Utilisation of Jatropha press cake as substrate in biomass and lipase production from *Aspergillus niger* 65I6 and *Rhizomucor miehei* CBS 360.62

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M. Ilmi[a,b], C. Hidayat[c], P. Hastuti[c], H.J. Heeres[a], M.J.E.C. van der Maarel[d,*]

[a] *Department of Chemical Engineering, ENTEG, University of Groningen, Nijenborgh 4, 9747AG Groningen, The Netherlands*
[b] *Faculty of Biology, Universitas Gadjah Mada, 55281, Indonesia*
[c] *Faculty of Agricultural Technology, Universitas Gadjah Mada, 55281, Indonesia*
[d] *Department of Aquatic Biotechnology and Bioproduct Engineering, ENTEG, University of Groningen, Nijenborgh 4, 9747AG Groningen, The Netherlands*

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ABSTRACT

Jatropha curcas L. is a tropical plant that grows well on poor soils in arid regions and produces oil-rich, unedible seeds. The oil from these seeds can be used in the production of biodiesel. In addition, the press-cake waste that is left after oil extraction contains approximately 40% of protein. The use of Jatropha press-cake as substrate for fungal growth and the production of lipase by *Aspergillus niger* 65I6 isolated from Indonesia and *Rhizomucor miehei* CBS 260.62 was investigated. Hydrolysis of the press-cake using 2.5% sodium hydroxide for 45 min was found to be the best pre-treatment method indicated by a high lipase activity. The alkaline hydrolysis increased the amount of reducing sugars and soluble protein to 133.3% and 40% of the initial amount, respectively. 5.7% of the protein fraction was hydrolysed by the alkaline pretreatment. The alkaline hydrolysed Jatropha press-cake was used as a growth substrate for *A. niger* 65I6 and *R. miehei* CBS 360.62. Both fungi grew well on the hydrolysed press cake. The addition of glucose or maltodextrin gave a substantial increase in fungal biomass but inhibited the lipase production, very likely due to catabolite repression by the sugars.

1. Introduction

Enzyme-catalysed biodiesel production from plant oil is considered to have several advantages over the chemical-catalysed conversion: the requirement of low reaction energy, the relatively easy removal of glycerol, and the complete conversion of the free fatty acids present in plant oils into alkyl esters (Fjerbaek et al., 2009; Meher et al., 2006). The use of enzymes also has several drawbacks, one of them being that enzymes are more costly compared to chemical catalysts (Fjerbaek et al., 2009). The high enzyme costs are mainly caused by the production costs of the enzymes, of which about one fifth comes from the costs of substrates such as meat peptone and yeast extract to grow the enzyme production host (Castilho et al., 2000).

Jatropha curcas L. is a tropical plant that is used for several purposes. Traditionally, Jatropha is used for hedges and contributes in controlling erosion. The biomass of the plant (wood leaves, and fruits) is used as firewood in rural areas. Jatropha produces non-edible oil which is an excellent feedstock for the production of biodiesel; the oil can also be used in producing soap, cosmetics, and in the dye industries (Moniruzzaman et al., 2016). Jatropha is being promoted widely and

large scale production is found mainly in South America, Africa, India, and Southeast Asia (Openshaw, 2000). In Indonesia, *J. curcas* plantation has been promoted by the national government and reached 256,545 ha in 2012, producing 513,082 t seed per year (Lestari et al., 2015; Wahl et al., 2012). Up to 50% of the plant oil is pressed from the oil-containing seeds of *J. curcas*, leaving a press-cake (JPC) as waste. This press cake contains a relatively high amount of protein, being approximately 60% of the cake's weight. This JPC is therefore an interesting product to be used as fertilizer, biogas feedstock or animal feed (Devappa and Swamylingappa, 2008; Silitonga et al., 2011; Singh and Singh, 2010).

