experimental data of each response factor (Y) were expressed in a second order mathematical model:

$$Y = \beta_o + \sum_i^n \beta_i X_i + \sum_i^n \beta_{ii} X_i^2 + \sum_i^n \sum_j^n \beta_{ij} X_i X_j \quad (1)$$

where i = 1 to 3, j = 2 to 3, and X = input variables. The number of runs (N) required to measure the responses in BBD is defined as $N= 2k(k - 1) + C_o$, where k is the number of input variables and C_o is the number of central points. Since there were three input variables tested (sulfuric acid concentration, time of pretreatment, and temperature) and four replicates in the central point of the design to calculate the experimental error, a total of 16 experimental runs were carried out. The response factors are pentose degradation, hexose degradation, pentose yield, hexose yield, sugar degradation, and sugar yield. The significant effects and two variable interactions were estimated by ANOVA. The relationship between the response factor and input variables was further elucidated using response surface plots.

2.2. Materials

All chemicals were of analytical grade and used as received. Termamyl and amyloglucosidase were purchased from Novo Nordisk, Bagsvaerd, Denmark. Cellulase of GC220 was purchased from Genencor, Rochester, NY, USA. *S. cerevisiae* CBS 8066, maintained on YPD agar medium, was used for ethanol fermentation.

2.3. Preparation and analysis fruit hull

Ripened jatropha fruits (with yellow color only, harvested in November 2009, Serpong, Indonesia) were peeled. The hulls were collected and dried at 60 °C for 48 h. The dried hulls were milled in a home blender (Philips HR 2071, Royal Philips Electronics, Amsterdam, The Netherlands) and sieved through a strainer with a 0.8-mm hole diameter. The dry matter of milled hull was 95.32% (*w/w*) (24 h, 105 °C). The milled hull material was kept in a sealed plastic container at 4 °C until used.

The chemical composition of the hull (Table 1) was analyzed in triplicate according to the following methods. The organic solvent extractives, the total water extractives, and the non-extractives (except protein) were measured by TAPPI methods (TAPPI, 2004a; TAPPI, 2004b; TAPPI, 2004c; TAPPI, 2004d; TAPPI, 2004e; TAPPI, 2004f), with minor modifications as described previously (Kootstra et al., 2009b). The protein content of fruit hull was determined using the Kjeldahl method, which consists of a destruction unit (Gerhardt Kjeldahlterm) and distillation unit (Gerhardt Vapodest). The amounts of amino acids in the water extractives were determined using a Dionex Ultra-HPLC instrument (Dionex Corporation, Sunnyvale, CA, USA) as described previously (Teng et al., 2011). For measuring monomeric sugars and water-soluble oligosaccharides in fruit hull, the samples were prepared as shown in Figure 1. Monomeric sugars were measured by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described earlier (Kootstra et al., 2009b).

Table 1. Jatropha fruit hull composition (% (*w/w*) dry matter).

Constituents	Content (% (*w/w*) dry matter)
Organic solvent extractives	
- Ethanol-toluene extractives	4.1 ± 0.02
- Ethanol extractives	2.0 ± 0.02
Water extractives	
- Glucose	4.0 ± 0.01
- Galactose, mannose, rhamnose, and arabinose	0.3 ± 0.00
- Glucan	7.2 ± 0.26
- Other oligosaccharide (galactan and mannan)	0.6 ± 0.00
- Amino acids	0.1 ± 0.00
- Others	24.0
Non-extractives	
- Glucan	20.4 ± 0.01
- Xylan	5.7 ± 0.01
- Galactan	2.0 ± 0.01
- Mannan	1.1 ± 0.01
- Rhamnan	0.8 ± 0.00
- Arabinan	0.8 ± 0.01
- Protein	4.9 ± 0.35
- Uronic acid	3.2 ± 0.16
- Acid soluble lignin (ASL)	0.8 ± 0.00
- Acid insoluble lignin (AIL)	11.7 ± 0.00
- Others	6.3
Total	100.0
Total pentose sugars[a]	6.5
Total hexose sugars[b]	35.6

[a] For response surface analysis, the data of pentose sugars (xylose and arabinose) were grouped to calculate pentose yield. [b]Similarly, the data of hexose sugars (glucose, galactose, mannose, rhamnose) were grouped to calculate hexose yield.

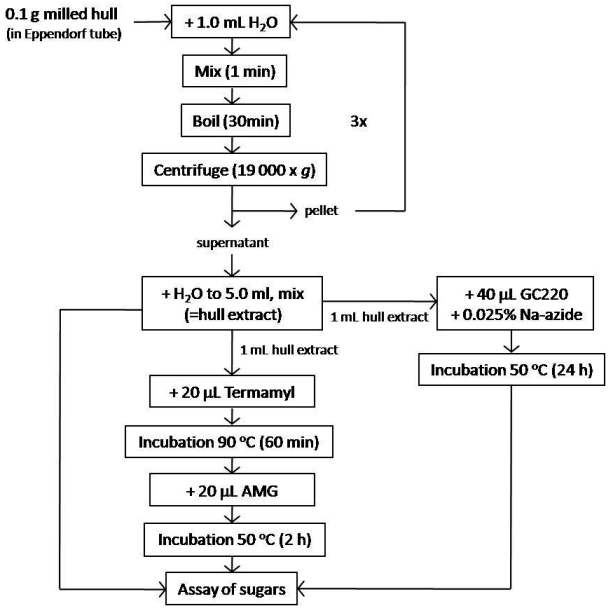

Figure 1. A scheme for determination water soluble sugars in fruit hull. A Hermle Z33M2 (HERMLE Labortechnik GmbH, Wehingen, Germany) was used in centrifugation. The amount of soluble oligoschharides in fruit hull was calculated by subtracting initial monosaccharide present in fruit hull from the total soluble sugar measured after enzymatic digestion.

2.4. Fruit hull pretreatment

Milled fruit hull (5.00 g; 4.76 g dry matter) was mixed with 45.0 mL of sulfuric acid solution (0.1%, 0.5%, or 0.9% (*w/v*)), resulting in 9.52% (*w/w*) dry hull solid loading. The hull/acid mixture was soaked for 20 to 24 h at room temperature and then transferred to a 316-SS (stainless steel) reactor (inner height × diameter, 90.0 × 40.0 mm; 5.0 mm-wall), fitted with thermocouples. Four reactors were heated at a time in an oil bath (Haake B bath filled with silicon oil of DC 200 fluid, 100 cSt, Dow Corning, Midland, MI, USA), equipped with a Haake N3 temperature controller (Thermo Fisher Scientific, Waltham, MA, USA). Sample core temperature was digitally recorded using a Picotech data collector and software (Picotech, Neots, Cambridgeshire, UK). Pretreatments were conducted at 140 °C, 160 °C, and 180 °C. Holding time was 30, 45, or 60 min, starting from when the desired core temperature was reached. The heating bath oil was preheated to between 1°C and 5°C above the desired sample core temperature; by this way the time until the desired core temperature was reached ranged between 16 and 20 min. During the holding time, the reaction temperature in the reactors oscillated at a maximum of 1°C from the desired temperature. The

reactors were cooled after the incubation to 25 °C in less than 10 min by quenching in ice water. After the pretreatment, the resulting material was transferred to pre-weighed 250-mL baffled shake flasks. The pH was adjusted to 5.0 with 10 M NaOH, and sodium azide solution (0.025% (*w/w*) final concentration; Merck KGaA, Darmstadt, Germany) was added. A 1-mL sample was taken for analysis. Monomeric sugars were measured by HPAEC-PAD method (Kootstra et al., 2009b). Furfural, 5-hydroxymethylfurfural (5-HMF), and acetic acid were measured by high performance liquid chromatography (HPLC) (Kootstra et al., 2009b). The monomeric sugar yield was calculated as follows:

$$Yield\,(\%) = \frac{S_L}{S_S} * 100\,(\%) \qquad (2)$$

where S_L is the amount of sugar (g) in the aqueous phase, and S_S is the amount of monomeric sugar present in the sample of dry hull (g sugar equivalents in polymeric sugar). The sugar degradation was calculated in a similar way in which S_L is accounted as the amount of sugar equivalents (g) to the amount of furfural or 5-HMF present in the aqueous phase, calculated in mole basis.

2.5. Enzymatic hydrolysis of pretreated fruit hull

To start enzymatic hydrolysis, GC220 (0.4 g (*w/w*) dry matter hull) were added into the baffled shake flask containing the pH-adjusted (pH 5.0) pretreated fruit hull. GC220 is a cellulase enzyme mixture (batch 4900759148, 7608 IU/mL cellulase activity). The amount of GC220 used corresponded to 46 FPU/g original dry matter hull. We used GC220 in excess (in the plateau region of the dose-effect curve of the enzyme mixture) to ensure that the effect of pretreatment on the sugar yield was measured, not the effect of the enzyme concentration. The weight of the material plus the flask was determined; after which, the flasks were closed with airtight plugs and placed in an Innova 44 incubator shaker (50 °C, 150 rpm, 2-in stroke; NBSC, NJ, USA). Samples of 1.5 mL were taken at t = 0, 24, and 72 h. GC220 was inactivated by incubation at 90 °C for 10 min. Samples were stored at −20 °C until analysis. Monomeric sugars were measured by HPAEC-PAD method (Kootstra et al., 2009b).

2.6. SSF of acid-pretreated hull hydrolysate into ethanol

Ten grams of milled fruit hull pretreated with 90 mL of sulfuric acid solution at the optimum conditions was used for ethanol fermentation tests with the yeast *S. cerevisiae* CBS 8066.

To prepare inoculums, yeast cells were grown in a 100-mL liquid medium of YPD (Merck) at pH 5.0 in a 250-mL Erlenmeyer flask. The culture was incubated for 24 h in an Innova 44 incubator shaker (37 °C, 150 rpm, 2-in. stroke; NBSC, NJ, USA). The yeast cake was prepared by centrifugation at 17,000×*g* for 15 min using the SORVALL RC6+ centrifuge (Thermo Fisher Scientific, Waltham, MA, USA).

Prior to fermentation, the pH of the acid-pretreated hull was adjusted to 5.0 using 10 M NaOH. Pre-hydrolysis was conducted to reduce the viscosity of the pretreated hull suspension and was performed by the addition of 0.95 g GC220 (equivalent to 0.1 g/g dry matter hull) followed by incubation for 6 h in an Innova 44 incubator shaker (37 °C, 150 rpm, 2-in. stroke; NBSC, NJ, USA). After pre-hydrolysis, the pre-hydrolysed substrate was enriched with the following (per liter): 4.2 g $(NH_4)_2SO_4$, 2.5 g KH_2PO_4, 0.42 g $MgSO_4.7H_2O$, trace elements (consisting of 15.0 mg Na_2EDTA, 4.5 mg $ZnSO4.2H_2O$, 0.84 mg $MnCl_2.2H_2O$, 0.3 mg $CoCl_2.6H_2O$, 0.3 mg $CuSO_4.5H_2O$, 0.4 mg $Na_2MoO_4.2H_2O$, 4.5 mg $CaCl_2.2H_2O$, 3 mg $FeSO_4.7H_2O$, 1 mg H_3BO_3, 1 mg KI, vitamins (consisting of 0.05 mg biotin, 1.0 mg calcium panthotenate, 1.0 mg nicotinic acid, 25 mg inositol, 1.0 mg thiamine, 1.0 mg pyridoxine HCl, 0.2 mg para-aminobenzoic acid), and fatty acids (10.0 mg ergosterol and 420 mg Tween 80 dissolved in 1.25 mL ethanol). Trace elements solution, vitamins solution, and fatty acid solution were prepared separately in stocks of 1000×, 1000× and 800×, respectively, as described previously (Verduyn et al., 1992). A 2.86-g enzyme mixture (GC220), equivalent to 0.3 g/g dry matter hull, was added to the substrate. The total volume of substrate just before fermentation was circa 100 mL. An initial yeast cake concentration between 0.50 and 0.55 g/100-mL substrate was applied in the SSF experiments (Cuevas et al., 2010). Fermentation was conducted at 37 °C and 70 rpm for 72 h. All SSF experiments were done in duplicate, and homogenous samples of 1.5 mL were withdrawn at 0 (just after yeast addition), 6, 11, 24, 35, 48, and 72 h of incubation for the analysis of monosaccharides, organic acids, and ethanol. For the analysis of ethanol and organic acids in SSF samples, the supernatant of centrifuged samples (5 min at 17,400× *g*) was

diluted 1:1 with 6 mM sulfuric acid and filtered in order to remove solids and precipitated proteins. Samples were analyzed using a HPLC system (Waters Corp., Milford, MA, USA) using the organic acid column IOA-1000 (Alltech, Deerfield, IL, USA) with 3.0 mM sulfuric acid as the mobile phase at 90 ^{o}C and a flow rate of 0.4 mL/min. Peaks were detected by using a RI detector (Waters 2414, Waters Corporation, Milford, MA, USA). SSF results of sugars, organic acids, and ethanol were reported in grams of product formed per liter fermentation medium. The theoretical ethanol yield was calculated from the total glucose consumed by the yeast, taking into account a fermentation yield of 0.51 (Ballesteros et al., 2008; Cuevas et al., 2010).

