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CONVERSION OF RICE STRAW TO FERMENTABLE SUGARS AND BIOETHANOL BY MFEX PRETREATMENT AND SEQUENTIAL FERMENTATION

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Abstract

The global rise in energy consumption, predicted increase in energy demands, price fluctuations, depletion and drawbacks of fossil fuels have converged to create an urgent need to develop more sustainable energy systems based on renewable feedstocks. Lignocellulosic materials are attractive feedstocks for bioethanol production. Rice straw is a promising feedstock for sustained production of biofuel. Bioethanol from lignocellulosic biomass could be a promising technology though the process has several challenges such as efficient pretreatment methods for delignification of lignocellulosics. Pretreatment is a prerequisite step for increasing the enzymatic digestibility for conversion to biofuels in biorefineries. The merits of a new and promising pretreatment called Multipurpose Fiber Expansion (MFEX) method for pretreating rice straw for bioethanol production was studied, where rice straw was treated with steam and carbon dioxide in sequence to make use of the synergistic effects obtained under pressure and moderate temperature for a few minutes and then rapidly releasing the pressure.A total reducing sugars of 645mg/g dry treated rice straw was achieved within 24 hours hydrolysis using laboratory prepared cocktail cellulolytic and hemicellulolytic enzymes. Of this total, 400 mg/g was glucose, which was rapidly fermented

within 24 hours by a genetically-engineered KlebsiellaoxytocaP2 leading to bioethanol yield of 375 mg/g dry treated biomass.Biofuels provide a potential and promising green alternative to avoid the global environmental crises that arise from dependence onfossil fuels. Conversion of glucose as well as xylose to bioethanol needs some improved co-fermentation technologies, to make the whole process cost effective.

Keywords

Rice Straw, MFEX Pretreatment, Enzyme Cocktail, Simultaneous Saccharification & Fermentation, Bioethanol

1. Introduction

To alleviate the increase in demand of energy, depletion of conventional sources of energy, the very speedily rising in fossil fuel prices, global warming, and climate changes, there is a need to find out an alternative source of energy, which fulfills the criteria of sustainable development, that means it not only enhances the world's economy, but also supports the environment.In 2008, United Nations defined Environmental Sustainability as meeting the needs of the present without compromising the ability of future generations to meet their needs. For a sustainable development, the development of economy and environment should go together as both are necessary for the development of human beings. For mitigation of climate change and for sustainable growth of economy. This can be achieved by promoting new renewable sources of energy such as solar , wind , and biomass based energy (Markou, Angelidaki, Nerantzis, &Georgakakis, (2013).Biofuels among other renewable and sustainable energy resources offer a worthy solution to counter depleting conventional fuel sources as well as to put a halt to climate changeJinfeng, Ming-Huang,&Yuh-Shan, (2011).The utilization of edible food crops such as corn or sugarcane for bioethanol production raises the question of food security and thus, it is not appropriate for sustainable development. So, there is a need to derive bioethanol from some other sources which do not put pressure on food crops. This led to the bioethanol production from inedible potential feed stocks (Sun & Cheng, 2002). Utilization of lignocellulosic biomass for bioethanol production is the better opportunity as it does not compete with the food crops and animal feeder and moreover these cellulosic materials contribute to environmental sustainability (Demirbas,2003).

Unsustainable use and open burning of rice straw in the field not only produces threat to environment by producing large amount of greenhouse gas (GHG) emission, but also make farmer's loose a very viable by-product. Rice straw can be used in bioethanol production and

bring additional income and sustainable utilization. This technology of waste to energy includes the pretreatment of biomass, subsequently converted to sugars, which thereafter transformed into bioethanol,but there occur several challenges and limitations in the process of converting biowaste to bioenergy, i.e. rice straw to bioethanol. One of the major challenges in developing technology for bioethanol production from rice straw is selection of an appropriate pretreatmenttechnique. The choice of pretreatment methods plays an important role to increase the efficiency of enzymatic saccharification thereby making the whole process economically viable. A major constraint in the enzymatic saccharification of biomass for bioethanol production is the cost of cellulase enzymes. Production cost of cellulases may be brought down by multifaceted approaches whichinclude the use of cheap lignocellulosic substrates for fermentation production of the enzyme, and theuse of cost efficient fermentation strategies like solid state fermentation (SSF). Laboratory prepared enzymes cocktail is one of the best methods to improve the process efficiency.