As JPC is abundantly available and due to its high protein content and protein composition, it is a potentially cheap substrate for enzyme production by submerged (SF) or solid-state fermentation (SSF). Several studies have been conducted to exploit JPC as a growth substrate using SSF for growing fungi or bacteria and the production of various enzymes. Mahanta and co-workers (2008) produced protease and lipase with *Pseudomonas aeruginosa* PseA in a SSF mode with JPC as substrate. Another example is the production of endo-β-1,4-glucanase and β-glucosidase (cellulose hydrolysis) by *Thermoascus*

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aurantiacus growing on JPC using SSF (Dave et al., 2012). Xylanase and cellulose could also be produced by *Aspergillus niger* FGSCA733 grown on JPC (Ncube et al., 2012).

The use of cheap agro-industrial waste as substrate in SSF is gaining more and more attention, but SF is still being used mostly as the easiest method in industrial bioprocessing due to its up and downstream process simplicity (Coradi et al., 2013; Hölker and Lenz, 2005). Several investigations have been done into SF using agro waste such as corn stover, bagasse, and husk (Nema et al., 2015; Oguntimein et al., 1992); de-seeded sunflower head (Patil and Dayanand, 2006); residual pant biomass from industrial processing of *Artemisia annua* (Chandra et al., 2010); and wheat straw and barn (Gomathi et al., 2012; Singh et al., 2009) as a carbon source. JPC was found to be a suitable substrate for proteases production by the bacterium *Bacillus tequilensis* P15 using SF (Bose et al., 2014). However, the use of (pretreated) JPC as substrate in SF to grow fungi and produce lipase has not been reported, so far.

The production of fungal lipases has been extensively studied. The best known lipase is the one from *Rhizomucor miehei*. It is produced commercially by overproduction in *Aspergillus oryzae* and sold in both liquid and immobilized form (Rodrigues and Fernandez-Lafuente, 2010). Some other fungi that are well known lipase producer are *A. niger, Candida rugosa, C. antartica*, and *Thermomyces lanuginosus* (Jaeger and Reetz, 1998; Salihu et al., 2012).

In this research we explored the suitability of alkaline pretreated JPC as substrate for the production of lipase in SF of *A. niger* 65I6 and *R. miehei* CBS 360.62. The *A. niger* strain was isolated from Indonesian soil samples and screened for its lipase producing capability. This strain showed promising potency in producing lipase using the SSF approach (Darmasiwi, 2010).

2. Materials and methods

2.1. Materials

Jatropha seeds were obtained from local farmers in Surakarta, Indonesia. *Aspergillus niger* 65I6 was obtained from the Faculty of Agricultural Technology, Universitas Gadjah Mada, Indonesia. *Rhizomucor miehei* CBS 360.62 was obtained from CBS-KNAW culture collection, Utrecht, The Netherlands. All fungus cultures were kept on Potato Dextrose Agar (Oxoid, Hampshire, UK). Maltodextrin was kindly donated by AVEBE (Veendam, The Netherlands). All other chemicals were purchased commercially.

2.2. Methods

2.2.1. JPC preparation

De-hulled Jatropha seed was first grinded using a commercial blender and then heated at 80 °C for three hours. The oil was extracted by pressing the grinded/heated seeds at approximately 200 kg/cm^2 for 15 min, followed by an extraction using hexane in a glass column. De-oiled press-cake (JPC; Jatropha press cake) was dried at room temperature and then stored at 4 °C. The carbohydrate, protein, and lipid composition of the cake was deduced from an elemental C, H and N analysis (Gnaiger and Bitterlich, 1984).

2.2.2. Sodium hydroxide hydrolysis of JPC

JPC was subjected to a sodium hydroxide hydrolysis following the protocol of (Apiwatanapiwat et al., 2009) with minor modifications. Briefly, fifty grams of JPC was suspended in 180 ml water, heated to 80 °C for 30 min, and then cooled down to 50 °C. Then 120 ml of 6.25% sodium hydroxide was added to this mixture to reach a final sodium hydroxide concentration of 2.5%. The mixture was heated at 50 °C for 15–90 min. Finally the hydrolysed mixture was neutralized to pH 7 using 4 M hydrochloric acid prior to drying. The protein content and degree of hydrolysis of the mixture were determined by the method

of Lowry (Lowry et al., 1951) and by the trinitrobenzenesulfonic acid (TNBS) reaction (Navarrete del Toro and Garcia-Carreno, 2004) respectively.