3. Results and Discussion

3.1. Raw material composition

Table 1 shows the composition of jatropha fruit hull. Carbohydrates account for 42.9% of the dry weight. This value is lower than those reported for other lignocellulosic materials such as wheat straw (59%), and cardoon biomass (52%) (Ballesteros et al., 2008; Kootstra et al., 2009a). A glucan (as glucose) content of 31.6% is comparable to those reported for agricultural residues such as cardoon, sunflower, or *Brassica* (Ballesteros et al., 2008; Ballesteros et al., 2002; Ruiz et al., 2006) but slightly lower than those for wheat straw and rice straw (36%) (Bak et al., 2009; Kootstra et al., 2009b). Unlike wood and straw, the hull contains about 12% water-soluble sugars. The total lignin value is 12.5% of which acid-soluble lignin (ASL) accounts for 0.8% only. The lignin value of the hull is less than 50 % of that of wood (Cara et al., 2006; Yang et al., 2002). ASL will solubilize during acid pretreatment, while acid-insoluble lignin (AIL) remains in solid form. Hemicellulosic sugars (xylose, galactose, mannose, rhamnose, and arabinose) account for 10.4% of the hull with xylose (5.7%) as the main hemicellulosic carbohydrate. No xylose was found after incubation of the water-extractable fraction with enzyme mixture GC220 (data not shown), indicating that the material does not contain soluble xylan. Together, the organic solvent extractables and water extractables account for 30% of the total dry matter, which may include non-structural components of fruit hulls such as waxes, fats, tannins, some resins, and soluble pectins (Cara et al., 2006; Winkler et al., 1997b). In comparison, the values for glucan and lignin content obtained are lower than those of the jatropha fruit hull

(39.3% and 14.2%, respectively) previously reported (Nallathambi Gunaseelan, 2009), which could be due to differences in cultivar, cultivation conditions, or ripeness of the fruit.

3.2. Pretreatment and enzymatic hydrolysis of fruit hull

The experimental conditions for the 16 runs according to the BBD can be seen in Table 2. Table 2 also summarizes the overall experimental results of sugar liberation from milled jatropha fruit hull after pretreatment and subsequent enzymatic hydrolysis for 24 h.

Both the highest pentose yield (34.4%) and pentose degradation (42.4%) after pretreatment were achieved at 0.9% sulfuric acid, 45 min, and 180°C, in which 57.4% pentose and 80.6% hexose were recovered after subsequent enzymatic hydrolysis (Table 2). After the subsequent 24-h enzymatic hydrolysis, the highest pentose yield (84.7%) was obtained from the biomass pretreated at less severe conditions (0.9% sulfuric acid, 30 min, and 160°C) in comparison to the highest hexose yield (80.6%) which was obtained at more severe conditions (0.9% sulfuric acid, 45 min, and 180°C).

In terms of total sugar liberation (Table 2), both the highest sugar yield (21.0%) and sugar degradation (24.4%) after pretreatment were achieved at 0.9% sulfuric acid, after 45 min, and at 180°C. After the subsequent 24-h enzymatic hydrolysis, the highest sugar yield of 77.0% was reached at this pretreatment condition.

Table 2. Experimental conditions according to Box-Behnken design and experimental results of sugar liberation from milled jatropha fruit hull.

	Run Number															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Pretreatment conditions																
Sulfuric acid (% *w/v*)	0.1	0.5	0.5	0.9	0.1	0.1	0.9	0.9	0.5	0.5	0.5	0.5	0.1	0.5	0.5	0.9
Time (min)	45	30	60	45	30	60	30	60	45	45	45	45	45	30	60	45
Temperature (°C)	140	140	140	140	160	160	160	160	160	160	160	160	180	180	180	180
Response factors																
A. Pentose (% *w/w*)																
Pretreatment																
Pentose yield	1.2	5.4	7.8	16.1	2.9	2.8	26.5	33.5	10.2	11.1	10.9	10.1	2.5	10.4	9.6	34.4
Pentose degraded	0.2	0.6	1.1	2.0	0.5	0.9	7.7	12.7	3.9	3.8	3.8	3.5	3.0	12.1	20.2	42.4
Total pentose liberated	1.3	5.9	8.9	18.2	3.4	3.7	34.1	46.2	14.1	15.0	14.7	13.6	5.5	22.4	29.8	76.8
After 24 h enzymatic hydrolysis																
Pentose yield	29.3	35.1	40.4	56.6	47.7	54.4	84.7	80.7	69.5	66.0	67.7	69.3	57.6	70.5	46.8	57.4
B. Hexose (% w/w)																
Pretreatment																
Hexose yield	12.5	14.0	14.5	15.2	8.8	6.6	17.8	18.1	13.7	12.8	13.0	13.9	1.5	8.3	6.0	18.6
Hexose degraded	0.1	0.5	0.6	0.9	0.2	0.3	1.8	2.5	1.3	1.3	1.3	1.3	0.4	2.7	3.6	6.4
Total hexose liberated	12.6	14.5	15.1	16.1	9.0	6.9	19.6	20.6	15.0	14.1	14.3	15.2	1.9	11.0	9.6	25.0
After 24 h enzymatic hydrolysis																
Hexose yield	57.6	58.7	57.6	57.6	54.0	51.2	65.6	69.9	59.9	58.3	59.0	59.6	51.2	64.8	63.7	80.6
C. Total sugars (% w/w)																
Pretreatment																
Sugar yield	10.7	12.7	13.4	15.3	7.8	6.1	19.1	20.5	13.1	12.5	12.7	13.3	1.7	8.6	6.5	21.0
Sugar degraded	0.1	0.5	0.9	1.5	0.4	0.6	4.7	7.6	2.6	2.6	2.5	2.4	1.7	7.4	11.9	24.4
Total sugar liberated	10.8	13.2	14.3	16.8	8.2	6.7	23.8	28.1	15.7	15.1	15.2	15.7	3.4	16.0	18.4	45.4
After 24 h enzymatic hydrolysis																
Sugar yield	53.3	55.1	55.0	57.4	53.0	51.7	68.6	71.5	61.4	59.5	60.4	61.1	52.2	65.7	61.1	77.0

Only data of 24 h enzymatic hydrolysis are presented because no increase of sugar liberation was observed between 24 h and 72 h of hydrolysis (data not shown).

3.3. Statistical analysis of sugar degradation and sugar yield

In the experimental Box-Behnken design, the influence of the three input variables (sulfuric acid concentration, time, and temperature of pretreatment) on pentose degradation, hexose degradation, pentose yield, and hexose yield were determined. The Box-Behnken model used to describe the experimental results is based on the effect of the three input variables studied, extended with parameters for interactions and squared factors. A square root transformation of the response factors was applied for improved model fit. To describe the interactive effects of the input variables on responses in the statistical analysis, one variable is set constant while the other two variables are varying in the design space. The input variables of X_1, X_2, and X_3 are the coded factors of sulfuric acid concentration, time, and temperature of pretreatment, respectively. The coded variables are defined as follows: $X_1 = (S_A - S_{A,C})/S_{A,S}$, $X_2 = (t - t_C)/t_S$, and $X_3 = (T - T_C)/T_S$, in which S_A = concentration of sulfuric acid (%), t = pretreatment time (min), and T = pretreatment temperature (°C); subscript C = center value and subscript S = step value; $S_{A,C} = 0.5\%$, $S_{A,S} = 0.4\%$, $t_C = 45$ min, and $t_S = 15$ min, $T_C = 160$°C and $T_S = 20$°C.

3.4. Pentose and hexose degradation after pretreatment

The starting point was a quadratic model which was found significant for the degradation of pentose into furfural and the degradation of hexose into 5-HMF during the pretreatment (both with $P < 0.0001$). The quadratic model was then adjusted by backward elimination: taking out terms that had no significant contribution ($P > 0.05$) one by one and then recalculating the model with the remaining terms. The adjusted regression models fit the data with the $R^2_{adjusted}$ of 0.99 for pentose degradation (Equation 3) and 0.97 for hexose degradation (Equation 4) as follows:

$$(Y_{PD,Pr})^{0.5} = 1.97 + 1.31X_1 + 0.29X_2 + 1.57X_3 + 0.94X_1X_3 + 0.19X_2X_3 + 0.51X_3^2 \quad (3)$$

$$(Y_{HD,Pr})^{0.5} = 1.13 + 0.56X_1 + 0.50X_3 + 0.30X_1X_3 - 0.15X_1^2 + 0.13X_3^2 \quad (4)$$

in which $Y_{PD,Pr}$ (% *w*/*w*) is the pentose degradation, and $Y_{HD,Pr}$ (% *w*/*w*) is the hexose degradation after pretreatment, respectively.

Figure 2 shows the response curves of pentose and hexose degradation as three-dimensional surfaces. As can be seen in Figure 2a and Equation 3, minimizing the degradation of pentose into furfural depends, for a large part, on sulfuric acid concentration and temperature. Degradation of pentose increases rapidly at higher sulfuric acid concentration and temperature. Because the degradation of pentose is, for a small part, influenced also by time, conducting the pretreatment step in a shorter time (30 min) is preferable than a longer pretreatment time if high pentose recovery is the target. A pentose degradation of 34% is obtained at 30 min, 0.9% sulfuric acid, and 180°C (Figure 2a) in which 33% of the pentose is simultaneously recovered (data not shown), resulting in a total pentose liberation from the biomass of 67%. A corresponding result has been reported in the pretreatment of corn stover at 20% (w/w) solid loading by using sulfuric acid (0.5% to 1.4% (w/w)) at 165°C to 195°C for 3 to 12 min, in which pentose degradation ranged between 5% and 31% (Schell et al., 2003).

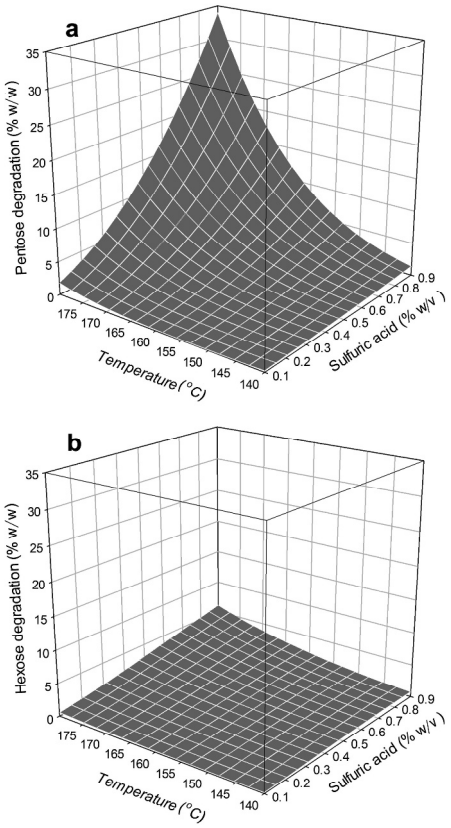

Figure 2. Three-dimensional response surfaces for (a) pentose degradation and (b) hexose degradation of jatropha fruit hull. The response surfaces were graphed after dilute sulfuric acid pretreatment. Pretreatment time is 30 min.

