

Lipid production from diverse oleaginous yeasts from steam exploded corn cobs

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Abstract. Corn cob hydrolysate was used as substrate for growth and lipid accumulation via oleaginous yeast species. A mass based suspension of 10 g 100 g⁻¹ corn cob hydrolysate contained 26.0 g L⁻¹ glucose, 8.5 g L⁻¹ xylose. The inhibitor concentrations were 0.16 g L⁻¹ acetic acid, 1.50 g L⁻¹ formic acid, 0.48 g L⁻¹ HMF and 0.06 g L⁻¹ furfural. These conditions reduced the cell growth of non-adapted yeast. Successful adaptation of the tested yeasts over several generations in corn cob hydrolysate was performed. The adapted yeast *Candida lipolytica* produced 19.4 g 100 g⁻¹ lipids in relation to the dry weight in 7.5 g 100 g⁻¹ dry matter corn cob hydrolysate in fed batch mode. The scale up was done up to a volume of 2.5 litres – here lipid accumulation up to 17.5 g 100 g⁻¹ was demonstrated with the quantitative GC/FID analyses. Predominantly oleic acid, palmitic acid, linoleic and palmitoleic acid were produced. This lipid spectrum is suitable for biodiesel production.

Key words: biodiesel, oleaginous yeast, corn cobs.

INTRODUCTION

Transportation is the largest energy consuming sector in Austria (BMFWF, 2014). Alternative to fossil fuels are needed because of climate change, energy security and the depletion of fossil fuels. Conventional biodiesel is produced from edible plant oils (mainly rapeseed in Austria), non-edible plants and waste oils and is therefore renewable and sustainable. The rising cost of the edible plant oils and the food versus fuel discussion intensify the development of alternative technologies.

Next generation biodiesel technology utilises lipid accumulating microorganisms like microalgae, bacteria, yeasts and fungi. These microorganisms produce and store lipids intracellular up to 70 g 100 g⁻¹ of biomass. At specific culture conditions the oleaginous yeasts convert diverse competitive substrates like sugars from lignocellulose or glycerol to lipids. These lipids can be used as feedstock for biodiesel production. However the specific culture conditions (C/N ratio, temperature, pH value, aeration, cultivation mode) of each oleaginous yeast species vary and must be determined. The oleaginous yeasts have several advantages: yeast is a well-known organism, the cultivation is simple and low-cost, year-round production of yeasts respectively lipids (assumed available year-round substrate) on land unsuitable for agriculture is practicable. About 70 yeast species accumulated lipid efficiently under specific conditions - often an excess of carbon and nitrogen depletion (reviewed in Li et al., 2008;

Meng et al., 2009; Subramaniam et al., 2010; Huang et al., 2013a; Thevenieau & Nicaud; 2013, Sitepu et al., 2014a).

Several reports of conversion of various lignocellulosic hydrolysates to lipid via oleaginous yeasts exist. The ability for lipid accumulation of different *Trichosporon* species was tested mainly in acid corn cob hydrolysate. The lipid content in these studies ranged from 32% up to 47% and the biomass from 20 g L⁻¹ up to 38 g L⁻¹ (Chen et al., 2012; Huang et al., 2012a; Chang et al., 2013; Huang et al., 2013b; Huang et al., 2014).

One aspect particularly effects the lipid production via oleaginous yeasts – in dependency of the used pretreatment method diverse inhibitors at different concentrations are released during this process (reviewed in Kumar et al., 2009; Alvira et al., 2010). These substances inhibit the cell growth and subsequent lipid accumulation of oleaginous yeasts. Altogether few studies about the effects of inhibitors on cell growth and subsequent lipid accumulation are available (Chen et al., 2009; Huang et al., 2012b; Jönsson et al., 2013; Sitepu e et al., 2014b). The conditions for cell growth and subsequent lipid accumulation must be determined for each substrate and each yeast species. Often extensive detoxification methods were performed to reduce the inhibitor concentration or adapted or inhibitor resistant yeast species were used (Parawira & Tekere, 2011).