1.1 Feedstock Pretreatment

The first step to the success of utilizing lignocellulosic materials for the production of biofuels is the efficiency of pretreatment methods. The challenge is to disarray lignin, which forms a protective wall, making cellulose and hemicelluloses inaccessible for further uses. A number of pretreatment processes (physical, chemical and biological) have been used to break the structural framework of plants and depolymerize lignin. Pretreatments that combine both chemical and physical processes are referred to as physico-chemical processes(Sun, Wen, Ma, & Sun, 2014).The RS is mainly composed of cellulose, hemicellulose, and lignin. The complex structure of lignin, hemicellulose, and cellulose in RS limits its effective saccharification. Hence, some pretreatment procedures need to be performed before its saccharification. Traditionally, the purpose of its pretreatment is to remove lignin, to reduce cellulose crystallinity, **and** to increase the porosity, thus improving its saccharification efficiency (García et al., 2014). Pretreatment is considered as a central process and remains a bottleneck in the process of lignocellulosic bioethanol production because the pretreatment step is known to be the most expensive and profoundly affects all downstream steps, such as enzyme hydrolysis, fermentation, waste residue handling, and ethanol recovery (Mosier et al., 2005).

1.2 Bio-Conversion Technology of Lignocellulose-to-Bioethanol

Glucose and xylose are the main sugars contained in lignocellulosic biomass, and effective extraction of these sugars is a very important stage factor in determining the effectiveness of bioethanol pathways (Cao &Aita, 2013). Bioenergy offers the opportunityto

reduce not only the carbon dioxide emissions but also thedependence of energy imports.Due to the worldwide energy crisis and global warming, there has been considerable interest in developing substitute renewable sources of energy. One of the promising candidates includes biomass-based biofuels. Bioethanol has received particular attention. The maximum utilization of all sugar fractions is essential to obtain an economic and viable conversion technology for bioethanol production.However, current grain feedstock faces intrinsic problems of sustainability and ethics. On the other hand, lignocellulosic biomass is regarded as one of the most viable options for bioethanol feedstock.An economical and eco-friendly bioethanol productionprocess for rice straw requires the recovery of lignin and silicafractions present in the rice straw and their subsequent use inthe manufacture of value-added products.

1.3 Bioethanol Usage and its Present Status

Production of bioethanol from rice straw, one of the most abundant agricultural wastes, has been extensively studied (Suriyachai, Weerasaia, Laosiripojana, Champreda, & Unrean, 2013). Therefore, it is of great importance in improving the present technology. The RS biorefinery is an effective way to achieve this goal by fully utilizing its components and co-producing high value-added chemicals (Song, Dotzauer, Thorin. & Yan, 2014). Utilizing lignocellulosic biomass resources offer the key advantage of minimizing the conflict between using crops for food production vs. bioenergy production (Menon, & Rao, 2012). Bioethanol has high octane number, high heat of vaporization and low cetane number; all these qualities make it a suitable fuel for blending with gasoline or use as neat alcohol in dedicated engines (Hahn-Hagerdal, Karhumaa , Fonseca , Spencer-Martins , &Gorwa- Grauslund , 2007) Bioethanol is used as an oxygenative additive as it contains 35% oxygen which reduces emission of particulate matter and oxides of nitrogen. It is blended with gasoline in different ratios. Bioethanol can also be a versatile chemical and organic solvent.