In case of hexose degradation, from the three input variables studied, the pretreatment time has no significant contribution on the degradation of hexose (Equation 4). The influence of the sulfuric acid concentration and temperature causes less than 6% hexose degradation in the design space as shown in Figure 2b. The low hexose degradation at pretreatment demonstrates that only a small fraction of the six-

carbon polysaccharides (mostly cellulose) of the biomass was completely depolymerized and degraded during the pretreatment, a positive property of a pretreatment, assuming the treatment's ability to facilitate the subsequent enzymatic hydrolysis.

3.5. Pentose and hexose yields after enzymatic hydrolysis

The starting point was a quadratic model which was found significant for the pentose yield and the hexose yield after the subsequent 24-h enzymatic hydrolysis of pretreated fruit hulls (both with $P < 0.0001$). The quadratic model was then adjusted by backward elimination: taking out terms that had no significant contribution ($P > 0.05$) one by one and then recalculating the model with the remaining terms. The adjusted regression models fit the data with the $R^2_{adjusted}$ of 0.91 for pentose yield (Equation 5) and 0.99 for hexose yield (Equation 6) as follows:

$$(Y_{PY,24h})^{0.5} = 8.19 + 0.75X_1 + 0.65X_3 - 0.53X_1X_3 - 0.50X_2X_3 - 1.23{X_3}^2 \quad (5)$$

$$(Y_{HY,24h})^{0.5} = 7.69 + 0.47X_1 + 0.22X_3 + 0.11X_1X_2 + 0.45X_1X_3 + 0.11{X_3}^2 \quad (6)$$

in which $Y_{PY,24h}$ (% *w*/*w*) is the pentose yield, and $Y_{HY,24h}$ (% *w*/*w*) is the hexose yield after the 24-h enzymatic hydrolysis, respectively. Figure 3 shows the response analysis of pentose and hexose yields as three-dimensional surfaces.

As can be seen in Figure 3a and Equation 5, maximizing the pentose yield is, for a large part, dependent on sulfuric acid concentration and temperature. The time of pretreatment on its own has no direct significant contribution on the pentose yield, but its interaction with the temperature has. In comparison to our results, it was reported that the yield of xylose (a major component of pentose) after a dilute sulfuric acid (0.5% to 2%) pretreatment of rapeseed straw at high temperature (180°C) is dependent on acid concentration and time of pretreatment (Lu et al., 2009).

Figure 3 shows that pretreatment of the fruit hull at the conditions studied results in a significant increase of the pentose yield and the hexose yield in the hydrolysis step.

Approximately 84% pentose yield after enzymatic hydrolysis can be achieved at 0.9% sulfuric acid (30-min pretreatment at 160°C) in which approximately 10% pentose is degraded (Figure 2a). Increasing the temperature above 160°C or the time of pretreatment for more than 30 min (data not shown) does not improve the pentose yield due to the degradation of pentose into furfural. The 30-min pretreatment results in a higher pentose yield than the longer pretreatment periods.

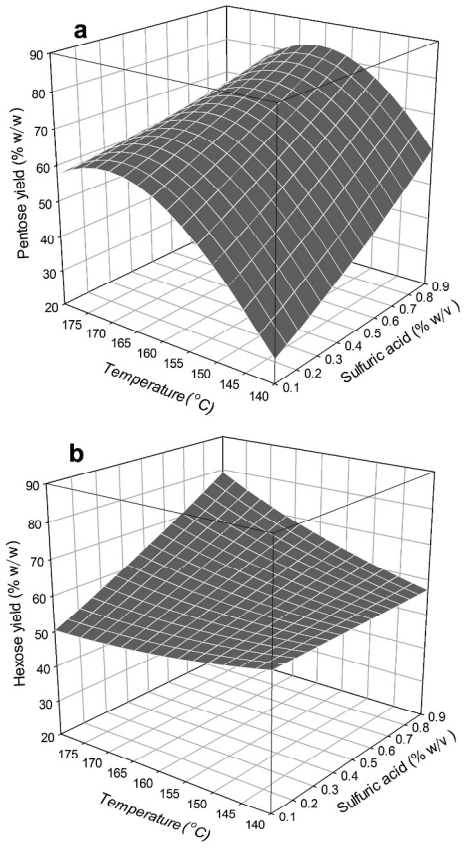

Figure 3. Three-dimensional response surfaces for (a) pentose yield and (b) hexose yield. The response surfaces were graphed after 24-h enzymatic hydrolysis, respectively, of the pretreated jatropha fruit hull. Pretreatment time is 30 min.

We found that without enzymatic hydrolysis, pretreatment alone of the fruit hull with 0.1% sulfuric acid at a temperature between 140°C and 180°C resulted in pentose yields of less than 4% (data not shown). However, high overall pentose yields obtained after enzymatic hydrolysis of the fruit hull pretreated with 0.1% sulfuric acid at 140°C to 180 °C (Figure 3a) demonstrate that the hydrothermal treatment itself contributes to hemicellulose digestibility, thereby enhancing the enzymatic hydrolysis. It has been reported that heating lignocellulosic biomass in aqueous media at a temperature above 150°C to 180°C will solubilize parts of the biomass, firstly the hemicellulose and shortly after the lignin (Hendriks and Zeeman, 2009).

From Figure 3b and Equation 6, the hexose yield ranged from approximately 52% at the least severe

pretreatment (0.1% sulfuric acid, 30 min, 140°C) to approximately 78% at the more severe conditions (0.9% sulfuric acid, 30 min, 180°C)

3.6. Total sugar yield after enzymatic hydrolysis

For the total sugar degradations (pentose plus hexose degradation) after pretreatment and the total sugar yield (pentose plus hexose yield) after the subsequent 24-h enzymatic hydrolysis of the pretreated fruit hull, the starting point of a quadratic model was found significant ($P < 0.0001$). The quadratic model was then adjusted by backward elimination: taking out terms that had no significant contribution ($P > 0.05$) one by one and then recalculating the model with the remaining terms. The adjusted regression models fit the data with the $R^2_{adjusted}$ of 0.98 for the total sugar degradation (Equation 7) and 0.96 for the total sugar yield (Equation 8) as follows:

$$(Y_{SD,\mathrm{Pr}})^{0.5} = 1.58 + X_1 + 0.21X_2 + 0.15X_3 + 0.69X_1X_3 + 0.38{X_3}^2 \quad (7)$$

$$(Y_{SY,24h})^{0.5} = 7.79 + 0.51X_1 + 0.28X_3 + 0.32X_1X_3 - 0.09{X_3}^2 \quad (8)$$

in which $Y_{SD,Pr}$ (% *w*/*w*) is the total sugar degradation after pretreatment, and $Y_{SY,24h}$ (% *w*/*w*) is the total sugar yield after the subsequent 24-h enzymatic hydrolysis.

Figure 4 shows the response curve of the total sugar yield and the total sugar degradation as three-dimensional surfaces. Statistical analysis of combined sugars (pentose plus hexose) showed that the time of pretreatment has no influence on the total sugar yield (Equation 8). Sulfuric acid concentration and temperature as well as their interaction have positive influences on the total sugar yield, contributing to the increase of overall sugar yield as sulfuric acid concentration and temperature increase. Because of the negative squared term of the temperature (Equation 8), an optimum point somewhere in the experimental space might be expected; however, the temperature coefficient seems too small to level off the curve. A yield of 78% was the maximum achieved value at the temperature of 180°C using 0.9% sulfuric acid, in which 21% sugar is degraded into furfural and 5-HMF (Figure 3).

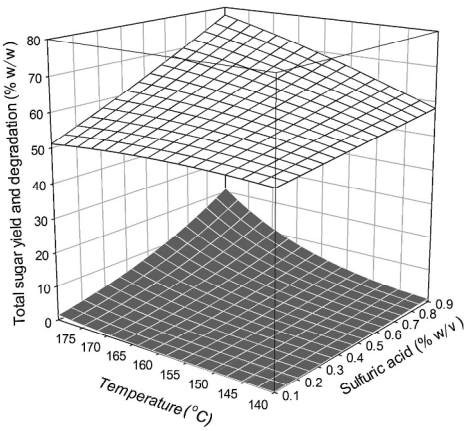

Figure 4. Three-dimensional response surfaces for total sugar degradation (dark) and total sugar yield (light). The response surface for total sugar degradation was graphed after pretreatment, and that for total sugar yield was graphed after 24 h enzymatic hydrolysis of pretreated jatropha fruit hull. Pretreatment time is 30 min.

3.7. Optimization and confirmation test

It has been shown that the maximum sugar yield as well as the hexose yield, respectively, after enzymatic hydrolysis can be achieved only if pretreatment of the fruit hull (9.52% solid loading) was performed using 0.9% sulfuric acid at a temperature around 180°C. Using the Design-Expert 8.0.3 software, optimization was conducted to obtain the highest hexose/sugar yields with minimum sugar degradations. The optimal conditions were as follows: sulfuric acid equals 0.9%, time equals 30 min, and temperature equals 178°C. Under these conditions, the model predicts the responses as follows: pentose degradation of 31%, hexose degradation of 6%, a pentose yield (after 24-h enzymatic hydrolysis) of 72%, and a hexose yield (after 24-h enzymatic hydrolysis) of 77%.

A confirmation test was performed under the pretreatment conditions described above, in duplicate. The experimental results with the deviations from the average were as follows: pentose degradation of 29.4 ± 1.27% (equivalent to 1.5 ± 0.05 g/L furfural), hexose degradation of 5.2 ± 0.14% (equivalent to 1.2 ± 0.03 g/L 5-HMF), a pentose yield of 70.8 ± 1.20% (equivalent to 4.4 ± 0.08 g/L pentose), and a hexose yield (24 h) of 78.3 ± 0.70% (equivalent to 26.5 ± 0.25 g/L hexose). These experimental sugar values agree well to those predicted by the model with deviations less than 1%. In addition, we found that acetic acid liberation was 1.5 ± 0.02 g/L.

3.8. SSF of hydrolysed fruit hull into ethanol

Fruit hull of 9.52% solid loading pretreated at optimum conditions (0.9% sulfuric acid, 178°C, 30 min) was applied in a SSF process using *S. cerevisiae* at 37°C. In a SSF

process, the glucose liberated by enzymatic hydrolysis is simultaneously converted into ethanol, thus reducing product inhibition of the enzyme by glucose. Figure 5 shows the course of ethanol formation for the 72-h fermentation in the standard medium containing 30 g/L glucose (Figure 5a) in comparison to the medium containing the pretreated fruit hull (Figure 5b).

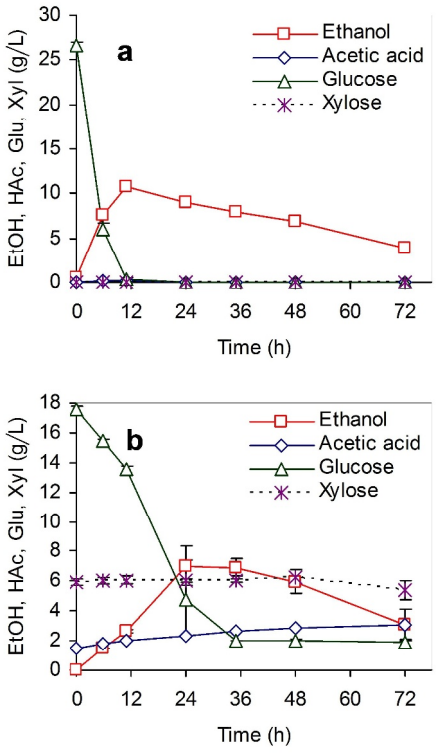

Figure 5. Course of ethanol formation. Formation of ethanol, liberation of acetic acid, and the consumption trends of glucose and xylose during fermentation by *S. cerevisiae* CBS 8066 of (**a**) standard medium containing glucose as a sole carbon source in comparison to (**b**) SSF of pretreated jatropha fruit hull.