For the first time, the ability of two oleaginous yeast species *Candida lipolytica* and *Yarrowia lipolytica* to produced lipids in steam exploded, enzymatically hydrolysed corn cob hydrolysate was explored. No extra nitrogen source was added to reduce the cost of the lipid production. Furthermore the influence of the inhibitors on cell growth of non-adapted and adapted yeasts was tested. The practicability of an industrial lipid production via oleaginous yeast was tested with scaled up experiments.

MATERIALS AND METHODS

Corn cobs hydrolysate preparation

A mixture of air dried corn cobs (*Zea mays*) was collected from local producers in Austria and chopped up by a garden shredder (Viking GE 260, Kufstein, Austria) in pieces of 2–3 cm length. The pretreatment was performed with the steam explosion unit (Voest Alpine Montage, Wels, Austria) at optimal condition (200 °C, 10 minutes) to achieve high sugar and low inhibitor concentrations. The steam explosion pretreatment unit was already described (Eisenhuber et al., 2013). The pretreated corn cobs were dried at 40 °C to a moisture content of about 70 g 100 g⁻¹. Different dry matter contents were used for hydrolysis: 7.5 g 100 g⁻¹ and 10 g 100 g⁻¹ in citrate buffer (c = 50 mmol L⁻¹; pH 5; adjusted with NaOH, c = 4 mol L⁻¹). The enzyme mixture Accellerase 1500 from DuPont™ Genencor® was used for hydrolysis at 30 FPU g⁻¹ cellulose. Hydrolysis was performed at 50 °C for 96 hours in a shaking incubator (speed 2.5 s⁻¹). The corn cob hydrolysate was filtered with a Büchner funnel (filter paper MN 640 m). The sugar and inhibitor concentration of the liquid was determined with HPLC. The liquid was used for medium preparation.

Microorganism, media, precultivation, cultivation and scale up

The cell growth and lipid accumulation of two yeast species *Candida lipolytica* and *Yarrowia lipolytica* (kindly provided from the strain collection from the company Agrana (<http://www.agrana.at/>)) were investigated in this study.

The precultivation was done with yeasts adapted over many generations in corn cobs hydrolysate medium (liquid filtered after hydrolysis plus 1.5 g L⁻¹ yeast extract, 3 g L⁻¹ peptone from casein, 2 g L⁻¹ potassium dihydrogen phosphate, 1 g L⁻¹ ammonium sulphate, 0.5 g L⁻¹ magnesium sulphate heptahydrate, pH 6, sterile filtered) or YGC medium (5 g L⁻¹ yeast extract, 20 g L⁻¹ glucose, autoclaved at 121 °C for 20 minutes) with inhibitor (at different concentrations ranging 0.5 g L⁻¹ up to 3.5 g L⁻¹). These yeasts were incubated for 12 hours at 25 °C or 30 °C in a shaking incubator (110 rpm). Ten percent preculture (v v⁻¹) was inoculated to corn cob hydrolysate medium without added chemicals. The samples were incubated at 25 °C or 30 °C for 7 days at 180 rpm in small scale (50 ml, no pH and no aeration control). The samples were centrifuged and resuspended in fresh corn cob hydrolysate medium without additional chemicals for fed batch mode. Samples were taken periodically for HPLC and GC analyses. The scale up was performed in Biostat C2 fermenter (Sartorius, Germany) with constant pH value 5.5, temperature 30 °C, 300 rpm, dragging air 2 litres min⁻¹) in batch or fed batch mode.

Testing the influence of inhibitors on cell growth

The precultivation was done in YPC medium with non-adapted or adapted yeasts. Ten percent preculture (v v⁻¹) was inoculated to YGC medium containing different inhibitors (acids and furans) at variable concentrations and incubated to a maximum of 48 hours at 30 °C at 110 rpm. Optical measurements at 600 nm with Spectrophotometer XION 500 (Hach Lange, Germany) were used to determine the cell growth and the influence of each inhibitor on cell growth.