This study aims to optimize process parameters for the innovative MFEX-pretreatment biotechnologies of rice straw, to study the production of cellulases and hemicellulase by a locally potent strains, to evaluate the application of these in-house prepared cocktail enzymes in enzymatic hydrolysis of MFEX-treated RS, besides bioethanol fermentation with genetically engineered *Klebsiellaoxytoca* P_2, and to establish such a RS biorefinery for decreasing its bioethanol production cost.

2. Materials and Methods

Bioethanol production from lignocellulosic biomass, such as rice straw, includes three main steps: pretreatment, saccharification, and fermentation (Chittibabu ,Ravoof , Pratheepa , & Supassri , 2012).

2.1 Biomass Feedstock (Ricestraw RS)Grinding

Biomass means all organic matter that grows by the photosynthetic conversion of solar energy, consequently biomass in the form of agricultural wastes provides a means of harnessing and storing solar energy. It represents an untapped source of fermentable sugars. Rice straw is an attractive lignocellulosic material for bioethanol production since it is one of the most abundant renewable resources. It has several characteristics that make it a potential feedstock for fuel bioethanol production. It has high cellulose and hemicellulose content that can be readily hydrolysed into fermentable sugars (Binod et al.,2010). There is growing interest in alternative uses of agricultural residues for energy applications. In this context, rice straw would be a potential candidate for future energy needs. Rice Straw wascollected from local crop fields during the harvest season;air dried and grinding into pieces 0.5-1.0 cm and was stored in sealed containers at 4^{o}C until use.

2.2 Characterization of Rice Straw

Agricultural residues are ubiquitous and generated in bulk quantities in developed as well as developing countries. The chemical properties of the components of these lignocellulosic materials make them a substrate of enormous biotechnological value. Cellulose is a polymer composed of chains of six-carbon sugars (primarily glucose). Hemicellulose is a very complex polymer composed of various five- and six-carbon sugars in a highly branched structure. Lignin is a complex,three-dimensional polymer composed of linked six-carbon phenolic rings.Before studying the valorization of agricultural wastes for fermentable sugar and bioethanol production, it is necessary to determine cellulose, hemicellulose, lignin, silica, and ash contents using methods reported byBelkacemi,Turcotte, Halleux, & Savoie, 1996. The chemical composition of agricultural residues varies depending on the growing location, season, and harvesting methods (Agblevor, Batz, Trumbo, 2003).The chemical composition of RS used in this study is shown in Figure1, where RS mainly consisted of: 35.01 % cellulose, 24.30 % hemicellulose, 17.73 % lignin, 11.20 % silica, 8.54 ash, and 3.22 % other extraneous materials. Based on chemical composition analysis, cellulose was the major component, followed by hemicellulose and lignin.