In the standard medium, glucose is depleted within 12 h. In that period, ethanol was rapidly produced and peaked at about 10.7 g/L. This ethanol concentration corresponds to 74% of the maximum theoretical yield.

In the pretreated fruit hull medium (Figure 5b), a 6-h pre-hydrolysis to reduce the viscosity of the hydrolysate suspension resulted in 18 g/L glucose initially available in the medium before the fermentation started. As can be seen in Figure 5b, glucose depletion in the fruit hull medium was slower than in the standard medium, reaching 2 g/L after 36 h and remaining constant from that point; the maximum ethanol concentration was 8.4 g/L at 24 h (corresponding to 71% of the maximum theoretical yield). This trend of ethanol formation, peaking at 24 h, corresponds well to the results previously reported (Cuevas et al., 2010). It is clearly seen that ethanol formation was slightly inhibited in the pretreated fruit hull biomass, leading to a lower ethanol yield (71% compared to 74%) and longer fermentation time (24 h compared to 12 h) at peak

points. In biomass hydrolysis, limiting concentrations of byproducts are important when the hydrolysate is to be used as a fermentation medium. For ethanol fermentation, it was reported that the presence of acetic acid (5 g/L), furfural (1.2 g/L), and 5-HMF (1.3 g/L) slightly decreased the ethanol yield in the fermentation process; however, a low acetic acid concentration (about 1 g/L) was found to have a positive effect on the ethanol production yield (Erdei et al., 2010). Due to pretreatment of the fruit hull at optimum conditions, 1.5 g/L furfural, 1.2 g/L 5-HMF, and 1.5 g/L acetic acid were also formed in the fermentation substrate. Therefore, inhibition of ethanol formation in the fruit hull substrate was probably caused by the presence of these compounds.

4. Conclusions

It is demonstrated that the model equations developed using the Box-Behnken design with the three input variables studied (sulfuric acid, time, and temperature) can be used to predict liberation and degradation of sugars from the jatropha fruit hull after dilute sulfuric acid-catalyzed hydrothermal pretreatment and subsequent enzymatic hydrolysis. A pretreatment at optimum conditions (0.9% sulfuric acid, 30 min, 178°C) followed by a 24-h enzymatic hydrolysis liberates nearly all of the sugars present, consisting of 71% pentose, 78% hexose, 29% pentose degradation into furfural, and 5% hexose degradation into 5-HMF. Therefore, higher sugar yields cannot be expected within the space studied. The SSF experiment of ethanol production showed that the sulfuric acid-pretreated fruit hull can be used to produce ethanol by *S. cerevisiae* in the simultaneous saccharification and fermentation process.

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CHAPTER 6

General discussion

1. Retrospection

The research questions for our research project stated in the Introduction were:

a) Which part of jatropha lignocellulosic biomass can be depolymerized to sugars and then valorized to chemicals /fuels, in particular to bioethanol?
b) Can a microbial method of jatropha oil extraction improve the oil yield in comparison to other known processes?
c) Can a microbial method of oil extraction preserve the original structure of jatropha protein?
d) Can jatropha fruit by-products after valorization give better added value products than only the seeds?

This thesis discusses related experimental results to answer these research questions, designs possible biorefinery routes based on our research findings combined with the other published methods of valorization, and calculates the revenue from products possibly valorized from the jatropha biomass.

1.1. The recalcitrance of jatropha fruit byproducts in pretreatment

Cellulosic biomass is generally an inexpensive resource that may be converted sustainably into large volumes of transportation fuels and chemicals. In many cases, pretreatment of highly recalcitrant cellulosic biomass to increase its enzymatic digestibility is essential to achieve high yields from biotechnological processes. In bioethanol production, as an example, pretreatment of cellulosic biomass is the most expensive processing step, representing approximately 20% of the total cost (Yang and Wyman, 2008). To achieve a low cost pretreatment technology, one must take into account technical aspects like sugar-release patterns, solid loading, the compatibility of pretreatment with the overall process, the availability of feedstock and enzymes, and the suitability of organisms to be used (Yang and Wyman, 2008)

In a jatropha oil production line abundant side-streams mainly derived from fruit are generated consisting of seed cake rich in valuable proteins, seed shell composed of mainly lignin, as well as polysaccharides and fruit hull with low lignin content but with high polysaccharides and water-soluble components. Owing to their differences in chemical and physical characteristics proper methods of hydrolyzing lignocellulose

from these by-products into sugars need to be thoroughly investigated. Some authors have highlighted factors that affect the ability of cellulose from plant biomass to be hydrolyzed, including porosity of the materials, the cellulose fiber crystallinity, and lignin and hemicellulose content (Hendriks and Zeeman, 2009). The presence of high lignin and hemicellulose in crop residues has been considered as the main barrier for cellulase enzymes to reach the reaction site within the cellulose chains, thus reducing the efficiency of hydrolysis (Sun and Cheng, 2002).

In our efforts to hydrolyze jatropha fruit polysaccharides by cellulases, we found that low concentrations of glucose were attained from sulfuric acid pretreated press cake and seed shell, although the sulfuric acid concentration has been increased up to 500 mM (equivalent to 4.9%) (Chapter 2). Even though the hemicellulose were successfully removed from both materials in the pretreatment, the cellulose of these materials was still hardly accessed by the enzyme molecules. From the fact that glucose release has a direct correlation with lignin content, in which lower glucose yields were obtained at higher lignin content, we concluded that the pretreatment parameters applied had a small effect on lignin degradation. Lignin in jatropha press cake and seed shell is highly resistant against the sulfuric acid concentrations and the operational temperatures applied (Chapter 2). The results suggest that lignin acts as the physical barrier in the cellulose conversion from jatropha press cake and seed shell.

We have demonstrated that the enzymatic digestibility of sulfuric acid pretreated fruit hull was improved significantly compared to sulfuric acid pretreated seed cake and seed shell (Chapter 2). For carbohydrate conversion, jatropha fruit hull therefore stands as one of the potential materials for bioconversion into sugar-based chemicals and fuels. We have shown that a pretreatment using 500 mM (or 4.9% *w/v*) sulfuric acid at a moderate operational temperature (120 ^{o}C) for 30 min released 100% xylan and 80% glucan. These yields look applicable as substrate for biological applications, but the use of high sulfuric acid concentratration (4.9% *w/v*) leads to increased acid neutralization cost thereby increasing the overall pretreatment costs. The question arises whether the use of more dilute sulfuric acid could result in comparable sugar yields. This question has brought us to optimize the pretreatment parameters

(temperature, dilute sulfuric acid, and time) of jatropha fruit hull (Chapter 5), which will be discussed later in this chapter.

1.2. The role of traditional coconut oil extraction (Java method) in jatropha oil extraction

In the traditional Java method of coconut oil extraction which uses a microbial process, mashed paddy-field crab is mixed with shredded coconut. After overnight incubation, the mixture is dried in the sunlight, then squeezed to collect the oil. In principle, the traditional Java method avoids the use of heat to preserve the coconut taste (Haryoto, 1983). This way is simple and very practical, in addition it requires less energy than the common ways of coconut oil extraction with heating. Inspired from this traditional practice we therefore investigated the possible application of the traditional Java method for the recovery of oil from jatropha seeds (Chapter 3).

For application on an industrial scale, the production of jatropha oil by way of mimicking the Java method is not practical nor economically feasible due to the limited number of crabs in nature. Therefore, the production of jatropha oil by utilizing the component originated from crabs that can be isolated and propagated in vitro with the appropriate method was our goal. For that reason, we investigated the possible use of bacteria isolated from crab, which are easily maintained and propagated in the laboratory, for the liberation of oil from jatropha kernels.

In terms of microbial oil extraction, we have elaborated the role of a bacterial strain, namely *Bacillus licheniformis* strain BK23, for the liberation of oil from shredded coconut or homogenized jatropha kernel (Chapter 3). Later we found that this strain hydrolyzed all protein present in jatropha kernel into soluble, smaller molecular weight fractions, an unfavorable trait if the protein is to be conserved and recovered for technical applications (Moure et al., 2006). With the objective of conserving the integrity of protein structure, we isolated new strains from the crabs, identified as either *Bacillus pumilus* or *B. altitudinis*, capable of releasing the jatropha oil largely by the destruction of hemicellulose in jatropha kernel (Chapter 4).

1.3. The fate of jatropha protein functionality during microbial oil extraction processes

Physico-chemical process and heating are generally involved in the separation and purification of vegetable proteins, which may affect the nutritional value of the final product, as well as the functional properties of protein when protein products will be used for food and non-food purposes (Moure et al., 2006). Processes in water media, either assisted or not by enzymes degrading polysaccharides of the cell wall structure to strengthen the oil extraction, can also improve nutritional value and functional properties of the proteins (Dominguez et al., 1994; Moure et al., 2006; Rosenthal et al., 1996). Several treatments done to remove the antinutritive compounds such as hydrothermal pretreatment, fermentation, soaking, and germination can improve the nutritional quality and the functional traits of the protein (Moure et al., 2006). These statements indicated that the hydrolysis of cell wall carbohydrate or the partial hydrolysis of high molecular weight protein molecules into smaller ones could improve the nutritional value, reduce or eliminate the antinutritive factors of the proteins of origin, or create new smaller proteins having totally new functional properties.

When the culture of *Bacillus pumilus* was directly applied to the jatropha kernel slurry for oil extraction (Chapter 4), the SDS-PAGE analysis in the residue after oil separation revealed the existence of three additional protein bands in the solid phase of the microbial-treated jatropha kernel slurry, bands which were not found in the NaOH extractable jatropha protein. There are two possible explanations: (1) microbial process gave higher number of jatropha protein subunits recovered than that of NaOH extraction, or (2) at least one jatropha protein subunit (88.5 kDa) that was missing in the solid phase might have been hydrolyzed by *Bacillus pumilus* proteases into smaller molecular weight of insoluble protein fractions. This partial hydrolysis were not detected by the agar plate method due to formation of insoluble fractions. The agar plate method, in fact, detected only a complete solubilization of whole available jatropha protein subunits. Further studies seem important to investigate the functional properties of the additional protein fractions which were extracted or formed by the action *Bacillus pumilus*.

1.4. Jatropha fruit byproduct conversion: ethanol case

In addition to oil as the main product, the biomass from jatropha plantation comprising wood, leaves, and fruit side stream may be transformed into chemicals and energy. The cellulosic biomass should be easily hydrolyzed into simple sugar-rich substrate suitable for fermentation, and sugar concentration must be high enough so that the conversion yield could generate sufficient economic value. We have not studied the conversion of jatropha wood. However, from the fruit parts our research showed that seed-cake and seed shell gave low sugar yield and therefore are not suitable for ethanol fermentation. Fruit hull, on the other hand, hydrolyzed more easily so it is very suitable as a substrate for bioethanol fermentation.

The fermentation of ethanol usually takes place at a temperature of 25-36 °C and lasts 6-72 hours depending on composition of hydrolyzate, type of the strain, the quantity of inoculum added and yeast activities; and in theory, 1 tonne of glucose will produce 511 kg of ethanol (Gnansounou and Dauriat, 2005). However, in practice the fermentation efficiency lies between 80-92% (Gnansounou and Dauriat, 2005; Mielenz et al., 2009). These high yields occur when the medium is unbalanced and something becomes limiting. In our experiments, we found lower fermentation efficiency (71-74%) for both the jatropha hull hydrolyzate and the standard media with glucose (Chapter 5). In our case the medium was complete resulting in significant amounts of biomass and glycerol and therefore a lower ethanol yield.

