Bio-processing of agricultural residues to bio-fuels using *Neurospora discreta*

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Abstract.

The use of lignocellulosic agricultural residues for production of biofuels can address two pressing concerns of waste treatment and energy crisis simultaneously. However, the lignin present in these residues poses a challenge due to its recalcitrance, resulting in low overall yields despite using multi-step processes employing strong chemicals or high energy input to degrade the lignin. 'Complete bioprocessing' of agricultural residues using microorganisms is in its nascent stages of development and offers a promising alternative to existing processes. In our lab, we have found that a locally isolated fungus, *Neurospora discreta* can be used for such a process as it has the enzymatic machinery required for complete degradation of lignocellulosic biomass and producing not only ethanol but also carotenoids which can serve as a value-added product. A proof of concept study for complete bioprocessing of two types of agricultural residues using *N. discreta* is discussed here.

Keywords: Biofuels, lignocellulosic biomass, complete bioprocessing, *Neurospora discreta*

1. Introduction

Lignocellulosic agricultural residues are some of the most abundant and sustainable sources available for production of bio-fuels and other value added materials or chemicals. There is a massive potential in agricultural countries such as India to produce bio-ethanol from biomass as they generate millions of tons per annum of agricultural residues. (Pappu, Saxena, & Asolekar, 2007). The major components of lignocellulosic biomass are cellulose, hemicellulose and lignin. Cellulose and hemicellulose are plant polymers synthesized from hexose and pentose sugars respectively. Lignin is an aromatic polymer synthesized from phenyl-propanoid precursors, which gives rigidity and strength to plants and is resistant to degradation. Conversion of biomass to biofuels involves the hydrolysis of cellulose and hemicellulose to their monomer sugars, followed by fermentation of the sugars to bioethanol. However, the presence of lignin makes the hydrolysis steps challenging, owing to its complex structure and recalcitrance. Removal of lignin is therefore a critical step to a viable process for converting biomass to biofuels. Current processes for delignification of biomass use strong chemicals or high energy inputs, which add to processing costs. Moreover, chemical delignification produces by-products such as furfurals and hydroxyl-methyl furfurals which could inhibit the fermentation of sugars to ethanol (Limayem & Ricke, 2012; Olofsson, Bertilsson, & Lidén, 2008). Microbial delignification offers a promising alternative as it does not involve the use of strong acids or alkalis and is carried out at mild operating conditions. Certain fungi are known to secrete lignin degrading enzymes such as laccases and lignin peroxidases (Bugg, Ahmad, Hardiman, & Rahmanpour, 2011; Lundell, Mäkelä, & Hildén, 2010) which can cleave specific lignin bonds and either oxidize or degrade the lignin, thereby releasing the cellulose and hemicellulose for hydrolysis. However, slow reaction kinetics of microbial delignification has prevented this from becoming commercially viable. Hydrolysis and fermentation of the de-lignified biomass is a well-researched area and although this is more straightforward compared to the removal of lignin, the fermentation of pentoses released from hemicellulose as well as the inhibitory products produced during delignification as discussed above still pose two critical challenges. Consortia of microbes or enzymes are used for these steps, which can be carried out in separate reactors for hydrolysis followed by fermentation or a single reactor wherein both hydrolysis and fermentation take place (Kádár, Szengyel, & Réczey, 2004; Olofsson *et al*., 2008). In our lab, we are developing a single step bioprocess for the complete conversion of lignocellulosic biomass to biofuels and value-added products. A locally isolated fungus, *Neurospora discreta* showed rapid growth on different types of biomass tested and an ability to degrade lignin effectively (Pamidipati & Ahmed, 2016). *Neurospora crassa*, a model organism has been reported to produce laccases that can degrade lignin, as well as the enzymes required for hydrolysis of the carbohydrate polymers (Dogaris, Vakontios, Kalogeris, Mamma, & Kekos, 2009; Froehner & Eriksson, 1974; Luke & Burton, 2001). Moreover, it has the capability of converting hexoses as well as pentoses to ethanol (Chandel, Chandrasekhar, Radhika, & Ravinder, 2011; Dogaris, Mamma, & Kekos, 2013; Rao, Mishra, Keskar, & Srinivasan, 1985). *N. discreta*, on the other hand, is much less studied and has never been explored as a potential candidate for complete bioprocessing of agricultural residues. In this paper, we have shown the capability of using a single robust fungal strain, *Neurospora discreta* for