Transmethylation and GC-FID analysis

For the analytical quantification of the fatty acid content and distribution, 5 mL yeast suspension was centrifuged at a rotational speed of 4,000 min⁻¹. The yeast pellet was washed once with 5 mL of deionized water and dried at 105 °C for 24 hours. The dry matter was resuspended and methylated with 5 mL of methanol/acetyl chloride with a volumetric dilution of 50:2 for 24 hours at 60 °C. Afterwards, the reaction was stopped by slowly adding 2.5 mL of a sodium carbonate solution with a concentration of 60 g L⁻¹. The resulting fatty acid methyl esters were extracted by adding 2 mL of hexane and shaking for 2 minutes. One mL of the upper phase, containing the methyl esters, was transferred in a 1.5 mL crimp vial and stored at -18 °C until measurement.

The hexane extract was injected in a Thermo Trace GC equipped with an autosampler AS 2000. The detection was carried out with a FID. The chromatographic conditions were the following: The injection volume was 2 µL, the injector temperature was set at 240 °C. Helium was used as carrier with 120 kPa constant pressure and a split flow at 30 mL min⁻¹. An Agilent J&W capillary column DB23 60 m, 0.25 mm ID and 0.25 µm film thickness was used for analytical separation. The oven temperature gradient was the following: 0–3 min 130 °C; 6.5 °C min⁻¹ to 170 °C. 2.8 °C min⁻¹ to 214 °C held 12 minutes. 3 °C min⁻¹ to 240 °C held for 15 minutes. The FID was set at a temperature of 280 °C, 450 mL min⁻¹ air flow, 45 mL min⁻¹ hydrogen flow and nitrogen as make up gas at 40 mL min⁻¹ were the torch conditions.

The data analysis was performed with the software Chrom Card Data System Ver. 2.8 from Thermo Finnigan. For calibration the external standard method has been used.

HPLC soluble contents

To determine the contents of sugars and inhibitors 1 mL of homogeneous yeast suspension was transferred into a 1.7 mL centrifugation tube and centrifuged for 5 minutes at an rotational speed of 13,000 min⁻¹. One mL clear supernant was transferred into a 1.7 mL screw cap vial and stored at -18 °C until measurement.

Saccharides and inhibitors were quantified by HPLC, using an Agilent Technologies 1200 Series equipped with a Varian Metacarb 87 H column (300 x 7.8 mm) at 65 °C, H₂SO₄ (c = 5 mmol L⁻¹) eluent and an isocratic flow rate of 0.8 mL min⁻¹ was used. The data acquisition was performed per refractive index detection and UV-detection at 210 nm. For calibration the method of external standard was applied. Data analysis was performed per Agilent Chemstation 04.03 b.

Determination of nitrogen with Dumatherm

Nitrogen was determined according to the Dumas method with a Dumatherm analyzer 7700 (Gerhardt GmbH & Co. KG, Königswinter, Germany, Dumas, 1831).

Determination of acquired yeast biomass

One mL of homogeneous yeast suspension was transferred into a 1.7 mL centrifugation tube and centrifuged for 5 minutes at an rotational speed of 13,000 min⁻¹. The clear upper phase was dismissed and the pellet was washed twice with 1 mL deionized water. The pellet was spilled quantitatively on a weighed aluminum pan with about 2–3 mL deionized water and dried for 2 hours at 105 °C in a drying closet. The difference in weight is the biomass content of 1 mL suspension.

RESULTS AND DISCUSSION

Corn cobs hydrolysate at different temperatures

The corn cobs mixture was pretreated with steam explosion at 200 °C for 10 minutes to achieve the optimal sugar – inhibitors relationship, dried and hydrolysed via enzyme at 7.5 g 100 g⁻¹ or 10 g 100 g⁻¹ dry matter. HPLC analyses after hydrolysis were shown in Table 1. After hydrolysis 26.0 g L⁻¹ glucose, 8.5 g L⁻¹ xylose, 1.50 g L⁻¹ acetic acid, 0.16 g L⁻¹ formic acid, 0.48 g L⁻¹ HMF (= 5-hydroxymethyl-2-furaldehyde) and 0.06 g L⁻¹ furfural were detected within 10g 100g⁻¹ corn cob hydrolysate (see Table 1). The hydrolysate at 7.5 10 g⁻¹ dry matter contained lower concentration of each substances (see Table 1).