2. Perspectives

Industrial products from biomass can only compete with petroleum based products if the biomass is converted optimally through an efficient biorefinery system, where high value chains are developed and put into action (van Ree and Annevelink, 2007). For the perspectives, we propose a general biorefinery route in such a way that all by-products are utilized and minimum or no further waste will be generated, referred to as a zero waste approach. Secondly, we propose to integrate our research findings in combination with the alternative methods of valorization into a whole crop biorefinery route. The alternative methods can be sourced from the research findings of our colleagues within the same jatropha project funded by KNAW-The Netherlands (KNAW, 2012) or from published articles. We will also elaborate the future prospect

of jatropha biomass biorefinery based on a simple economy analysis of chemical and fuel products derived from jatropha biomass.

2.1. Zero-waste approach in the refinery of jatropha biomass

We propose a comprehensive zero-waste approach of biorefinery for jatropha biomass as seen in Figure 1. In a jatropha plantation, the jatropha plants require sunlight, carbon dioxide, nutrients, and water for growth. As a result of cultivation, harvesting, and pruning, biomass in the form of fruit, wood and leaves will be produced. In the first stage of the refinery (primary refinery), fruit, wood and leaves can undergo the process of separation, extraction, pretreatment, and hydrolysis to generate primary products namely oil, proteins, carbohydrates, lignin, and bioactive compounds (Devappa and Swamylingappa, 2008; Dhyani et al., 2011; King et al., 2009; Lestari et al., 2010; Makkar et al., 2008; Openshaw, 2000; Singh et al., 2008). Several additional fractions like the highly acidic/alkaline resistant seed shells and liquid waste are also generated from those processes.

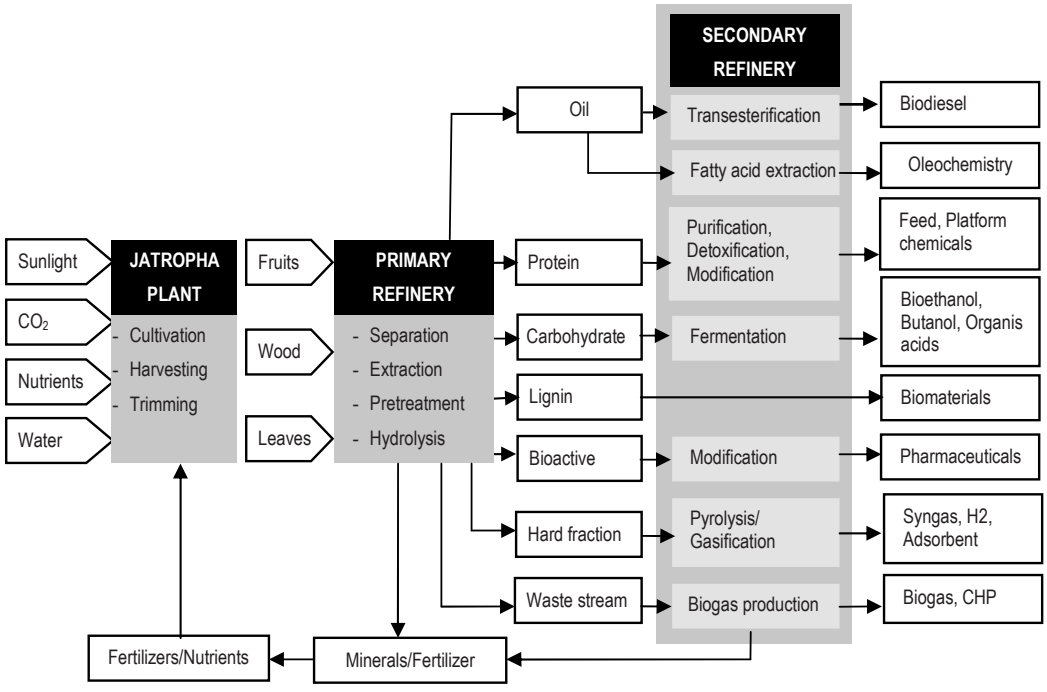

Figure 1. A flow diagram of zero-waste approach in jatropha biorefinery

In a secondary refinery, biogas can be produced by anaerobic digestion of the liquid waste (Gunaseelan, 2009; Staubmann et al., 1997). Energy sources for the refinery processes can be sourced from the biogas or from pyrolysis and gasification fuels made from the hard fraction like seed shells (Goyal et al., 2008; Vyas and Singh, 2007). Fermentation of carbohydrate fractions will produce ethanol, which can also be used as an additional source of energy or as the reagent for transesterification of oil into biodiesel (FACT-Foundation, 2010). Proteins isolated from the seeds can be separated, purified, and or modified into animal feed and platform chemicals (Hamarneh et al., 2010; King et al., 2009; Kootstra et al., 2011; Lestari et al., 2010; Makkar and Becker, 2009; Moure et al., 2006). Bioactive compounds from the leaves can be purified or modified to produce pharmaceutical compounds (Devappa et al., 2010). Finally, primary and secondary refineries will produce minerals and organic fertilizer that can be recycled back to the plantation as nutrients for the Jatropha plant (FACT-Foundation, 2010).

2.2. Process design in biorefinery of jatropha fruit

Our research was focused on the utilization of jatropha fruit. Fruits are dehulled to produce the fruit hull and seeds. In Indonesia jatropha plantations commonly have a population of 2500 plants/ha. A productivity of 2 kg seed/plant/yr has been reported (Purwaamijaya et al., 2007). One hectare of jatropha plant therefore would yield 5000 kg seed annually (containing 25% to 33% oil).

For designing the process, calculating the quantity of products valorized, and estimating the economic value of each product, we take one hectare jatropha with the seed yield of 5000 kg/ha/yr as the basis in process design. This yield has been reported as the minimum plant productivity to be economically feasible (Syakir, 2010). In a traditional practice of jatropha cultivation, we calculate that production of 5000 kg/ha dry seed will dump as much as 9300 kg/ha fresh fruit hull into the land. This fruit hull contains 2140 kg/ha dry matter. The total energy content (GJ/ha/yr) of jatropha plantation can be seen in Table 1.

Table 1. Energy content of jatropha byproducts, assuming seed productivity of 5000 kg/ha/yr

Components	Total protein (% DM)	Total carbohydrate (% DM)	Lipid (% DM)	Energy content (MJ/kg)	Total energy content (GJ/ha/yr)
Fruit hull	5.0	42.9	nd	13.0[b]	27.8
De-oiled seed cake	21.5	37.4	0	12.9[b]	44.2
De-oiled kernels	40.2[a]	28.0[a]	0	11.4[c]	20.2
Seed shells	nd	46.3	nd	17.2[d]	28.4

Nd = not detected
[a] Calculated from chemical compositions data of de-oiled seed cake and seed shell
[b] Cited from published article (Gunaseelan, 2009)
[c] Calculation based on 16.7 MJ/kg for carbohydrate and protein (Gunaseelan, 2009)
[d] Cited from published article (Openshaw, 2000)

2.2.1. Valorization of jatropha seeds

Valorization of the 5000-kg seeds is shown in Figure 2. We include mass balance in valorization so that the quantity and the economic value of the intermediates or final products can be calculated. The 5000-kg seed is fractionated into 3350 kg kernel (67%) and 1650 kg shell (33%). The kernel contains 47% oil (Chapter 3). By microbial extraction of the whole kernel followed by hexane extraction of the residual kernel cake, 100% oil (1575 kg) is extracted from the kernel. The advantages of a microbial process are: some kernel cell components are solubilized (good to increase extractability/yield of the protein in NaOH extraction); the residual oil can be extracted with much less volume of organic solvent (hexane); and the process in protein isolate production, via NaOH extraction for example, will give higher protein purity than that without microbial treatment. The SDS-PAGE presented in Chapter 4 proves good separation of proteins in the solid phase of the microbial protein extraction.

We need the hexane extraction step to remove the residual oil from kernel cake to improve the protein isolate quality in the next steps. After residual oil extraction with hexane, protein is isolated with 0.055M NaOH (Lestari et al., 2010). The protein isolate is collected by precipitation at pH 5, centrifuged and then dried. From this operation, 607 kg protein isolate (85% overall protein yield) is obtained. The aqueous and solid residues are sent to anaerobic digester. This residues contain carbohydrate, protein/amino acids, lignin, and other kernel components, accounted for 1168 kg DM, which altogether has 96% VS (volatile solid) suitable for biogas production

(Gunaseelan, 2009). The quantity biogas produced from this residue (deoiled kernel) is calculated as follows:

Total solid = 1168 kg
Energy content of de-oiled kernel (Table 1) = 11.4 MJ/kg
Total energy = 1168 kg × 11.4 MJ/kg = 13.32 GJ
CH_4 yield from kernel = 53%*
1 m^3 CH_4 has 33.81 MJ*
CH_4 produced = 0.53 × 13.32 GJ = 7.1 GJ
= 7100 / 33.81 m^3
= 210 m^3
*(Gunaseelan, 2009)

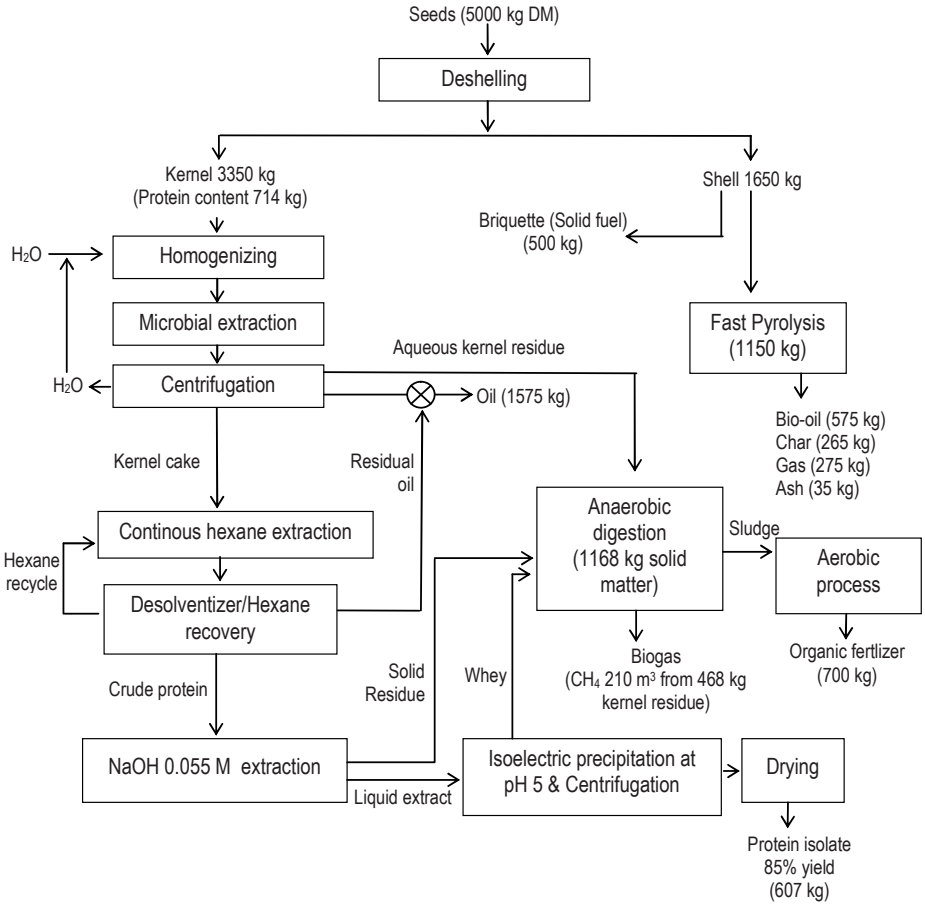

Figure 2. A flow diagram of jatropha seed valorization per hectare plantation (kg DM)

The sludge from anaerobic digester can be aerobically fermented as fertilizer. The fertilizer has a higher nutrient content than the cattle manure, and in addition, it is free from pathogens, which have been killed during fermentation and therefore becomes a very healthy natural fertilizer (FACT-Foundation, 2010). Based on process scheme of biogas digester proposed by FACT-Foundation (2010), we estimate that 60% of the sludge from the digester can be fermented to fertilizer. Apparently, about 700 kg fertilizer can be produced from the digester sludge. This fertilizer is estimated to contain 2.4% N, 9.2% P, and 6.7% K.

