EXTRACTION OF CELLULOSE AND BIOFUEL PRODUCTION FROM GROUNDNUT SHELLS AND ITS APPLICATION TO INCREASE CROP YIELD

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ABSTRACT
India is the second largest producer of groundnuts. Groundnut shells which remain as a waste after the separation of groundnut seeds is mostly dumped or burned. They can be used as a renewable source of energy. Fossil fuels are non-renewable sources of energy which need an alternative. In present study groundnut shells were collected from Mirewadi village in Maharashtra. They were cleaned, dried, powdered and used for biofuel production. Pure culture of \textit{Saccharomyces cerevisiae} was used for fermentation of the hydrolysate with an addition of peptone as protein source. MIC of alcohol and glucose for \textit{S. cerevisiae} was carried out. Aliquots were removed at different time intervals and subjected to distillation. Distilled samples were subjected to glucose and ethanol estimation by DNSA and Dichromate method respectively. The yield of alcohol was found to be highest on 4\textsuperscript{th} day of fermentation. Cellulose was extracted from groundnut shell powder. Muffle furnace was used to make ash of groundnut shells and used to check its applications to increase crop yield.

KEYWORDS: Acid hydrolysis, Fermentation, Biofuel production, distillation, ethanol estimation, cellulose extraction.

INTRODUCTION
Fossil fuels immensely contribute to environmental pollution, degradation and also enhance greenhouse gas emission leading to depletion of ozone layer (Rabah et al., 2014). Biofuel is a renewable source of energy and hence can be used as an alternative to conventional fossil fuels.
fuels. It burns up to 75% cleaner than fossil fuels (Oniya et al., 2014). Use of agricultural waste having no economic value for biofuel production gives a better way of efficiently utilizing agricultural land. Sugarcane molasses, groundnut shells, rice husks, straw, corn cobs, etc. are being studied as substrates for biofuel production. The groundnut (Arachis hypogaea), is a species in the legume family (Fabaceae) and is an annual herbaceous plant growing 30 to 50 cm (1.0 to 1.6 ft) tall (Nyachaka et al., 2013). Groundnut shells contain high cellulose (37%) and hemicellulose (18.7%) content and also other carbohydrates about 2.5%, which increase the efficiency of fermentation and provide better yield (Jaishankar et al., 2014).

Yeast, fungi, certain microalgae and genetically modified microorganisms are used as feedstock for biofuel production (Gohel et al., 2013). The yeast, Saccharomyces cerevisiae, produces ethanol by fermentation of glucose. But it is unable to ferment pentose sugars. Sufficient biomass of yeast can be produced using fermentors and other advantages include smaller area for production as compared to plants, easy extraction method and ability to grow on a wide variety of media (Gohel et al., 2013).

Cellulose is the most abundant biopolymer in the world (Lavanya et al., 2011). Cellulose is found in a wide range of species and present along with hemicelluloses, lignin, pectin, wax and resins (Abbakar et al., 2015). It can be obtained from numerous resources, such as wood, eucalyptus, sisal, cotton, coconut fibers, and non-plant sources, including forms produced by bacteria and found in tunicates. The structure of cellulose is organized into fibrils, which are surrounded by a matrix of lignin, extractive inorganics and hemicelluloses (Costa et al., 2013). The use of groundnut shell ash, an agricultural waste, helps in proper waste management. The ash is also used as soil stabilizer to increase the strength of soil and moisture content (Sujatha et al., 2015).

In the present study, Saccharomyces cerevisiae was used for fermentation of acid hydrolysate of groundnut shell powder. Cellulose was extracted from groundnut shell powder and groundnut shell ash was checked to increase crop yield.

MATERIAL AND METHOD
Sample collection
Groundnuts (Arachis hypogaea) were collected directly from a farm in Mirewadi village, Maharashtra. The collected groundnut shells were washed thoroughly with tap water to
remove soil particles. They were then oven dried for several hours to remove the moisture completely. Oven dried groundnut shells were powderized using a blender.

A) Biofuel production
Selection of organism
Based on the ability of organism to ferment sugars, *Saccharomyces cerevisiae* was selected for fermentation. Baker’s yeast granules were used for obtaining pure culture of *S. cerevisiae* on *Sabouraud* agar media (4% glucose, 1% peptone, 0.5% NaCl, 3% agar-agar). Colony characters were noted and morphological characteristics were studied using Monochrome staining. The growth characteristics were also studied.