2.2.3. Lipase production using hydrolysed JPC

The composition of the medium to grow the fungi and produce lipase was modified from a previous study (Adham and Ahmed, 2009). 50 ml medium containing 3% (w/v) hydrolysed JPC, 0.05% (w/v) MgSO$_4$, 0.05% (w/v) KCl, 0.2% (w/v) K$_2$HPO$_4$, 1% (v/v) olive oil (to induce lipase production), and 1% (w/v) glucose was placed in 250 ml baffled Erlenmeyer flasks (Corning Inc., New York); these flasks were inoculated with a spore suspension containing approximately 1×10^5 spores/ml from five days old sporulating cultures of *A. niger or R. miehei* grown on Potato Dextrose Agar. The flasks were incubated at 30 °C and 100 rpm shaking. After five days, the entire content of the flasks was filtrated using Miracloth filter (Merck Millipore, Massachusetts). The liquid filtrate was kept at 4 °C for lipase activity assay. All experiments were done in duplicate. The solids retained on the Miracloth filter were dried and the N-acetyl glucosamine content, as a measure of the amount of fungal biomass, was determined using the method described previously (Zamani et al., 2008).

2.2.4. Lipase activity assay

Lipase activity of the liquid filtrate was determined by incubating 100 µl filtrate with 1 ml isooctane containing 0.25 M oleic acid and 0.25 M ethanol for 20 min at 30 °C. The amount of oleic acid at 0 min and after 20 min was determined using the cupric-acetate pyridine colorimetric assay (Kwon and Rhee, 1986). One unit of activity (U) is defined as the amount of oleic acid (µmol/ml) converted to product per minute.

3. Results and discussion

3.1. Composition of JPC

Protein was the highest constituent in deoiled JPC (44.92%), followed by carbohydrate (22.28%) (Table 1). The protein content was lower compared to the average protein content reported in kernel cake of Jatropha seed (58.13%), while carbohydrate content was close to the reported average (21.71%) (Achten et al., 2008). The carbohydrate composition from this sample was not analysed, but according to Staubmann et al. (1997) the amount of sugars, starch, and cellulose commonly found in JPC is 9.36%, 0.68%, and 8.37%, respectively.

3.2. Effect of hydrolysis time on the amount of reducing sugars, soluble protein, and the degree of hydrolysis of the protein fraction

The colour of the hydrolysed JPC became darker with increased hydrolysis time (Fig. 1.), very likely as a consequence of Maillard reactions (Hodge, 1953). The amount of reducing sugars increased linearly to 66.7–78.9% while the amount of soluble protein increased from 8.6% to 70.9% (Fig. 2). Hydrolysis also resulted in an increase of the amount of reducing sugars going from 3% to 20.5% (Fig. 2).

The suitability of agricultural waste as substrate for fermentation has been studied extensively; almost all of these studies focused on

Table 1.
Chemical composition of de-oiled Jatropha press-cake on dry weight basis. Standard deviation based on duplicate measurements of each sample.

Component	Content (%, w/w)
Protein	44.9 ± 0.37
Carbohydrate	22.3 ± 0.94
Water content	13.7 ± 0.02
Ash	6.1 ± 0.19

Fig. 1. Visual appearances of hydrolysed JPC. Numbers indicate hydrolysis time in minutes.