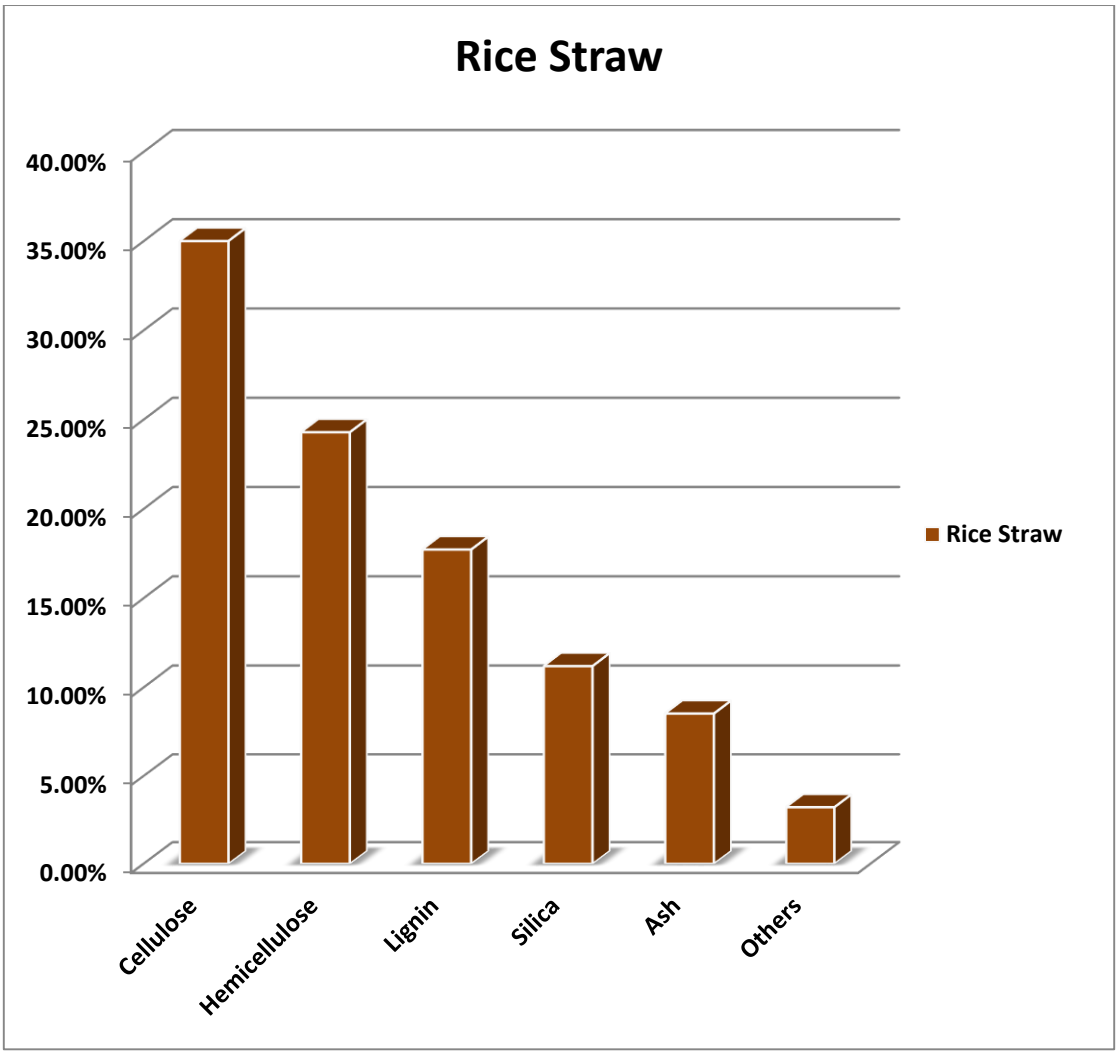

Figure 1: *Composition of Untreated Rice Straw Expressed as Percentage Content of the Main Fractions on a Dry Weight Basis*

2.3 Innovative Pretreatmentbiotechnologies

The conversion of lignocellulosic biomass to bioethanol has many technical and economical challenges. To make the biomass conversion economical, it is necessary to lower the cost of the pretreatment and enzymatic hydrolysis.Optimum pretreatment technique must include: (1) Reduction in cellulose crystallinity. (2)Reduction in lignin content. (3) Increase the porosity of the material, which reflectson increase in surface area.A good pretreatment process must: (1) Be efficient and cost-effective.(2)Improve the formation of fermentable sugars. (3) Avoid the degradation or loss of carbohydrates. (4)Avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes. (5) Pretreated material is most efficiently hydrolyzed using lowenzyme loading. (6) Be environmentfriendly. Up to now, no single pretreatment technique has yet found effective, efficient, and widespread commercial application, i.e. all the previous pretreatment methods

have limitations. Hence, the development of more effective and efficient pretreatment is hindered by: (1) Knowledge about lignocellulosic material structure. (2) Nature of interactions between lignocellulose components and pretreatment chemicals.(3) The factors controlling enzymatic hydrolysis.