In seed shell valorization, although the shell contains high polysaccharide (44.3%), our experiments proves too difficult to split the shell polysaccharide via biochemical approach into monomeric sugars and chemicals/fuels, most likely because of the high levels of lignin acting as physical barrier. Therefore, a thermochemical approach for valorization of seed shell is a rational choice. From a 1650-kg seed shell separated from 5000 kg dry seeds, we propose to convert 30% shell (equals to 500 kg; 8.6 GJ) to briquette as solid fuel, which can be used as energy source for the process or as cooking fuel in the rural area. Fractionation of the other portion of the shell (equals to 1150 kg, 19.8GJ) through a thermochemical approach namely fast pyrolysis, which is beyond our research scope, can be conducted as better conversion yields in the production of liquid fuel (bio-oil), pyrolysis gas, activated charcoal (as adsorbent), and ash rich in minerals have been established recently (Hidayat, 2014).

The conversion yields the quantity and energy value of the products obtained from fast pyrolysis of 1150 kg seed shell that can be seen in Table 2. The bio-oil can be used for transportation fuels. The gas phase contains 36.5% CO (72 kg), 51.9% CO_2 (102 kg), 8.9% CH_4 (17 kg), and 2.7% C_2+ (C_2H_4, C_2H_6)(5 kg) (Hidayat, 2014) may be used for energy generation or for synthesis of bulk-chemicals, such as methanol and FT-diesel (Bridgwater et al., 1999). The char has excellent properties as adsorbent for separation processes (Vyas and Singh, 2007; Wever et al., 2012). The minerals found in the ash include Al (9100 ppm), Fe (4420 ppm), Na (5400 ppm), Ca (45000 ppm), Mg (12200 ppm), P (11000 ppm) (Hidayat, 2014). The ash rich in important plant nutrients (P, N, Ca, Mg) has potential application as a fertilizer.

Table 2. The quantity and energy value of products from fast pyrolysis of 1150 kg seed shell

Products	Yield[a] (% *w/w*)	Quantity (kg)	Energy value (GJ)
Bio-oil	50	575	10.1[b]
Gas	17	196	1.82[c]
Char	23	264	-
Ash	3	35	-
Others	7	80	-

[a] Hidayat (2014)
[b] Calculated from higher heating value (HHV) of pyrolysis oil 17.5 MJ/kg (Hidayat, 2014)
[c] Calculated from the lower heating value (LHV) of CO (10.1 MJ/kg), CH4 (50.0 MJ/kg), C_2H_4 & C_2H_6 (average 47.5 MJ/kg) (from http://en.wikipedia.org/wiki/Heat_of_combustion, accessed on 16-03-2014)

2.2.2. Valorisation of jatropha fruit hull

Valorization of the hull is shown in Figure 3. Production of a 5000-kg dry seed will concurrently generate 9300 kg fresh fruit hull (2140 kg DM) as a waste. With respect to the high humidity content in jatropha fruit hull (77% wet weight), in preparing the materials for industrial biorefinery process, we recommend to firstly expell the water content from the fresh hull using expeller machine to remove humidity and water soluble fractions. Water expelling will reduce the feedstock volume and energy required for feedstock drying. This operation will also prevent excessive formation of fermentation inhibitors like 5-HMF and furfural (due to pretreatment) from the free sugars initially available in the hull (Chapter 5). After expelling, the dewatered hull biomass (1370 kg DM) is collected and ready for acid pretreatment. The soluble fraction (7800 kg) which contains polysaccharides, small amount of amino acids, and presumably pectin and tannin can be pumped to anaerobic digester for biogas production.

After dilute acid pretreatment of the hull biomass (using 30% solid loading) followed by filtration, the acidic filtrate (3600 kg) containing xylose, amino acids, acid soluble lignin, minerals, and other soluble components may be neutralized, enriched, and then fermented to lactic acid by *Rhizopus oryzae* (Maas et al., 2006; Maas et al., 2008). However, we do not propose this route in fruit hull valorization because fermentation with fungus will require costly and sophisticated equipment like a fermenter and recovery unit. Additionally, with 30% solid loading in pretreatment, a maximum of only 3% of C-5 sugars can be produced in the pretreated liquor, which is too low to

produce lactic acid that is economically feasible. Instead, we propose to use the neutralized filtrate for biogas production.

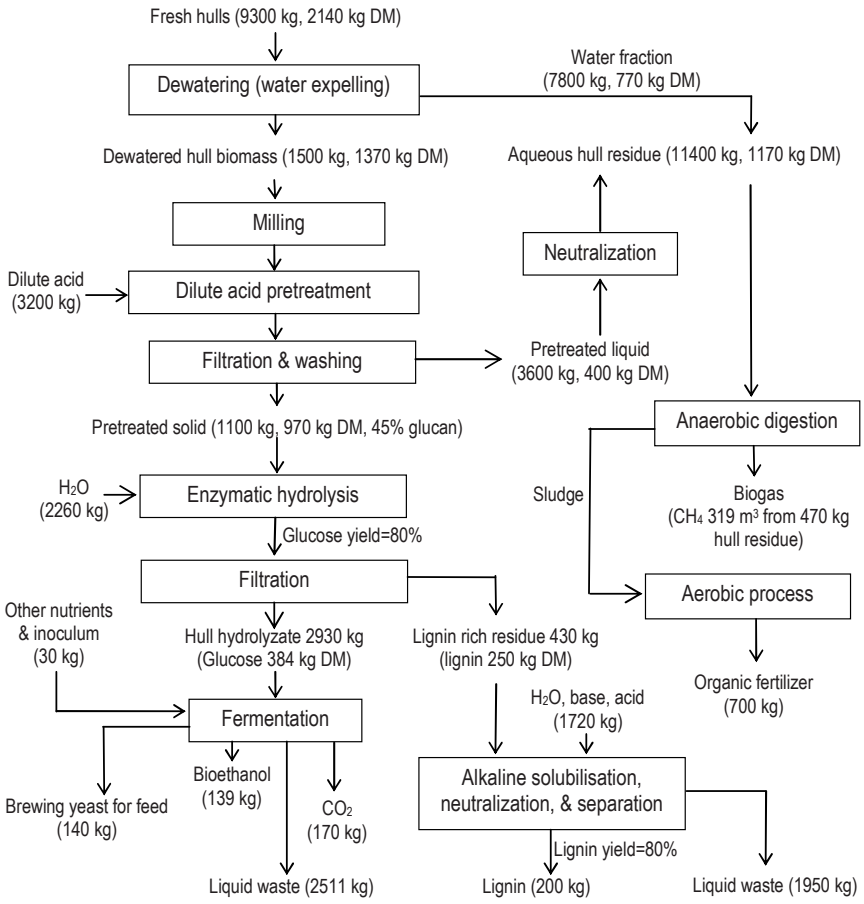

Figure 3. A flow diagram of jatropha fruit hull valorization per hectare

After filtering, the resulting acid pretreated solid (1100 kg, 970 kg DM, 45% glucan) undergoes enzymatic hydrolysis where 80% glucan will hydrolyze to 384 kg glucose, leaving the acid insoluble lignin (250 kg) in solid form that can be separated by filtration. By this method, we calculate that 137 g/L glucose is obtained in the hydrolyzed liqour, which is suitable as fermentation substrate for bioethanol

production. Assuming the ethanol yield from hull biomass is 71% of theoretical yield (Chapter 5), as much as 139 kg bioethanol in the fermentation broth is achieved. Additionally, the yeast cake from the fermentation process is a good source of protein as feed supplement.

After enzymatic hydrolysis, the lignin can be purified by alkaline solubilization, precipitation in neutral condition, and drying. Assuming 80% yield, as much as 200 kg lignin can be recovered. The purified lignin can be directly used as a biomaterial, such as adhesive.

The liquid from expelling operation (7800 kg, 770 kg DM) and the neutralized liquid from pretreatment (3600 kg, 400 kg DM) are combined as the aqueous hull residue (11400 kg, 1170 DM) for biogas production. The quantity biogas produced from this hull residue is calculated as follows:

Total solid = 1170 kg
Energy content of the hull (Table 1) = 13.0 MJ/kg
Total energy = 1170 kg × 13.0 MJ/kg = 15.2 GJ
CH_4 yield from hulll = 71%*
1 m^3 CH_4 has 33.81 MJ
CH_4 produced = 0.71 × 15.2 GJ = 10.8 GJ
= 10800 / 33.81 m^3
= 319 m^3
*(Gunaseelan, 2009)

The sludge from anaerobic digestion of the hull residue can be used to produce organic fertilizer. About 700 kg fertilizer can be produced from the digester sludge.

2.3. A view of future prospect in the jatropha biorefinery

2.3.1 The economy benefit of jatropha seeds

The current total area of jatropha plantation in Indonesia is difficult to obtain because the plantations are small in size and scattered throughout the country. The market value of jatropha seed could range from IDR 1000 to 3000 per kg seeds depending on seed availability. However, for calculation of the revenues compared to other food crops, we may assume the jatropha seed price of IDR 1500/kg with minimum seed yield of 5000 kg/ha, which is required to be economically feasible (Syakir, 2010).

Table 3 shows total revenue of jatropha seeds compared to other food crop in Indonesia. The low revenue from jatropha seeds is probably the main cause of the jatropha farmers turned their agricultural business to other food crops, which in turn contributed to the fast declining amount of jatropha plantations in Indonesia. Hence, valorization of jatropha side-stream into valuable products is the answer to improve the economic profit and benefit of this biofuel crop.

Table 3. Yield and price of jatropha seeds compared to other food crops in Indonesia

Feedstock	Yield[1)] (kg/ha/yr)	Market Value (IDR/kg)[2)]	Market Value EUR/kg[3)]	Total revenue (EUR/ha/yr)
Jatropha seeds	5000	1500	0.10	500
Rice	5146	8600	0.57	2933
Corn	4799	6000	0.40	1920
Cassava	22418	2000	0.13	2914
Soybeans	1457	10500	0.70	1020
Peanuts	1743	19000	1.27	1742

Note: data was accessed on March 2014 from:

1) http://www.bps.go.id/tnmn_pgn.php; the market value is the national price on Dec 2013

2) http://pusdatin.setjen.pertanian.go.id/publikasi-361-analisis-perkembangan-harga-komoditas-pertanian-januari-2014.html

3) 1 EUR = IDR 15000 (on 11 March 2014)

2.3.2 The economic benefit of jatropha seeds with valorization

To generate the maximum value per hectare jatropha plant, the potential total revenue from a 5000-kg oilseed only with only pressing and with valorization as previously proposed are compared (Table 4).

Table 4 shows the estimation of the economic potential in the valorization of jatropha biomass. Using plant productivity of 5000 kg seed/ha/yr, a total potential revenue can reach around 2808 EUR/ha/yr. This value is 4-fold the total revenue obtained by only pressing the seed (699 EUR/ha/yr). From our estimate, it is clearly seen that a total valorization through biorefinery processing of jatropha biomass could significantly improve the economic value of this biofuel crop.

Although the total potential revenue of jatropha after valorisation is comparable to some important food products such as rice and cassava, without valorization (Table 3)

current intensive studies and research on improving jatropha plant productivity would bear a tremendous achievement in increasing plant productivity from 1575 kg oil/ha/yr to at least 2000 kg oil/ha/yr. Productivity increase will open opportunity to gain more profit and benefit to the farmers as well as to the biorefinery factory site.