Fig. 3. Total amount of lipase produced by *A. niger* growing on 3% alkaline treated Jatropha press cake. Standard deviation based on duplicate measurements of each sample.
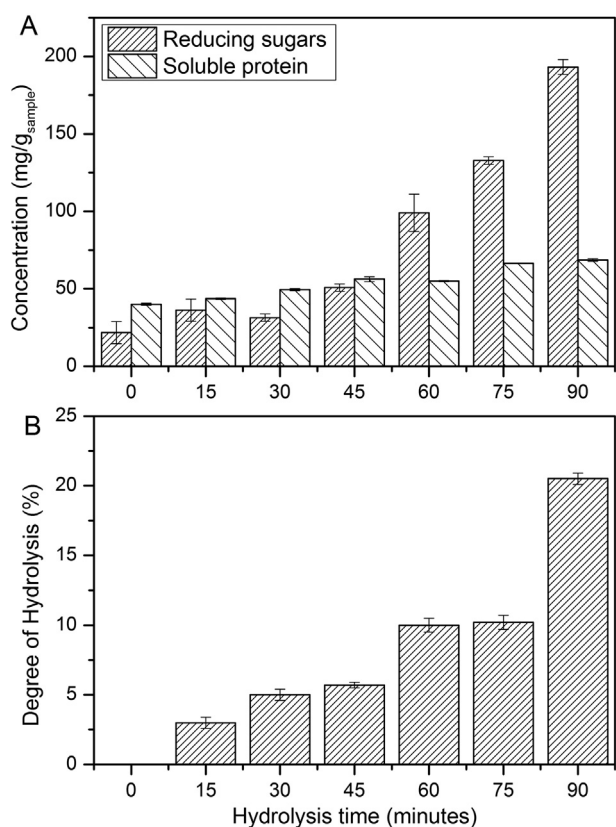

Fig. 2. Effect of hydrolysis time on the reducing sugars content and soluble protein content (A), and degree of hydrolysis (B). Standard deviation based on duplicate measurements of each sample.

Fig. 4. Growth profile of *A. niger* (A) and *R. miehei* (B) grown on alkaline hydrolysed JPC alone (□), alkaline hydrolysed JPC with glucose (■), and alkaline hydrolysed JPC with maltodextrin (▲). Standard deviation based on duplicate samples.

solid state fermentation (SSF) (Hölker et al., 2004). SSF can be defined as cultivation of microorganisms using dry or moist solid substrate such as wheat straw as carbon and energy source (Pandey et al., 2000). Although SSF is an attractive method it is known to give difficulties on a large scale. The sharp increase of the temperature of the ferment as a result of the microbial growth is a major problem in large volume SSF, especially in the production of temperature sensitive substances such as enzymes; the lack of a free aqueous phase in the SSF process makes it difficult to remove the heat. Another problem is water loss due to evaporation; replenishing the evaporated water will increases water activity and thus raises the risk of an unwanted microbial contamina-
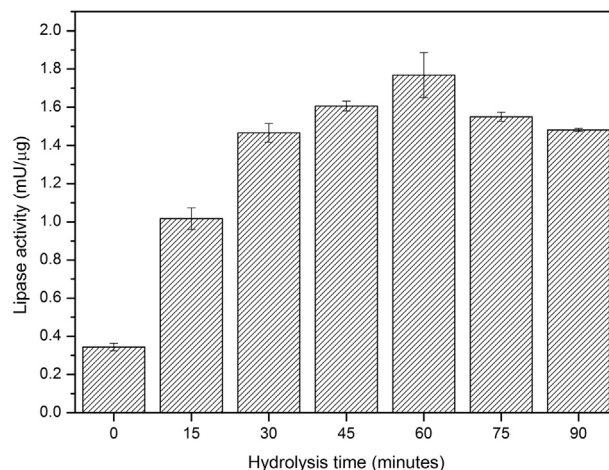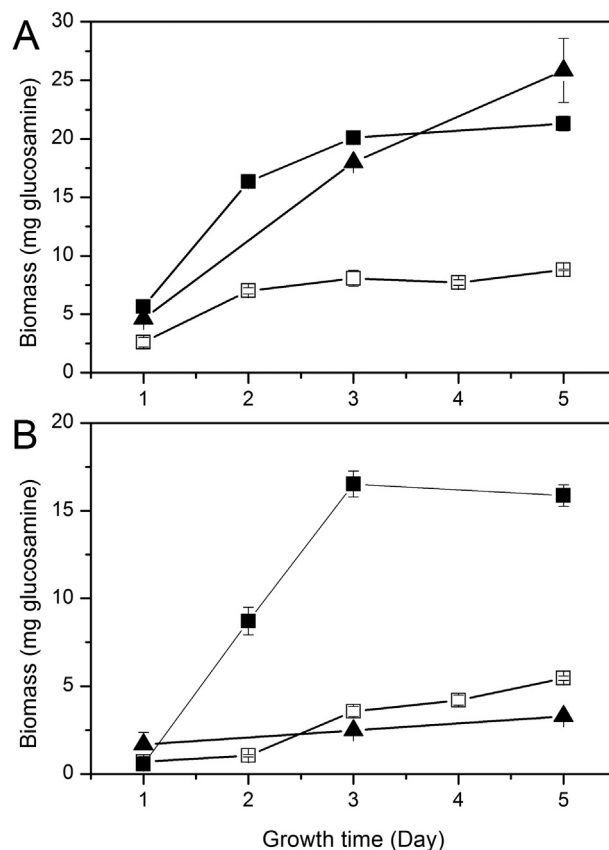
tion. Submerged fermentation (SF) does not have these problems and could be a more preferred method (Hölker and Lenz, 2005). One factor affecting substrates suitability in SF is the accessibility of the substrate to the microorganisms. In this study, JPC was pretreated by alkaline hydrolysis to increase the accessibility by reducing the degree of polymerisation (DP) of the protein chains. Sodium hydroxide gave the highest degree of protein hydrolysis (19.9%) when compared to