Table 1. HPLC results from steam exploded corn cobs after hydrolysis with 7.5 g 100 g⁻¹ or 10 g 100 g⁻¹ dry matter. Values of sugars and inhibitors in (g L⁻¹). Standard deviations varied between 0.01 and 0.04

Sample (g 100 g ⁻¹)	Glucose	Xylose	Formic acid	Acetic acid	HMF	Furfural
7.5	22	7.5	0.11	1.1	0.35	0.04
10	26	8.5	0.16	1.5	0.48	0.06

In other lipid production studies corn cobs were typically pretreated with acid – resulting in similar inhibitor concentrations but other sugar concentrations (Chen et al., 2012). No deeper study about steam exploded, enzymatically hydrolysed corn cobs exists and therefore no direct comparison is feasible.

Influence of the inhibitors on cell growth

The composition of the used corn cob hydrolysate was complex. Diverse substances within the corn cob hydrolysate inhibited the cell growth and lipid accumulation (Chen et al., 2009; Huang et al., 2012b; Jönsson et al., 2013; Sitepu et al., 2014b). Therefore the cell growth and the tolerance of the yeast to the different inhibitors were tested in synthetic medium. Different concentrations of acetic and formic acid were added alone or in combination to the YGC medium ranging from 1 up to 3 g L⁻¹. Purposely higher concentrations of the inhibitors were chosen to cover the higher inhibitor concentrations detected in hydrolysate from wet steam exploded corn cobs. The results were shown in Table 2.

Table 2. Optical density (= OD) at 600 nm from the non-adapted and adapted yeast *Candida lipolytica* after incubation in the presence of acetic and formic acid. Values of acids in (g L⁻¹). Standard deviations varied between 0.01 and 0.04.

	Acetic acid	Formic acid	OD _{600 nm}
Non-adapted	-	-	17
	1	1	17
	2	2	0
		2	0
	3		13
Adapted	-	-	9
	1	1	8
	2	2	6
	3	3	0
	3		8
		3	6

The adapted *Candida lipolytica* grew slower than the non-adapted yeast. Non-adapted *Candida lipolytica* tolerated the combination of 1 g L⁻¹ of acetic and formic acid. Two g L⁻¹ formic acid inhibited total the cell growth whereas 3 g L⁻¹ acetic acid slightly reduced the cell growth. Overall the adapted *Candida lipolytica* was more tolerant – only the combination of 3 g L⁻¹ of acetic and formic acid prohibited the cell growth (see Table 2). The same approach with *Yarrowia lipolytica* revealed the same effects (data not shown).

The cell growth of non-adapted *Candida lipolytica* and *Yarrowia lipolytica* was tested in the presence of the inhibitors furfural and HMF. Unfortunately no furfural and/or HMF adapted yeast existed at the time. Furfural was investigated in the range of 0.5 to 1.4 g L⁻¹ and 5-HMF 1 to 4 g L⁻¹. The optical density of the samples after 48 hours incubation was shown in Table 3.

Table 3. Optical density (= OD) at 600 nm from the non-adapted yeast *Candida lipolytica* after incubation in the presence of furfural and HMF. Values of furfural und HMF in (g L⁻¹). Standard deviations varied between 0.01 and 0.04

	Furfural	HMF	OD _{600 nm}
<i>Candida lipolytica</i>	-	-	12
	1		0
		0.5	12
		1.6	17
	1	0.5	0

HMF did not inhibit the cell growth even at higher concentration but 1 g L⁻¹ furfural together with 0.5 g L⁻¹ HMF prohibited the cell growth. The same experiments with *Yarrowia lipolytica* revealed the same effect (data not shown).