Table 4. The comparison of the potential market value and gross profit margin of the products from the only jatropha seed pressing and valorization

Products	Productivity (kg product/ha/yr)	Potential applications	Market value (EUR/kg) [a]	Revenue (EUR/ha/yr) [e]
Seeds as feedstock	5000		0.10	500
Current situation: only pressing oil for biodiesel productions				
Oil	1575	Biodiesel	0.40	630
Press cake	3425	Biogas/fertilizer	0.02	69
Total potential revenue				699
Potential gross profit				199
Future prospect: valorisation of jatropha biomass into more value added products				
Oil	1575	Biodiesel	0.55	866
Protein isolate	607	Adhesives/emulsifier	1.80	1092
Bioethanol	139	Transportation fuel	0.80	111
Lignin	200	Adhesives	0.40	80
Biogas (m^3/ha/yr) [b]	534	Cooking	0.15	80
Bio-oil	575	Transportation fuel	0.27 [d]	155
Char	265	Adsorbent	1.00	265
Ash	35	Fertilizer	0.15	5
Brewing yeast [c]	140	Feed supplement	0.40	56
Organic fertilizer	1400	Fertilizer	0.07	98
Total potential revenue				2808
Potential gross profit [f]				2308
Estimated production cost [g]				808
Estimated net profit [h]				1500

[a] All market value are Indonesian prices except for Jatropha protein isolate ~ soy protein isolate (>90% protein) of 1.3 – 1.9 EUR / kg

[b] Productivity in m^3/ha/yr; market value in EUR/m^3

[c] The market value is taken from http://www.alibaba.com/showroom/dry-yeast-for-animal-feed.html; accesed on 16-03-2014

[d] Bio-oil market value is calculated from the energy content ratio of bio-oil (19 MJ/kg) and crude fossil oil (42 MJ/kg) then multiply by the price of crude fossil oil (EUR 0.75/kg, on 14 March 2014) and by 0.8 (correction factor)

[e] Revenue = Productivity × Market value

[f] Potential gross profit = Total potential revenue – Seed cost

[g] Assume the production cost comprise 35% of potential gross profit

[h] Estimated net profit = Potential gross profit - Estimated production cost

3. General conclusion

Our research aims were to develop sustainable technologies of jatropha oil extraction and biomass fractionations within a framework of bioconversions (enzymatic and microbial processings) that could suit the requirement for in situ (local) application, in this case Indonesia. Our findings show that microbial extraction of oil is a simple process and can be applied to extract oil from seeds on the jatropha plantation site. Microbial process yields 70% oil, and this is comparable to the known processes such as by using expeller or by enzymatic extraction. Microbial processes use whole kernels whereby better protein quantity and quality can be obtained. Microbial process can alter the protein structures, but this might be an advantagoues trait since the alteration could provide protein fractions with totally new functional properties. In valorization of fruit biomass into variuos products (oil, protein isolate, lignin, biogas, bio-oil, etc.) most known techniques (pretreatment, hydrolysis, fermentation, extraction, separation, anaerobic digestion, pyrolysis) are applicable for local conditions. From our economic analysis it is clearly seen that a total valorization through biorefinery processing of jatropha biomass could significantly improve the economic value of this biofuel crop. For future research, our specific recommendations are: (1) to investigate functionalities of the additional protein fractions formed by the action *Bacillus pumilus.* (2) to isolate enzymes from *Bacillus pumilus*, in this case xylanase, and to study its properties in the extraction of jatropha oil and in conserving the original structure of jatropha protein.

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Summary

Jatropha curcas, L has been introduced as one of the most promising candidates for future bioenergy production because it has seeds with a high oil content which are suitable for power generation or for the production of biodiesel. In 2006, Indonesia launched - due to energy crisis - a new renewable energy program, which aimed to source 17 percent of its energy needs from renewable resources by 2025. Since then, the Indonesian national policy began introducing Jatropha plant as the favored biofuel source. However, jatropha oil has not been traded at price levels that satisfactorily reward growers/farmers. Moreover, jatropha plantation suffer from low oil productivity and intensive labor. Therefore, one of the strategies to improve the economics of this potentially profitable plant is to increase the value of the side streams of oil production (trimmed stems/branches, seed or kernel cake, seed shells, and fruit hulls).

Our research objectives were to develop sustainable technologies of jatropha oil extraction and jatropha biomass fractionations within a framework of bioconversions (enzymatic and microbial processings) that could suit the requirement for in situ (local) application, in this case Indonesia. The questions to answer include, firstly, which part of jatropha lignocellulosic biomass can be depolymerized to sugars and then valorized to chemicals/fuels, in particular to bioethanol? Secondly, can a microbial method of jatropha oil extraction improve the oil yield in comparison to other known processes? Thirdly, can a microbial method of oil extraction preserve the original structure of jatropha protein? Fourthly, can jatropha fruit by-products after valorization give higher added-value products than just the seeds?

Initially, we investigated the effect of dilute sulfuric acid pretreatment on enzymatic digestibility of jatropha seed shells, fruit hulls, and seed-cake (Chapter 2). The aim was to estimate the feasibility of releasing monomeric sugars from the several Jatropha fractions. Jatropha fruits consist of (in *w/w* dry fruits) 50% seed kernels, 20% husks (seed shells) and 30% hulls (fruit exocarp). The fruit contains 23.5% of vegetable oil. Fractionation of the fruit byproducts, mainly protein and carbohydrate, will increase the economy of jatropha processing factory. In this study, we investigated the effect of pretreatment using dilute sulfuric acid (0 – 500 mM) at 120

°C for 30 minutes on enzymatic digestibility of milled jatropha biomass: seed shell, seed-cake or fruit hull at 10% (*w/w*) solids loading by determining glucose and xylose release. We found that the lignocellulose fractions of the jatropha seed shell and the jatropha seed cake were relatively recalcitrant to dilute sulfuric acid pretreatments suggesting that more intense pretreatment is necessary to disrupt lignin barriers sufficiently to improve enzymatic digestibility. However, dilute sulfuric acid pretreatment solubilized 65% of the available protein in the jatropha seed cake. After dilute acid pretreatment, the lignocellulose fraction of the jatropha fruit hull proved relatively more susceptible to hydrolysis by cellulases (GC220). As much as 70% glucose and 100% xylose was obtained from the jatropha fruit hull after a pretreatment with 500 mM sulfuric acid. A pretreatment at higher temperature may resolve the need of more diluted acid concentration and a shorter time to achieve comparable or higher sugar yields.

Based on our findings that the jatropha fruit hull was relatively more susceptible to hydrolysis by cellulases (GC220), we optimized the experimental variables (sulfuric acid concentration, time, and temperature) for jatropha fruit hull pretreatment and its hydrolysis into sugars (Chapter 5). Simultaneous saccharification and fermentation of jatropha fruit hull hydrolyzates by *Saccharomyces cerevisiae* for ethanol production was also studied. Dilute sulfuric acid pretreatment of jatropha fruit hull at high temperatures (140 to 180 °C) performed in a 110-mL stainless steel reactor was investigated to enhance the enzymatic digestibility of its lignocellulosic components. Carbohydrates accounted for 43% of the dry matter of jatropha fruit hull biomass. The goal of the study was to optimize the pretreatment conditions (acid concentration, time, and temperature) in order to obtain the highest sugar yield after subsequent enzymatic hydrolysis. A Box-Behnken Design was applied to the experimental set up in order to reduce the number of experiments. The optimal pretreatment conditions are 30-min incubations at a temperature of 178 °C with a sulfuric acid concentration of 0.9% (*w/v*). Using these pretreatment conditions for a fruit solid loading of 9.52% followed by a 24-h enzymatic hydrolysis resulted in a liberation of 100% of all pentoses present (71% yield and 29% degradation to furfural) and 83% of the hexoses (78% yield and 5% degradation to 5-hydroxymethylfurfural). The simultaneous saccharification and fermentation experiment showed that acid-

pretreated fruit hull can be used as a substrate for *Saccharomyces cerevisiae* to produce ethanol.

A microbial method of jatropha oil extraction to improve the oil yield in comparison to other known processes is elaborated in Chapter 3. A traditional Java method of coconut oil extraction assisted by paddy crabs was investigated to find out if crabs or crab-derived components can be used to extract oil from jatropha seed kernels. Using the traditional Java method the addition of crab paste liberated 54% *w/w* oil from grated coconut meat. Oil extraction using crab paste carried out under controlled temperatures and in the presence of antibiotics showed that at 30 ^{o}C or 37 ^{o}C enzymes from crab played a dominant role in liberating oil from grated coconut meat and aqueous jatropha kernel slurries. However, at higher temperature (50 ^{o}C), thermophilic bacterial strains present inside crabs played a significant role in the extraction of oil from both oilseeds tested. A thermophilic bacterial strain isolated from crab paste and identified based on 16s rRNA sequence as *Bacillus licheniformis* strain BK23, when added as starter culture, was able to liberate 60% *w/w* oil from aqueous jatropha kernel slurry after 24h at 50 ^{o}C. Further studies of BK23 and extraction process optimization are the challenges to improve Jatropha oil extraction yield and process economy.

Because *Bacillus licheniformis* strain BK23 degraded protein in liberating the oil, we investigated alternative microbial methods of oil extraction with the aim to preserve the original structure of jatropha protein (Chapter 4). We investigated the use of bacterial cells isolated from paddy crab for the extraction of oil from jatropha seed kernels in aqueous media while simultaneously preserving the protein structures of this protein-rich endosperm. A bacterial strain – which was marked as MB4 and identified by means of 16S rDNA sequencing and physiological characterization as either *Bacillus pumilus* or *Bacillus altitudinis* – enhanced the extraction yield of jatropha oil. The incubation of an MB4 starter culture with preheated kernel slurry in aqueous media with the initial pH of 5.5 at 37 ^{o}C for 6 h liberated 73% *w/w* of the jatropha oil. Since MB4 produces xylanases, it is suggested that strain MB4 facilitates oil liberation via degradation of hemicelluloses which form the oil-containing cell wall structure of the kernel. After MB4 assisted oil extraction, SDS-PAGE analysis showed that the majority of jatropha proteins were preserved in the solid phase of the

extraction residues. The advantages offered by this process are: protein in the residue can be further processed for other applications, no purified enzyme preparation is needed and the resulting oil can be used for biodiesel production.

Chapter 6 presents the retrospectives of our research findings and the perspectives of the future utilization of jatropha, specifically in Indonesia. In valorization of the jatropha fruit biomass into variuos products (oil, protein isolate, lignin, biogas, bio-oil, etc.), most known techniques (pretreatment, hydrolysis, fermentation, extraction, separation, anaerobic digestion, pyrolysis) are applicable for local (Indonesia) conditions. From our economic analysis, it is clearly seen that a total valorization through whole crop biorefinery processing of jatropha biomass could improve the economy value of this biofuel crop significantly.

Scriptie titel:

Valorisatie van biomassa van jatropha vruchten voor energietoepassingen

Samenvatting

Jatropha curcas, L wordt beschouwd als een van de beste kandidaten voor toekomstige bio-energie productie omdat het zaad een hoog oliegehalte heeft waardoor het geschikt is voor energieopwekking of voor de productie van biodiesel. In 2006 lanceerde Indonesië een nieuw programma met als doel om in 2025 17 procent van zijn energiebehoefte uit hernieuwbare bronnen te halen. De Jatropha plant werd hierin gezien als de favoriete biobrandstofbron. Boeren en telers kunnen echter onvoldoende verdienen aan jatropha olie als gevolg van de lage olie productiviteit en hoge arbeidskosten in de plantage. Eén van de strategieën om de economische aspecten van dit potentieel winstgevende gewas te verbeteren is om de waarde van de zijstromen van de olieproductie (takken, zaden, perscake, zaadschillen en fruit) te verhogen.

De doelstelling van ons onderzoek was om duurzame bioconversie technologieën voor de extractie van Jatropha olie en fractionering van jatropha biomassa te ontwikkelen, met als eis dat deze in Indonesie zou kunnen worden toegepast. De vragen die beantwoord moesten worden waren:

1) Hoe kan lignocellulose bevattend jatropha biomassa gedepolymeriseerd worden tot suikers en vervolgens omgezet in chemicaliën en brandstoffen (met name ethanol)?
2) Kunnen we de olieopbrengst verbeteren ten opzichte van andere bekende werkwijzen door middel van een waterige extractie met behulp van microorganismen?
3) Kan deze microbiële extractie de originele structuur en waarde van jatropha eiwit in stand houden?
4) Draagt de verwaarding van de zijstromen bij aan de economie van de jatropha plantage?