Table 2

Activity of lipase and biomass produced from *A. niger* and *R. miehei* grown for five days on alkaline-hydrolysed JPC, alkaline-hydrolysed JPC with glucose, and alkaline-hydrolysed JPC with maltodextrin. Standard deviation based on duplicate samples.

		JPC without glucose	JPC with glucose	JPC with maltodextrin
A. niger	Lipase (U/ml)	5.53 ± 0.9	7.4 ± 0.62	2.5 ± 0.26
	Biomass (µg/ml)	175.99 ± 1.16	511.4 ± 51.14	517.1 ± 54.9
	Lipase/biomass (U/µg)	0.03 ± 0.005	0.015 ± 0.0002	0.005 ± 0.00001
R. miehei	Lipase (U/ml)	14.57 ± 0.99	10.47 ± 0.85	1.7 ± 0.21
	Biomass (µg/ml)	49.86 ± 12.42	337.5 ± 9.74	65.7 ± 3.6
	Lipase/biomass (U/µg)	0.31 ± 0.06	0.03 ± 0.002	0.026 ± 0.002

enzyme and acid treatment after 90 min of incubation (Apiwatanapiwat et al., 2009). Applying similar conditions, a degree of protein hydrolysis of 20.5% was found in this study, a value very close to the one found by Apiwatanapiwat et al. (2009). However, only 2.9% increase of soluble protein per gram sample was detected in our study after 90 min compared to 52.7% protein increase found by Apiwatanapiwat et al. (2009). The low increase indicates that the hydrolysis method we used is not optimal to solubilize insoluble protein present in the press cake, resulting in a large amount of valuable insoluble protein left intact and thus having a negative impact on the efficiency of the hydrolysis process. Optimization of the alkaline hydrolysis method needs to be done to overcome this problem.

In order to quickly screen which of the six hydrolysates gave the highest lipase production, *Aspergillus niger* was grown on each of them in SmF and the amount of lipase produced after five days of growth was determined. The amount of lipase produced increased up to 45 min of hydrolysis; more than 45 min of hydrolysis did not give a higher amount of lipase (Fig. 3).