Few studies exist about the effects of inhibitors on cell growth and subsequent lipid accumulation. These studies revealed similar effects – *Cryptococcus curvatus* was also more sensitive to furfural than HMF, another study with *Yarrowia lipolytica* reported similar results. For example, *Trichosporon fermentans* tolerated relative high concentrations acetic and formic acid (Chen et al., 2009; Yu et al., 2011; Huang et al., 2012b; Sitepu et al., 2014b). However each yeast species differs in tolerance of inhibitors and must be tested.

The cell growth of non-adapted and adapted yeasts was also tested at the inhibitor concentrations of the corn cob hydrolysates. The cell growth of non-adapted yeast was reduced at tested inhibitor concentrations. The adapted *Candida lipolytica* tolerated the inhibitors very well (data not shown). Adapted yeasts in corn cob hydrolysates were used in the subsequent approaches. The inhibitors were partially utilised via the yeast (data not shown).

Corn cob hydrolysate as substrate for lipid production

Initial experiments were performed unsuccessful in corn cob hydrolysate with additional nitrogen sources (yeast extract and peptone). Nitrogen determination was done from corn cob hydrolysate with Dumatherm – 10 g 100 g⁻¹ dry matter corn cob hydrolysate contained 0.045 g total nitrogen per 100 g⁻¹ biomass – enough nitrogen for cell growth. Therefore no external nitrogen was added to the corn cob hydrolysate in the subsequent experiments. Adapted yeast was tested in corn cob hydrolysate with fed batch mode. Fresh corn cob hydrolysate was added after seven days and fermentation was undertaken for further seven days. The lipid contents and cell biomass were shown in Table 4.

Candida lipolytica accumulated the highest lipid content (19.4 g 100 g⁻¹) in corn cob hydrolysate at 30 °C. *Yarrowia lipolytica* reached lipid contents similar to non-oleaginous yeasts. Fed batch experiment with 10 g 100 g⁻¹ dry matter corn cob hydrolysate resulted in lipid content ranging from 4.5 g 100 g⁻¹ to 8 g 100 g⁻¹ (data not shown). Obviously the inhibitors within the corn cob hydrolysate did not interfere with the cell growth but with the lipid accumulation.

Table 4. Lipid content and cell biomass of the two yeast species in corn cob hydrolysate (fed batch mode, 7.5 g 100g⁻¹ dry matter)

Yeast species	Dry matter (g 100 g ⁻¹)	Lipid content in medium (mg L ⁻¹)	Lipid content (g 100 g ⁻¹)
<i>C. lipolytica</i> 25 °C	1.3	1470	11.3
<i>C. lipolytica</i> 30 °C	0.83	1600	19.4
<i>Y. lipolytica</i> 25 °C	1.27	1430	11.3
<i>Y. lipolytica</i> 30 °C	1.72	1600	9.3

Several studies about lipid accumulation were performed with corn cobs as substrate. The used yeast species produced higher lipid contents but in contrast to this study the total yeast lipids were extracted according the procedures described by Folch et al. (1957). The different extraction method or the use of other yeast species may explain the lower lipid content in this study. No studies about steam exploded corn cobs as substrate for lipid production via oleaginous yeasts existed. A direct comparison was therefore not possible.

Scale up experiments

Scale up to 2.5 litres in fermenter was done with the yeast *Candida lipolytica* to test the industrial scale of lipid production via yeast. The lipid content (gray bars) of the yeast *Candida lipolytica* in corn cob hydrolysate increased from 2.6 g 100 g⁻¹ after 24 hours up to 17.5 g 100 g⁻¹ after 168 hours incubation then the lipid content decreased due to depletion of the carbon source (black bars) (see Fig. 1). At the beginning 7.5 g 100 g⁻¹ corn cob hydrolysate contained about 29 g L⁻¹ total sugar (glucose plus xylose).