Acid hydrolysis
Groundnut shell powder (1g) was subjected to acid hydrolysis using sulphuric acid (20ml) of various concentrations *i.e.* 2%, 4%, 6%, 8%, and 10%. The mixture was autoclaved at 121°C, 15psi for 20 min and further cooled to room temperature. The hydrolysate was filtered to remove the residue. The hydrolysate was neutralized using Sodium hydroxide. The total amount of total carbohydrates, glucose and xylose present in filtrates was checked, which helped to narrow down the concentration of H$_2$SO$_4$ at which best hydrolysis was obtained.

Estimation of total carbohydrates
Total amount of carbohydrates present was estimated by Phenol-Sulphuric acid method using glucose as a standard having a concentration of 1 mg/ml and the colorimetric reading was taken at 490nm (Sadashivam, 2004).

Estimation of glucose and xylose
The amount of glucose present in the neutralized hydrolysate was estimated by DNSA method and the colorimetric reading was taken at 540nm (Sadashivam, 2004). Estimation of Xylose present was carried out by Phloroglucinol assay and the colorimetric reading was taken at 540nm (Ayudhya *et al.*, 2007). Glucose and xylose sugars were used as standards having a concentration of 1mg/ml and 0.5 mg/ml resp.

Based on the results, further 8g of Groundnut shell powder was hydrolysed in 160 ml of 4% H$_2$SO$_4$ neutralised and used for further tests.
Preparation of Fermentation medium
1.6g of Peptone was added to the neutralized hydrolysate (pH 5.6) obtained. It was autoclaved at 121°C, 15 psi for 15 mins and used as fermentation medium. The amount of glucose was estimated by DNSA method.

Fermentation process
Pure culture of *S. cerevisiae* was inoculated into the sterile *Sabouraud* broth. Flasks were incubated at room temperature and kept at both static and shaker conditions. The cell density of the *S. cerevisiae* suspension was checked using Haemocytometer count. 2ml of culture (0.529 x 10^6 cells/mm^3) was then added to the fermentation medium and allowed to ferment. Aliquots were removed at different time intervals and subjected to distillation. Distilled samples were subjected to ethanol estimation by Dichromate method (Zimmermann, 1963).

Minimum inhibitory concentration (MIC) determination of alcohol and sugars
MIC of alcohol: Minimum Inhibitory Concentration of alcohol for *S.cerevisiae* was found by using 30% alcohol by tube turbidity method. A standard concentration of 30% alcohol solution was made in sterile *Sabouraud* broth. The diluent was plain sterile *Sabouraud* broth. The tubes were incubated at 37°C for 24 hrs. The tubes were checked for visible growth (Mazzola et al., 2001).

MIC of glucose
Minimum Inhibitory Concentration of glucose for *S.cerevisiae* was found by using 80% glucose by tube turbidity method. A standard concentration of 80% glucose solution was made in sterile *Sabouraud* broth. The diluent was plain sterile *Sabouraud* broth. The tubes were incubated at 37°C for 24 hrs. The tubes were checked for visible growth ((Mazzola et al., 2001).

B) Extraction of cellulose
A dried shells of groundnut was obtained and grinded into powder for the extraction. 8g of sample was taken to which 400 ml of 2 % w/v Sodium hydroxide was added and digested for 4 hrs at 80°C in a water bath. Following thorough washing and filtration, it was bleached with 200 ml of a 1:1 aqueous dilution of Sodium hypochlorite for 15min at 80°C. The material was then washed sufficiently with water and repeated the process for purification. The resulting α-cellulose was washed thoroughly with distilled water. The presence of cellulose was confirmed by Phenol Sulphuric acid method (Achor et al., 2014).
C) Biofertilizer

Preparation of groundnut shells ash
The groundnut shells were washed, dried and grinded to make fine powder. Fix amount of powder was taken into crucibles and heated in Muffle furnace (480°C) to make ash for two days. The ash was weighed on each day till constant weight was obtained.

Use of groundnut shells ash to increase crop yield
1 g of groundnut shells ash was added into 25 g of autoclaved soil. 26 g soil was taken as Control. To this surface sterilized moong beans (Vigna radiata) were inoculated. The seeds were allowed to germinate and grow. After seven days, carbohydrate and protein content of the plantlets were checked by Anthrone test and Folins-Lowry method respectively (Sadashivam, 2004).