A new technique calledMultipurpose Fiber Explosion [MFEX] [Bahaa T.Shawky, Patent ASRT No. 24507 / 2009], has been developed as an effective and efficient pretreatment for increasing cellulose hydrolysis.Among all the previous pretreatments, MFEX process gave the most promising results in terms of the hydrolysis rate and sugar yield. Moreover, it is easy to operate and clean (eco- friendly), i.e. the [MFEX] has been developed and meets all these requirements.

The pretreatment was performed in a 20 L (stainless steel 316) reactor (10L working volume) equipped with a temperature and pressure sensor (Fig. 2). After mixing the grinding rice straw with a suitable moisture, the substrate was treated with steam and carbon dioxide in sequence to make use of the synergistic effects obtained under pressure(10 atm), moderate temperature (80 ^{O}C), and mixing using electric stirrer (100 r.p.m.) for 15 minutes. Nitrogen gas was additionally loaded into the reactor for explosion before the pretreated rice straw process was ended by rapidly releasing the pressure.

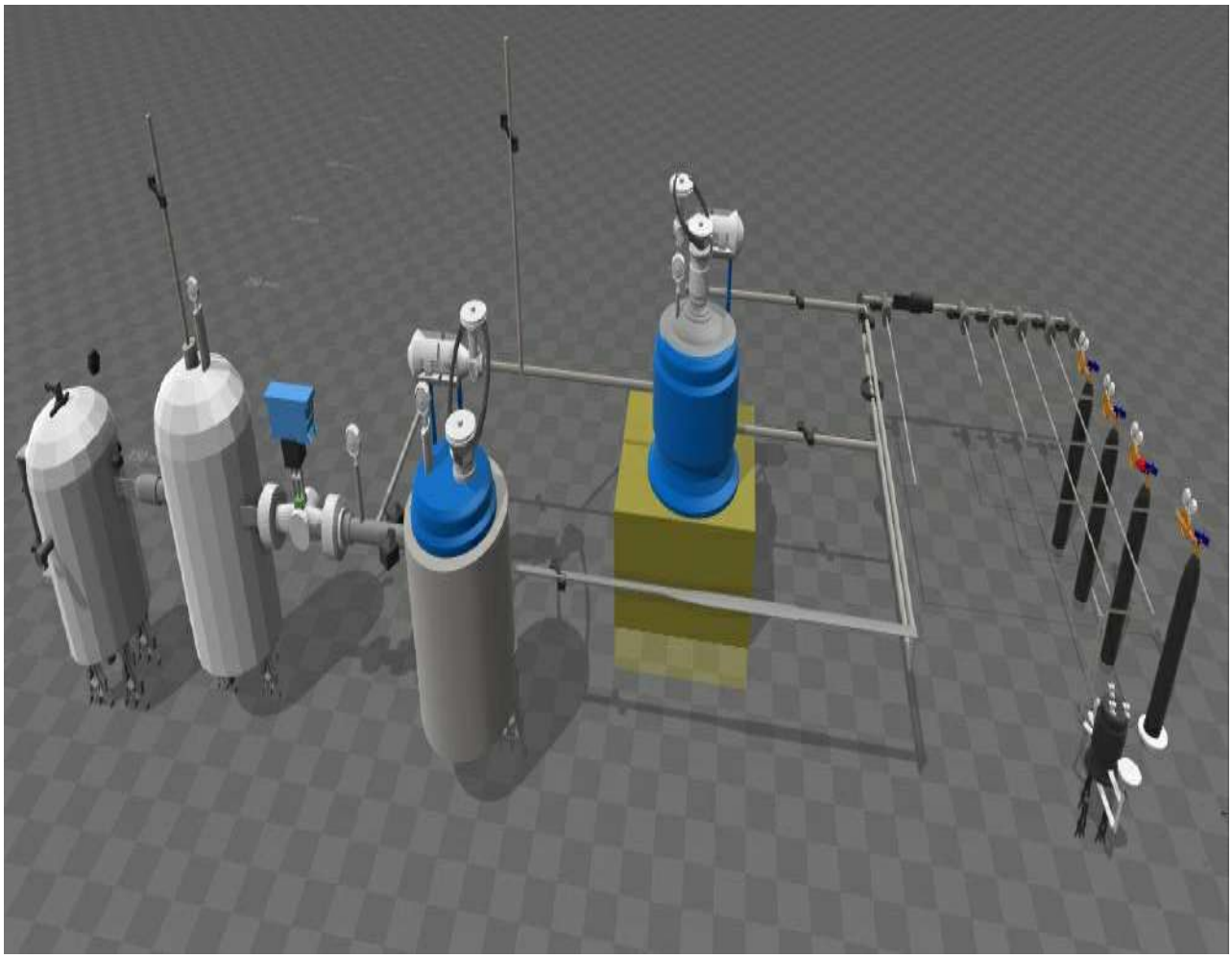

Figure 2: *Prototype: Smart MFEX: Multipurpose Fiber Explosion: As an Effective, Efficient, and Eco-Friendly Pretreatment to Improve the Production of Low-Cost Fermentable Sugars Fromagricultural* **wastes** *(RS) and Bioethanol as Biofuel there from.*

The MFEXpretreatment holds the advantage of being flexible to operate and easy to scale up and it seems to have the potential to be practically applied in large-scale bioethanol production from lignocellulosic wastes. An analysis of the economic benefits of this innovative MFEX model prove its feasibility by using suitable decrystallizing (swelling) agents and agricultural waste as feedstock which will enable a bioethanol producing system to gain a maximum annual profit with an annual return rate. In this study, optimum integration system will be recommended, which take into account for various feedstock, pretreatment processes, and fermentative method to produce high value-added products. Especially thistechnology of biowaste to bioenergy.