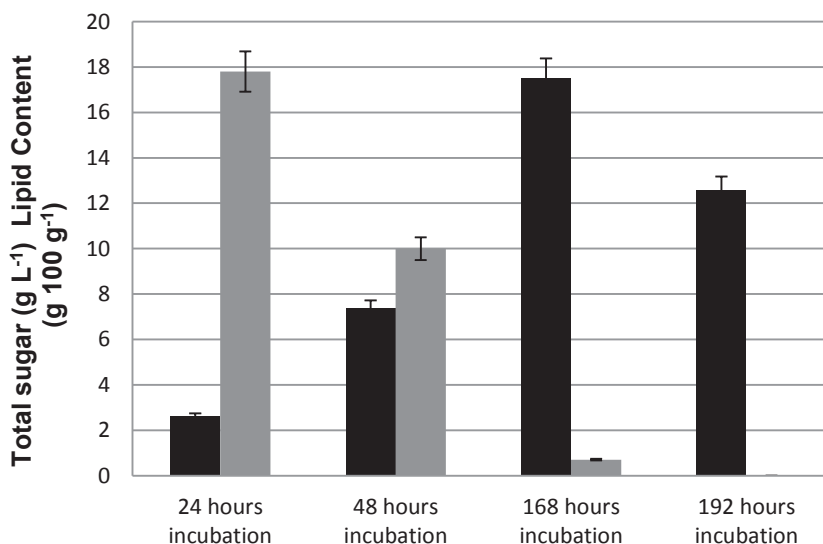


Figure 1. Lipid content (g 100 g⁻¹) and total sugar (g L⁻¹) of the yeast *Candida lipolytica* in corn cob hydrolysate during incubation time in fermenter.

More or less the same lipid content as before was achieved in fermenter with exact cultivation conditions – 17.5 g 100 g⁻¹ lipid content in shorter time (7 days) in batch mode. Lower lipid content was reached with fed batch mode in fermenter (maximum 14.23 g 100 g⁻¹; data not shown). Other scale up experiments in corn cob hydrolysate were not reported in diverse studies (Chen et al., 2012; Huang et al., 2012a; Chang et al., 2013; Huang et al., 2013b; Huang et al., 2014).

The composition of the lipids produced in the scale up experiment was mainly palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid which is suitable for biodiesel production. The values of these acids increased during the incubation time (see Table 5). The same acids were also detected in the other studies with corn cob hydrolysate (Chen et al., 2012; Huang et al., 2012a; Chang et al., 2013; Huang et al., 2013b; Huang et al., 2014).

Table 5. Values of the mainly produced acids in the scale up experiment during the incubation time. Values in (mg L⁻¹)

Time	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid
24 hours	39	12	0	113	51
48 hours	226	53	34	598	143
168 hours	624	188	81	1100	329
192 hours	708	279	120	1038	440

The lipid production increased during the incubation time however this experiment was discontinued due to the substrate depletion. In summary, steam exploded, enzymatically hydrolysed corn cobs will be a potential substrate for microbial lipid production due its availability.

CONCLUSIONS

Hydrolysate from steam exploded corn cobs was successfully used as substrate for lipid accumulation with specific oleaginous yeasts. Meanwhile hydrolysate from lignocellulose are produced competitive and is used as substrate for second generation bioethanol production replacing petrol (Forth International Conference on Lignocellulosic Ethanol, Straubing, 2014). Conventional biodiesel must be completed or replaced like bioethanol first generation production. Therefore my and several working group and companies worked worldwide with specific oleaginous microorganisms, often with yeasts due their numerous advantages.

Our results are lower than reported from other studies and differed probable due to the different extraction method or used yeast species. The corn cob hydrolysate mixture was complex, maybe further unknown inhibitors prevented higher lipid accumulation. Further studies concerning this issue will be done.

At the moment excessive costs prevent marketability of biodiesel from oleaginous yeast. However an economical process requires - an oleaginous yeast species which is tolerant to inhibitors and osmotolerant, grows fast and exhibits high oil accumulation; year-round available and competitive substrate; competitive extraction method and economic coproducts.

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