'complete bioprocessing' of two lignocellulosic biomass residues: coco-peat and sugarcane bagasse which have widely different lignin content and structures. This fungus was capable of: 1) delignification, 2) hydrolysis and 3) fermentation of sugars to ethanol. Moreover, we have also shown the ability of this fungus to produce 'carotenoids' as a by-product of its growth, which can serve as an important value added product and further improve the process economics (Figure 1).

2. Methods

2.1 Agricultural residues

Sugarcane bagasse and coco peat were obtained from local vendors. They were thoroughly washed, dried and chopped and sieved to obtain 20-30 mm fraction. They were stored in air tight containers at 2-4^{o}C.

2.2 Fermentation set-up

Submerged fermentations were set up using sub-cultures of the fungus *N. discreta*, which is a locally isolated fungal strain. Each 250-ml flask contained 1 gm of substrate with 0.5 g of sucrose in 100 ml Vogel's minimal media. The inoculation of the flasks was done as reported in our earlier paper (Pamidipati & Ahmed, 2016), where the final spore count of the cells was adjusted to approximately 10^{7}cells/ml and 1 ml suspension was used to inoculate each flask. The flasks were incubated at 30^{o}C for 30 days. During the 1st stage, i.e., for 15 days after inoculation, the flasks were aerated every alternate day for accelerating the plant polymer degradation. In the 2nd stage, microaerobic conditions were maintained for maximizing the ethanol production.

2.3 Sample preparation

At the end of the fermentation, the flask content was filtered through a double-layered muslin cloth to separate the liquid and solid fractions. The solid fractions were thoroughly washed with de- ionized water, filtered and dried at 105^{o}C till constant weight. The dried samples weight was noted. The sample was stored for further processing and analysis of the residual lignin, and residual cellulose. The liquid fractions were centrifuged at 10,000 rpm for 10 min and the clear supernatant was stored at 4^{o}C for further analysis.

2.4 Lignin estimation by Klason's method:

Lignin content in the solid biomass samples was measured using standard Klason's method, (Sluiter *et al*., 2008) before and after fungal treatment.

2.5 Cellulose estimation by Updegraff method:

Cellulose content in the solid biomass samples was estimated by Updegraff method (Bauer & Ibáñez, 2014). 30 mg of biomass was weighed into 15ml centrifuge tubes with screw caps. 3 ml of acetic acid/water/nitric acid in 8/2/1, v/v/v ratio was added and the samples were heated in a boiling water bath for 30 min with occasional stirring. While the hemicellulose and lignin fractions from the biomass would get hydrolyzed and dissolved in this solvent, the intact crystalline is left behind as a solid fraction. The tubes were then centrifuged at 3000 g rpm for 10 mins and the supernatant was removed without disturbing the pellet. The pellet was re-suspended in 7 mL of water, centrifuged and the liquid was discarded. This washing step was repeated one more time. Equal amounts were discarded each time, ensuring the solid pellet does not get disturbed. The remaining pellet was then incubated with 72% sulphuric acid for 1 hour with occasional stirring. The solution was then made up to 10 ml using deionized water. The pH of the samples was carefully adjusted to 7.0 using 5N NaOH and 0.1N NaOH. The total glucose content was estimated by di-nitro salicylic acid assay method. A calibration curve was plotted using varying concentrations of pure glucose.

2.6 Estimation of Ethanol content by Gas chromatography

Gas-chromatography (Shimadzu GC – 2010 Plus) with Flame Ionization detector was used to estimate ethanol and acetic acid in the liquid supernatants. The capillary column had following dimensions: 30m, 0.25 mm ID and 0.2um thickness (Make: Spinco-tech, Model: EB-1). The operating conditions were as follows: injector, 120 °C; oven, 150 °C; and detector, 160 °C with a carrier gas flow of 16 mL min $^{-1}$.