2.4 Isolation, Purification, and Preparation of Cellulolytic Microorganismsinocula

Cellulolytic strains were isolated from the samples of rotten rice straw, vegetable decays, and infected hay. The isolation was done by dilution plate method

(Shawky&Hickisch,1984a). The inoculated plates were incubated at 30 ^{o}C for 72 h. The pure cultures were maintained on cellulose agar slants at 4 ^{o}C. For preparation of fungal inocula, either about 2 ml of sterile distilled water containing 0.1% Tween 80 or normal saline was introduced into the sporulated slants of each fungus or the spores were dislodged intothe liquid by gentle pipetting. The spore suspensions containing 10^7 spores/mL were used as inoculums in the subsequent experiments.

2.5 Cellulolytic Enzymes Production under Solid Statefermentation (SSF)

Ten gram of the solid biomass (MFEX-treated RS), taken in 250 mL Erlenmeyer flasks was well moistened with 47.5 mL so as to achieve either mineral salts solution (Shawky&Hickisch, 1984a) or augmented with yeast extract 0.1%,and wheatbran 1.0% (pH 4.8) and autoclaved at 121.5 ^{o}C for 15 min. After cooling, the flasks were inoculated with *Trichodermareesei* BTS1990/or *Aspergillusniger* BTS 2004/or *Phanerochaetechrysosporium* BTS 44(1 x10^7spores/mL) and incubated at 30 ^{o}C for 2, 4, 6 days. Samples were withdrawn as whole flask and the contents were mixed with 100 mL of 50 mM citrate buffer (pH 4.0) on a shaker for 60 min, and then filtered through cheesecloth. The filtrate was centrifuged at 10,000g at 4^{o}C for 20 min and the supernatant was used as crude enzyme extract. All the experiments were carried out in four replicate and the results have been presented as the mean of four.