The increased intensity of the brown colour of the hydrolysates suggests an increase concentration of melanoidins as a result of the Maillard reaction, a typical reaction taking place between reducing sugars and amino/acids proteins at elevated temperatures (Hodge, 1953). Melanoidins are carbohydrate-induced protein oligomers with a high molecular weight (Hofmann, 1998). The oligomerisation decreased the soluble protein content in the hydrolysates, making less soluble protein available to the fungus to grow and thus resulting in lower amounts of total lipase per culture. Melanoidins, although containing protein, are not accessible to fungi to be used as substrate for growth.

3.3. Effect of glucose and maltodextrin on fungal biomass and lipase production

The addition of glucose to the growth medium gave a significant increase on the amount of biomass of both *A. niger* and *R. miehei* compared to when both fungi were grown on JPC alone. Only *A. niger* grew well on hydrolysed JPC and maltodextrin; *R. miehei* did not grow better when maltodextrin was added (Fig. 4.). The amount of lipase produced by *A. niger* and *R. miehei* did not differ significantly when glucose or maltodextrin were present (Table 2.). On JPC alone *A. niger* produced 0.03 U of lipase per µg biomass while *R. miehei* produced almost ten times more (0.31 U/µg biomass). Although more biomass was produced by both fungi when glucose or maltodextrin was present, the amount of lipase was significantly lower; *A. niger* produced 0.01 U/µg biomass and *R. miehei* 0.03 U/µg on glucose while on maltodextrin this was 0.005 U/µg for *A. niger* and 0.026 U/µg for *R. miehei*. Very likely catabolite repression by glucose and maltodextrin shuts down lipase production by both fungi.

Carbon catabolite repression in *Aspergilli* is well known. The protein CREA plays an important role in mediating the repression; it acts as a repressor protein binding to specific short sequence of the promoter of target systems and prevents transcription of these target genes (Ruijter and Visser, 1997). The gene encoding CREA protein,

creA, has been found widely in *Aspergilli* including *Aspergillus nidulans*, *A. tubingensis*, and *A. niger* (Ruijter and Visser, 1997).

Even though the carbon catabolite repression mechanism in *R. miehei* has not been completely described, some studies showed that the present of glucose inhibits lipase production (Davranov and Khalameizer, 1997; Marek and Bednarski, 1996). Also in the related fungus *Rhizopus delemar* carbon catabloite repression of lipase by glucose was found (Martinez Cruz et al., 1993). Another study found that in *Fusarium oxyporum* glucose repressed the expression of lipase even in the presence of olive oil (Rapp, 1995). The authors suggested that the inhibition takes place at the transcriptional level. Very likely a similar mechanism takes place in *A. niger* and *R. miehei* when glucose or maltodextrin are present in the growth medium.

The results also suggest that *A. niger* is able to convert maltodextrin into glucose probably by the enzyme glucoamylase. It is well known that *A. niger* produces glucoamylases explaining the growth on maltodextrins (Svensson et al., 1986; Pedersen et al., 2000; Yuan et al., 2008) (Table 2.). In the genome of *R. miehei* a range of genes encoding enzymes involved in starch and hence maltodextrin degradation are present, explaining its growth on maltodextrins in this study (Zhou et al., 2014).

4. Conclusions

Alkaline-hydrolysed Jatropha press cake is suitable as a nitrogen source for growing filamentous fungi and the production of lipase. The hydrolysis should be performed under strict conditions for a maximum of 45 min to maximize soluble protein amount and minimize undesirable Maillard products. The addition of glucose and maltodextrin as a carbon source stimulates the amount of fungal biomass formed but decreases the amount of lipase produced as a consequence of catabolite repression; further research to optimize lipase production using various carbon source is needed to overcome catabolite repression and maximize the amount of fungal biomass as well as the amount of lipase produced.

Authors contribution

MI, CH, PH, HH and MM wrote the manuscript and agree with the content. MI and MM designed the experiments; MI, CH and PH interpreted the results.

Declaration of interest

All authors declare no potential conflict of interests.

Acknowledgments

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