2.7 Estimation of carotenoids by UV-Visible spectroscopy

Carotenoids were extracted from liquid supernatants by solvent extraction and estimated using UV-visible spectroscopy at 450 nm (Barredo, 2009; de Carvalho *et al*., 2012). To 5ml of the liquid sample, 5 ml of acetone was added and vigorously stirred for 10 minutes. 5ml of di-ethyl ether was later added and the solution was again vigorously mixed. Carotenoids, which are hydrophobic in nature, are extracted into the ether layer. The two layers were separated in a separating funnel. Carotenoid content in the di-ethyl ether layer was estimated using UV-Visible spectrophotometer (HITACHI U-2900) at 450 nm wavelength. A calibration curve was plotted with varying concentrations of pure β-carotene (Make: Sigma Aldrich) solution prepared in di-ethyl ether.

3. Results and discussion

3.1 Analysis of solid samples

The capability of lignin degradation in cocopeat and sugarcane bagasse samples which have widely different lignin structures by *N. discreta* has already been discussed in our earlier paper (Pamidipati & Ahmed, 2016). Klason's test, UV, HPLC and FTIR analysis were conducted to study lignin degradation in these samples. We have further analyzed our solid samples for cellulose content by Updegraff method. Figure 2 shows cellulose and lignin content in solid samples before and after fungal treatment. *N. discreta* was found to degrade bagasse more easily than coco-peat which is in accordance with our earlier results owing to the differences in lignin structures (Pamidipati & Ahmed, 2016).

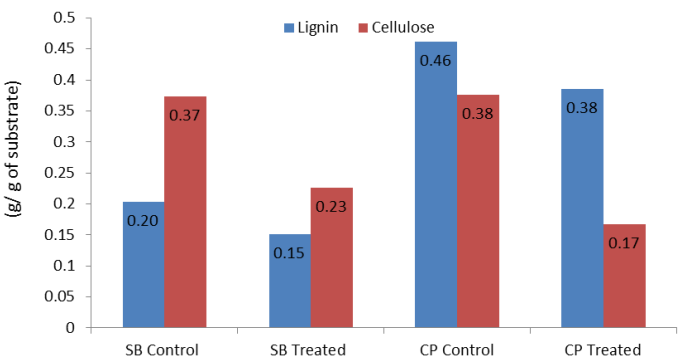

Figure2. Lignin and cellulose content (g/g of substrate) in sugarcane bagasse (SB) and coco-peat (CP)