Enzyme assays and units:

- CarboxymethylCellulase activity (CMC-ase) (Endoglucanase) was estimated using 0.5 mL of 1% carboxymethyl cellulose as substrate (Mandels,Andreotti, & Roche, 1976). One unit of CMC-ase activity released 1 μmol/min of reducing sugar measured as glucose under the standard assay conditions.
- Filter Paper activity (FP-ase) (Exoglucanase) (Cellobiohydrolase) (Avicelase) was determined using Whatman No 1 filter paper strips (3 x 1 cm, 25 mg) as substrate (Mandels, Medeiros, Andreotti, &Bissett, 1981). One filter paper unit (FPU) is that amount of enzyme which yields 1μmol of glucose/min. Units of filter paper activity (FPA) were converted to international units (IU) by using the multiplication factor (1 filter paper unit = 0.185 IU) (Duff, Cooper, & Fuller, 1985,&Ghose, 1987)**.**
- β-Glucosidase activity (β-Gl.) (Cellobiase) (BGL) was measured by using p-nitrophenyl-β-D-glucoside (pNPG) (5mM) as a substrate. One β-Gl. IU is that amount of enzyme, which forms 1 μmolp-nitrophenol/min. (Pal, Banik, Ghorai, Chowdhury, &Khowala, 2010).

- Xylanase activity (X)(1,4-β-D-xylanxylanohydrolase), was determined using birch wood xylan as substrate according to Bailey,Biely, &Poutanen, 1992,using 1% xylan as a substrate. The reducing sugars were measured as xylose equivalents. One X IU is that amount of enzyme, which produces 1 μmol xylose/min.
- ß-Xylosidase (E.C.3.2.1.37) was assayed similarly

Using p-nitrophenyl-d-xylopyranoside (pNPX) as substrate
(Bhattacharyya, Khowala, Kumar, &Sengupta, 1997).

- The reducing sugars in the samples were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Extent of hydrolysis was calculated and expressed as:

Saccharification(%)= reducing sugar / carbohydrates in substrate × 0.9 × 10 (1)

2.6 Enzymatic Saccharification

The enzymatic hydrolysis experiments were carried out in 250 mL screw capped flasks containing 3 g of MFEX-treated RS. The reaction volume was made up to 30 mL with 1 M citrate buffer (pH 4.8) and tween 80 (0.05% w/w) along with the different enzyme combination. The cellulase, ß -glucosidase and xylanase enzymes used were in-houseprepared. The mixtures were incubated at 50 ^{o}C for 48 h with agitation at 200 rpm. Samples were removed after 48 h, centrifuged for 10 min at 10,000g and quantified for the glucose and xylose released. All hydrolysis experiments were carried out using a 10% (w/w) solid loading, in terms of MFEX-treated RS.. All hydrolysis experiments were carried out in triplicate.

2.7 Enzymatic Hydrolysis and Fermentation of MFEX- Treated RS Forbioethanol Production

The enzymatic saccharification is executed which involves the cleaving of polymers of cellulose and hemicelluloses with the help of enzymes. The hydrolysis of cellulose gives rise to glucose while the hemicelluloses releases several pentose and hexose (Taheradeh & Niklasson, 2004).The sugar released during hydrolysis is converted into the bioethanol through fermentation by Simultaneous Saccharification and Fermentation (SSF). The ethanol production depends on percentage of sugar recovery, type of simple sugar (pentose or hexose) and production of inhibitors (Singh et al., 2014). The engineered bacterium *Klebsiellaoxytoca* P_2 was grown at 30 ^{O}C in Luria broth (Luria & Delbrück, 1943). It is well known microorganism for bioethanol production, which produce high bioethanol yields,

about 90% of the theoretical (De La Rosa et al., 1994, Reshamwala, Shawky, & Dale, 1995, Bahaa et al., 1996).

2.8 Statistical Analysis

Obtained data were statistically analyzed according to the method described by Snedecor & Cochran, 1980. All values are means of four replicates. The significance of differences among means was carried out using the least-significant-difference (LSD) test at $p < 5\%$ level.

3. Results and Discussion

Production of bioethanol from biowaste

3.1 Availability of Lignocellulosic residues viz. Rice Straw

In an effort to find sustainable alternative energy sources and to fulfill the ever increasing energy demand. Bioethanol production from RS presents itself as a promising solution.

