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Isolation of fungi from dung of wild herbivores for application in bioethanol production

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ABSTRACT

Producing biofuels such as ethanol from non-food plant material has the potential to meet transportation fuel requirements in many African countries without impacting directly on food security. The current shortcomings in biomass processing are inefficient fermentation of plant sugars, such as xylose, especially at high temperatures, lack of fermenting microbes that are able to resist inhibitors associated with pre-treated plant material and lack of effective lignocellulolytic enzymes for complete hydrolysis of plant polysaccharides. Due to the presence of residual partially degraded lignocellulose in the gut, the dung of herbivores can be considered as a natural source of pre-treated lignocellulose. A total of 101 fungi were isolated (36 yeast and 65 mould isolates). Six yeast isolates produced ethanol during growth on xylose while three were able to grow at 42 °C. This is a desirable growth temperature as it is closer to that which is used during the cellulose hydrolysis process. From the yeast isolates, six isolates were able to tolerate 2 g/L acetic acid and one tolerated 2 g/L furfural in the growth media. These inhibitors are normally generated during the pre-treatment step. When grown on pre-treated thatch grass, *Aspergillus* species were dominant in secretion of endo-glucanase, xylanase and mannanase.

Introduction

Plant biomass represents the largest source of renewable energy in nature. The search for renewable sources of energy requires a global effort in order to reduce the harmful consequences of global warming and to meet future energy demands.[1] Second Generation biofuels are emerging as a new source of energy that is produced from biomass. The production of biofuels through advanced process technologies could aid in reducing greenhouse gas emissions. These technologies would also allow the production of renewable fuels without negatively impacting directly or indirectly on food production.[2]

Plant biomass is composed of lignocellulose, which generally consists of up to 45% cellulose, 30% hemicelluloses and 25% lignin.[3] Cellulose and hemicelluloses are polysaccharides, while lignin is an aromatic heteropolymer binding the two polysaccharides together. Lignocellulosic biomass includes agricultural residues such as corn stover, straw, sugarcane bagasse, herbaceous energy crops, wood residues (sawmill and paper mill discards), and municipal waste.[4] These materials could serve as a cheap, abundant and renewable energy

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feedstock that is essential to the functioning of industrial communities and critical to the development of a sustainable global economy.[5]

The products of cellulose and hemicellulose hydrolysis are substrates for fermentation in the production of biofuels/bioethanol. Hydrolysis of lignocellulosic biomass is a slow process due to the resistant crystalline structure of cellulose and the physical barrier of lignin surrounding cellulose, thus limiting the sites for enzymatic attack.[6] Novel organisms are needed to provide improved enzymes and reduce the cost of converting lignocellulosic material to fermentable sugars. Also required are organisms that are able to ferment pentose sugars effectively.[7] These organisms should also be able to resist inhibitors released during the pretreatment of lignocellulose.[8]

It is estimated that up to 70% cellulose present in natural feed is excreted by herbivores making their dung a rich source of "pre-treated" lignocellulolytic material for the isolation of lignocellulosic organisms.[9] Coprophilous fungi are dung-loving and encode many enzymes needed for the hydrolysis of cellulose.[10] A number of studies report the isolation of cellulase producing fungi from the dung of domestic animals, however, few studies have been done on the dung of wild herbivores. One study report the isolation of six different xylose fermenting yeasts species from the dung of a number of herbivores in Thailand.[11] Other studies focused on the breakdown of elephant dung,[12] the production of hydrogen using thermophilic anaerobes in elephant dung,[13] the isolation of cellulolytic fungi from the dung of elephants,[14] the antimicrobial compounds produced by fungi from the dung of elephant, tiger and rhinoceros[15] and lastly the isolation of a β-glucosidase from a fungus associated with the rumen of buffalo.[16]

Yeasts currently considered for the fermentation of pentoses, especially xylose, are mainly *Scheffersomyces stipitis, Kluyveromyces marxianus, Candida shehatae* and *Pachysolen tannophilus*. Ethanol production by these yeasts utilizing xylose as carbon source is generally five times lower when compared to *Saccharomyces cerevisiae* fermenting glucose.[17] Other factors to consider in searching for an ideal xylose fermenter are resistance to inhibitors, such as furfural and acetic acid, ability to carry out fermentation at low pH and high temperatures conditions.[18]

The aim of this study was to isolate xylose utilizing yeasts and cellulolytic moulds from decomposed dung of various herbivore species found in the Kruger National Park, South Africa. Yeast isolates were evaluated for their xylose fermentation capabilities, while mould isolates were screened for cellulolytic enzyme production.