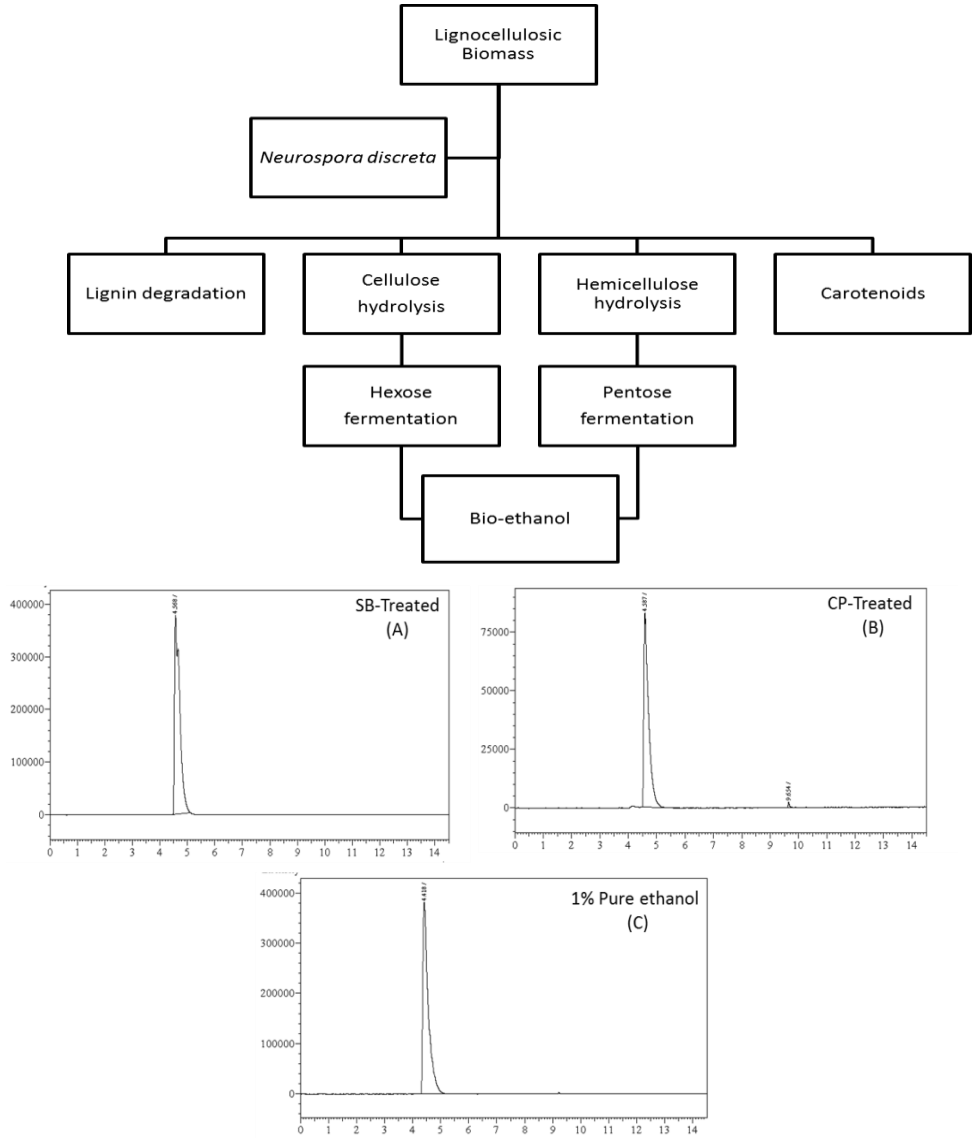

Figure 1. Complete bioprocessing of lignocellulosic biomass residues to bio-ethanol and value- added products

Figure 3. Gas chromatograms of liquid supernatant samples: (A) Fungal treated sugarcane bagasse (B) Fungal treated cocopeat and (C) 1% v/v pure ethanol in water.

3.2 Analysis of liquid samples

3.2.1 Ethanol content

Figure 3 shows chromatograms of liquid supernatant samples of sugarcane bagasse and cocopeat. A chromatogram of pure ethanol of 1% (v/v) concentration is shown in Figure 2(C), which has a retention time at 4.42. Ethanol concentration was measured in the liquid samples of cocopeat and sugarcane bagasse using a calibration curve plotted using varying concentrations of pure ethanol in water from 0.5% to 2% (v/v). The liquid supernatant of sugarcane bagasse showed higher ethanol concentration (1% v/v) as compared to cocopeat (0.22% v/v). Due to the higher guaiacyl content in the lignin of cocopeat, the degradation products could have inhibited the fermentation of degraded sugars to ethanol.

3.2.2 Carotenoids

The concentration of carotenoids in di-ethyl ether extracts of liquid supernatant samples was estimated at 450 nm in UV-visible spectrophotometer. The concentration of carotenoids was found to be around 0.025 µg/ml in both coco-peat and sugarcane bagasse fungal treated liquid supernatants. Carotenoids were not detected in the solvent extracts of untreated biomass samples.

4. Conclusion

In this study, we have demonstrated for the first time the capability of isolated fungal strain *N. discreta* to degrade lignin and cellulose polymers and produce ethanol from the hydrolyzed sugars. Owing to its rapid growth rate, robustness and ease of adaptation it is a suitable candidate for 'complete bioprocessing' of agricultural residues. Moreover, we have also shown that *N. discreta* secretes carotenoid pigments that can serve as a value-added product. A more detailed study will be carried to out to study the parameters that can increase the degradation of

the plant polymers and also improve the titer values of ethanol and carotenoids.

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