RESULT AND DISCUSSIONS
A pure culture of Saccharomyces cerevisiae was isolated. A monochrome staining was performed to check for its purity and the growth characteristics were studied. Pure cultures of Saccharomyces cerevisiae were maintained on Sabouraud’s agar slants. Regular subculturing was carried out in 1- 2 weeks. 24- 48 hr grown cultures were used for the study.

MIC of Glucose and Alcohol
Minimum Inhibitory Concentration of glucose and alcohol for S.cerevisiae was found by using 80% glucose 30% alcohol respectively by tube turbidity method and found to be in the range of 56-64% for glucose and 3-6% for alcohol.

A) Biofuel production

Estimation of total carbohydrates, glucose and xylose
The total amount of carbohydrates present was estimated by Phenol-Sulphuric acid method and the optical density was measured at 490 nm. The total carbohydrate content was highest in 4% hydrolysate and was found to be 3.9mg/ml (Figure 1).
The total amount of glucose and xylose present in the neutralized hydrolysate was estimated by DNSA method and Phloroglucinol assay resp. Maximum amount of glucose and xylose was estimated in 4% hydrolysate and was found to be 3.2 mg/ml and 1.7 mg/ml resp (Figure 2).

Glucose estimation during fermentation
The amount of glucose during fermentation was estimated by DNSA method. The amount of glucose was found to be decreasing from day 1 to day 6 of fermentation i.e. from 4.2 mg/ml to 2.5 mg/ml. The decrease is due to the utilization of glucose as a carbon source by Saccharomyces cerevisiae. It is seen that yeast does not use pentose sugars as their carbon source (Kumar et al., 2014) (Figure 3).
Fig 3: Decrease in glucose level during fermentation.

**Alcohol estimation by Dichromate method**

The amount of alcohol produced was estimated by Dichromate method. There was gradual increase in the amount of alcohol produced as the fermentation days increases. A sharp drop was seen after the fourth day. This may be due to the decrease in glucose in the medium. Maximum amount of Alcohol was estimated on the fourth day and was found to be 0.5mg/ml (Figure 4).

Fig 4: Day wise alcohol estimation.

**B) EXTRACTION OF CELLULOSE**

The extraction of cellulose was carried out from the powdered shells of groundnuts. The presence of cellulose was confirmed by Phenol Sulphuric acid method. 0.41g of cellulose per gram of groundnut shell powder was obtained (Figure 5).
C) Increase in crop yield
Groundnut shell powder was heated in a muffle furnace at a temperature of 480°C for two days. 40g of Groundnut shells powder gave 3.1g of Groundnut shells ash. This ash obtained was mixed in soil and the plants grown were checked for the increase in protein and carbohydrates content (Figure 6).
Growth of seeds was observed in both Control and Test and its efficiency was calculated by the formula (AOAC, 2005).

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\text{% Efficiency} = \left( \frac{T-C}{C} \right) \times 100
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Estimation of total carbohydrates and proteins
Total carbohydrate and protein content of plantlet was estimated by Anthrone test and Folin-Lowry test respectively. An increase in the carbohydrates and protein contents of the plants were seen which were grown in the presence of groundnut shell ash. The carbohydrate content in the test plants was found to be 0.72 mg/ml and protein content was found to be
1.09 mg/ml. There was a 12.5% increase in the carbohydrate content whereas a 71.11% increase in the protein content (Figure 7).

CONCLUSION
Groundnut shells have high amount of lignin, hemicellulose and cellulose. These components can be used for various purposes like production of Biofuel, Bioadhesives, Biofertilizer. The cellulose can be hydrolysed to simple sugars. Acid hydrolysis was carried out and the neutralized hydrolysate was subjected to total carbohydrate, glucose and xylose estimation respectively. The maximum amount of carbohydrate and glucose was found in 4% hydrolysate which was 3.9 mg/ml and 3.2mg/ml respectively. S.cerevisiae was used for fermentation. The alcohol estimation of the distilled samples was carried out using Dichromate method and maximum alcohol content was found to be 0.5mg/ml on Day 4. Cellulose was extracted and 0.41g of cellulose per gram of groundnut shell powder was obtained. The effect of groundnut shell ash to increase crop yield was checked and efficiency of GNS ash for increase in Carbohydrate and protein content was found to be 12.5% and 71.11% respectively. The bioethanol produced can be used along with gasoline as gasohol and the cellulose extracted can substitute plant cellulose leading to reduction in deforestation.

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