Material and methods

Sample collection

Fifty decomposed dung samples, from wild herbivores, were collected from the Kruger National Park, South Africa. Forty dung samples were collected near the Phalaborwa rest camp and 10 samples were collected from the proximity of the Skukuza rest camp. An experienced game ranger aided with the identification of the sources of the dung samples. All samples were collected into plastic bags and processed within 48 h.

Isolation of fungi

Approximately 1 g of the dung samples were sprinkled directly on agar plates containing 10 g/L xylose, 10 g/L beechwood xylan, 10 g/L avicel cellulose or 10 g/L locust bean gum (mannan), as a sole carbon source, 6.7 g/L YNB (yeast nitrogen base, Difco), 15 g/L bacteriological agar and 0.2 g/L chloramphenicol to inhibit bacterial growth. The fungal isolates (yeasts and moulds) were purified through repeated streaking on fresh YM (10 g/L glucose, 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone and 15 g/L bacteriological agar) plates and pure cultures were stored on YM agar slants.

Fermentation of xylose by yeast isolates

Fermentation media (20 g/L xylose, 10 g/L yeast extract, 2 g/L KH_2PO_4, 10 g/L NH_4SO_4, 2 g/L $MgSO_4{\cdot}7H_2O$ and 0.2 g/L chloramphenicol) in a 250 ml Erlenmeyer flasks each containing 25 ml of media was inoculated with a yeast isolate and incubated at 30 °C and 150 rpm for 24–120 h. The above mentioned culture was used to inoculate 3 × 100 ml of the same media in 500 ml Erlenmeyer flasks to an OD_{600nm} of 0.2 and incubated at 30 °C and 150 rpm for 96 h. Samples of 2 ml were taken every 24 h. All the samples were centrifuged for 5 min at 2000 x g and 4 °C after which the supernatants were filtered through a 0.22 μm syringe filter and stored at −20 °C until analysis.

Tolerance to inhibitors and elevated temperatures

Xylose fermenting yeast isolates were further tested for their ability to grow in the presence of 1, 2, 3, 5, 7, and 10 g/L acetic acid and 1, 2, 3 and 4 g/L furfural in YM agar plates. All plates were incubated at 30 °C for 48 h. The maximum growth temperatures for all the yeast isolates were determined using YM slants. The slants were incubated at 35, 37, 40, 42, and 45 °C. The maximum temperature for growth is considered the highest temperature where growth occurred.

Production of enzyme by mould isolates on thatch grass based medium

Mould isolates were screened for endoglucanase, xylanase and mannanase activity in liquid media containing 20 g/L pre-treated thatch grass (*Hyparrhenia sp*), 4 g/L KH_2PO_4, 10 g/L $(NH_4)HPO_4$, 10 g/L peptone, 3 g/L yeast extract and 0.1 g/L chloroamphenicol in 100 ml deionized water. Pre-treatment of thatch grass was performed by grinding air dried grass to a fine powder. Dilute acid pre-treatment using 1.2% sulphuric acid was performed at 120 °C for 60 min at a biomass concentration of 10% (w/v). After pre-treatment, 5 M sodium hydroxide (NaOH) was used to adjust the pH of the thatch grass suspension to 6. Erlenmeyer flasks (250 ml) containing 50 ml of the thatch grass based media were inoculated with ten 4 mm plugs of agar from a freshly cultured plate. All isolates were inoculated in duplicate and incubated in a rotary shaker for 5 days at 30 °C and 150 rpm. Samples were taken at 24 h intervals.

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