In eerste instantie hebben we onderzocht wat het effect van een voorbehandeling met verdund zwavelzuur is op de enzymatische hydrolyse van jatropha zaadschillen, vruchtvlees, en perscake (Hoofdstuk 2). Het doel was om een indruk te krijgen van de

mogelijkheden om de monomere suikers uit de verschillende jatropha fracties te halen. In deze studie werd het effect van voorbehandeling met verdund zwavelzuur (0-500 mM) bij 120 °C gedurende 30 minuten op de enzymatische verteerbaarheid van gemalen jatropha biomassa door het bepalen van de hoeveelheid glucose en xylose die werd vrijgemaakt. De lignocellulose fracties van de Jatropha zaadschillen en de perscake waren relatief recalcitrant hetgeen indiceert dat een intensere voorbehandeling nodig is om de lignine barrières voldoende te verbreken. Deze voorbehandeling hydrolyseerde 65 % van het beschikbare eiwit in de Jatropha perscake. Het hydrolysaat dat rijk is aan aminozuren zou verder gefractioneerd kunnen worden tot enkele aminozuren voor de bereiding van N-chemicals. Het Jatropha vruchtvlees bleek relatief meer gevoelig voor hydrolyse door cellulases: 70% van het glucose en 100% van het xylose kon worden vrijgemaakt na een voorbehandeling met 500 mM zwavelzuur. Lagere zwavelzuurconcentraties en kortere incubatietijden kunnen toegepast worden door de voorbehandeling bij hogere temperatuur toe te passen.

Op Java wordt een traditionele methode gebruikt om kokosolie te extraheren, waarbij gebruik wordt gemaakt van krabben uit rijstvelden. In hoofdstuk 3 wordt het onderzoek beschreven waarin deze methode wordt toegepast op het vrijmaken van olie uit Jatropha zaadkernen. De traditionele Java-methode, waarbij krabpasta wordt toegevoegd aan het kokosvlees, bevrijdt 54% w/w olie. Bij incubatie bij 30 en 37 °C had de toevoeging van antibiotica nauwelijks effect op het proces, zowel bij kokosvlees als bij Jatropha zaadkernen, hetgeen aangeeft dat enzymen uit de krabpasta verantwoordelijk waren voor het vrijmaken van de olie. Het bleek echter dat bij 50 °C de olie grotendeels werd vrijgemaakt met behulp van thermofiele bacteriestammen die in de krabpasta aanwezig waren. Een thermofiele bacteriestam werd geïsoleerd uit de krabpasta en werd op basis van 16S rRNA-sequentie geidentifieerd als *Bacillus licheniformis* BK23. Wanneer deze stam werd toegevoegd als starter culture aan een waterige slurrie van jatropha zaadkernen, werd 60% van de olie vrijgemaakt na 24 uur bij 50 °C. Verdere studies van BK23 en optimalisatie van de extractie zijn nodig om het extractie rendement te verbeteren en de proces kosten te verlagen.

Omdat de *B. licheniformis* stam BK23 bij het vrijmaken van de olie ook het eiwit dat aanwezig is in de zaadkernen afbrak, onderzochten we alternatieve methoden voor microbiële olie extractie met als doel de oorspronkelijke structuur van jatropha eiwit te behouden (hoofdstuk 4). We onderzochten het gebruik van bacteriële cellen geïsoleerd uit rijstveld krab voor de winning van olie uit jatropha zaadkernen in waterige media, waarbij tegelijkertijd de eiwit structuren van deze eiwitrijke endosperm werd behouden. Een bacteriestam - die werd gekenmerkt als MB4 en geïdentificeerd door middel van 16S rDNA sequencing en fysiologische karakterisering als ofwel *Bacillus pumilus* of *Bacillus altitudinis* - verbeterde het extractie rendement van jatropha-olie. De incubatie van een MB4 starterculture met voorverwarmde kernel suspensie in waterige media met de initiële pH van 5.5 bij 37 ° C gedurende 6 h bevrijdde 73% w / w van de jatropha olie. MB4 produceert xylanases en dit suggereert dat het vrijmaken van de olie gebeurt via de afbraak van hemicellulose in de oliehoudende celwand structuur. SDS-PAGE analyse toonde aan dat het Jatropha eiwit grotendeels ongeschonden bleef in dit proces. De voordelen van deze werkwijze zijn: dat het eiwit in het residu verder verwerkt kan worden voor andere toepassingen, dat er geen gezuiverd enzympreparaat nodig is en dat de verkregen olie gebruikt kan worden voor biodiesel.

Gebaseerd op onze bevindingen dat het voorbewerkte jatropha vruchtvlees relatief gevoeliger is voor hydrolyse door cellulases, optimaliseerden we de experimentele variabelen (zwavelzuur concentratie, tijd en temperatuur) voor voorbehandeling en hydrolyse van jatropha vruchtvlees tot suikers (hoofdstuk 5). Ook werd de gelijktijdige versuikering en fermentatie van jatropha vruchtvlees hydrolysaten door *Saccharomyces cerevisiae* voor de productie van ethanol bestudeerd. Voorbehandeling van jatropha vruchtvlees met verdund zwavelzuur bij hoge temperaturen (140-180 ° C), uitgevoerd in een 110 ml roestvrij-stalen reactor werd onderzocht om de enzymatische verteerbaarheid van de lignocellulose componenten te verbeteren. Het doel van de studie was de voorbehandelingsomstandigheden (zuurconcentratie, tijd en temperatuur) te optimaliseren om na enzymatische hydrolyse de hoogste suikeropbrengst te verkrijgen. De Box-Behnken method werd toegepast om het aantal experimenten te verminderen. De optimale condities voor de voorbehandeling zijn 30 minuten incubatie bij een temperatuur van 178 °C met een zwavelzuur concentratie van 0.9% (w/v). Door deze voorbehandeling condities te gebruiken bij 9.52% (w/w)

fruit, gevolgd door een 24-h enzymatische hydrolyse werd 100% van alle aanwezige pentoses vrijgemaakt (71% opbrengst en 29% afbraak tot furfural) en 83% van de hexoses (78 % rendement en 5% afbraak tot 5-hydroxymethylfurfural). Het gelijktijdige saccharificatie en fermentatie experiment toonde aan dat met zuur voorbehandeld vruchtvlees gebruikt kan worden als substraat voor *Saccharomyces cerevisiae* om ethanol te produceren.

Hoofdstuk 6 presenteert de retrospectieven van onze onderzoeksresultaten en de perspectieven van de toekomstige benutting van jatropha, met name in Indonesië. In valorisatie van de jatropha fruit biomassa tot verscheidene producten (olie, eiwit isolaat, lignine, biogas, bio-olie, etc.), zijn de meeste bekende technieken (voorbehandeling, hydrolyse, fermentatie, extractie, scheiding, anaërobe gisting, pyrolyse) toepasbaar onder lokale (Indonesië) condities. Uit onze economische analyse wordt duidelijk dat een totale valorisatie van jatropha biomassa via bioraffinage, de economische waarde van deze biobrandstof gewas aanzienlijk kan verbeteren.

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AHMAD MARASABESSY

Curriculum vitae

Ahmad Marasabessy was born on October 11th, 1965 in Ambon, Indonesia. He attended the local primary school (1973 –1979) and the local secondary school (1979 – 1984), then moved to another secondary school in Bandung to finish his 6th year (1984–1985). From 1985 to 1991, he continued his bachelor and graduated in the Pharmacy Department of Institut Teknologi Bandung (ITB), Indonesia. During his studies, he specialized in cell fermentation and process technology. His thesis project was on Penicillin G fermentation in feed-batch mode by regulating glucose addition. From 1991 to 1992, he completed professional education in the field of pharmacy with the degree 'pharmacist'. After his pharmacy graduation, from 1992 until now he is working in Badan Pengkajian dan Penerapan Teknologi (The Agency for The Assessment and Application of Technology) Jakarta as researcher on the development of fermentation technology for the production of microbial-derived products such as antibiotics and enzymes. From 1997 to 1999 he went to Palmerston North New Zealand for diploma and master degrees. He obtained Diploma of Technology (DipTech) in Biotechnology, Institute of Technology and Engineering, Massey University, New Zealand, in 1998. His diploma dissertation entitled 'Lactose hydrolysis by immobilized whole cells of *K. Lactis* CBS 2357'. He obtained Master of Technology (MTech) in Bioprocess Engineering, Institute of Technology and Engineering, Massey University, New Zealand in 1999. His master thesis entitled 'Ethanolic fermentation of cassava starch in a two-stage continuos bioreactor'. In 2007, he came to Wageningen, the Netherlands, to start his PhD in the Department Valorisation of Plant Production Chains at Wageningen UR, under the supervision of Prof. Dr. Johan P.M. Sanders. The objective of his PhD research 'Valorization of jatropha fruit biomass for energy applications' is to develop sustainable technologies of jatropha oil extraction and jatropha biomass fractionations within a framework of bioconversions (enzymatic and microbial processings) that could suit the requirement for in situ (local) application, in this case Indonesia, which the results are described in this thesis.

List of publications

Peer reviewed

Marasabessy, A., Moeis, M.R., Sanders, J.P.M., Weusthuis, R.A., 2010. Coconut oil extraction by the traditional Java method: An investigation of its potential application in aqueous jatropha oil extraction. *Biomass and Bioenergy*, 34(8), 1141-1148.

Marasabessy, A., Moeis, M., Sanders, J.M., Weusthuis, R., 2011. Enhancing jatropha oil extraction yield from the kernels assisted by a xylan-degrading bacterium to preserve protein structure. *Applied Microbiology and Biotechnology*, 90(6), 2027-2036.

Marasabessy, A., Kootstra, A., Sanders, J., Weusthuis, R., 2012. Dilute H_2SO_4-catalyzed hydrothermal pretreatment to enhance enzymatic digestibility of *Jatropha curcas* fruit hull for ethanol fermentation. *International Journal of Energy and Environmental Engineering*, 3(1), 1-11.

Oral/ poster presentations

Marasabessy, A., Moeis, M.R., Sanders, J.P.M., Weusthuis, R.A. Microbial modification of *Jatropha curcas* fruits for energy and feed applications. International Conference on *Jatropha curcas*, 1-2 Nov 2010, Groningen, the Netherlands.

Weusthuis, R.A., Marasabessy, A., Sanders, J.P.M. Biorefinery of *Jatropha curcas*. 4th International Conference on Renewable Resouces and Biorefineries (RRB4), 1-4 June 2008, Rotterdam, the Netherlands.

Marasabessy, A., Lestari, D., Weusthuis, R.A., Sanders, J.P.M. *Jatropha curcas*: Applying the biorefinery concepts to a new biofuel crop. Indonesian Student's Scientific Meeting, 13-15 May 2008, Delft, the Netherlands.

Marasabessy, A., Weusthuis, R.A., Sanders, J.P.M. Microbial modification of *Jatropha curcas* seedcake for energy applications. Summmer Course Glycoscience (+ poster presentation), 9-12 June 2008, Wageningen, the Netherlands.

Overview of completed training activities

Discipline specific activities

Courses

Summmer Course Glycoscience (+ poster presentation), WU-VLAG, 2008, Wageningen, The Netherlands

Renewable Bioresources and Biorefineries (+ field excursion), UNI GRAZ – EU, 2008, Graz, Austria

Renewable Resource of Bulk Chemical Industry, WU-VLAG, 2009, Wageningen, The Netherlands

Conferences

Indonesia Students' Scientific Meeting (ISSM, 2008), Delft, The Netherlands

4th International Conference on Renewable Resources and Biorefineries, (RRB4, 2008) Rotterdam, The Netherlands

International Conference on Jatropha curcas (ICJC, 2010), Groningen, The Netherlands

General courses

Information Literacy, WGS, 2008

Philosophy and Ethics of Food Science & Technology, WU-VLAG, 2008

Techniques for Writing and Presenting Scientific Papers, WGS, 2009

Optionals

Regular Porgress Meeting and Theme Meeting, WU-VPPC Group, 2007-2010

Progress Meeting (Project Group), WU-RuG-KNAW, 2007-2010

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