3.2 Potential of Rice Straw for Bioethanol Production

Bioethanol from biomass has become an increasingly popular alternative to gasoline. However, the first generation biofuels has resulted in an undesirable direct competition with food supply. A switch to second-generation biofuelsshould help to reduce pressure on the food crops. Large parts of these plant materials are made up of complex carbohydrates such as cellulose and hemicelluloses, which can be converted to fermentable sugars. Ethanol fermenting microorganisms can utilize these sugars and convert into bioethanol.

3.3 Importance of Pretreatment

Pretreatment has been viewed as one of the most expensive processing steps in lignocellulosic biomass-to-fermentable sugars conversion (Mosier et al., 2005). A Steam – added CO_2explosion pretreatment was performed for bioethanol production from RS. The pretreatment conditions, such as steam concentration, CO_2 loading level, residence time, mixing, and temperature were optimized using response surface methodology (RSM). The response for optimization was defined as the glucose conversion rate. Thus, the present study suggests that Steam–added CO_2 explosion pretreatment is an appropriate process for bioethanol production from RS. The optimized pretreatment conditions resulting in maximal glucose yield (90 %) were determined.

The present study highlighted the merits of a new and promising pretreatment called Multipurpose Fiber Explosion (MFEX)[Bahaa T. Shawky, Patent ASRT No. 24507 / 2009], which has been developed as an attractive method for pretreatment of agricultural wastes,

yielding high digestible cellulose, where biomass is treated with steam and carbon dioxide in sequence to make use of the synergistic effects obtained under pressure and moderate temperature for a few minutes and then rapidly releasing the pressure.It was obvious that a combination of Steam Fiber Explosion(SFEX) treatment, and Carbon Dioxide Fiber Explosion (CDFX) treatment, was more effective than the individual treatment alone due to synergistic effects. It was hypothesized that the carbon dioxide dissolves in the steam condensate and dissociates to form carbonic acid "mild acid hydrolysis" according to:

$$CO_2 + 2H_2O \; H_3O^+ + HCO_3^- \quad \rightleftharpoons \quad (2)$$

That would increase the auto hydrolysis rate, which makes the MFEX process more effective. Reagent carbon dioxide is known to penetrate the biomass under high pressure, resulting in pore size increase in the lignocellulosic complex (Kim & Hong, 2001).CO2 can be collected during the pretreatment process and recycled for various uses (Quadrelli, Centi, Duplan, & Perathoner, 2011).

3.4 Identification, and Screening of CellulolyticandHemicellulolyticorganisms

The purified fungi were maintained on Shawky&Hickisch, 1984b medium supplemented with 1% (w/v) cellulose powder, ca 20 micron as slants at 30 ^oC for 5 days and stored at 5 ^oC until use and recultured bimonthly. Screening of pure cultures were carried according to the measurement of cellulase and hemicellulase activities.The pure screened isolates were identified, using criteria, based on the Manual of Microbiological Methods, 1957. Of a number of pure selected isolates having relatively high cellulolytic and hemicellulolytic activities, the selection of*Trichodermareesei*BTS 1990, *Aspergillusniger* BTS 2004, and *Phanerochaetechrysosporium* BTS 44 as the most potent cellulolytic fungi have been reported.

Although *Trichodermareesei*BTS 1990is one of the best sources of endoglucanase (CMC-ase) andexoglucanase (FP-ase), its production of β-glucosidase (cellobiase) is low. While, *Aspergillusniger*BTS 2004produces β-glucosidasein large quantities. This observation runs parallel with the findings by Srivastava, Ramachandran, &Gopalkrishnan, 1981 and Schell, McMillan, Philippidis, Hinman, &Riley, 1992. The hydrolytic potential of the cellulase complex produced by *Trichoderma* is, however, greatly enhanced by the addition of supplemental β-glucosidase (Bisset& Sternberg, 1978). Reports have demonstrated the production of the cellulase complex with enhanced β-glucosidase activity in mixed cultures

of *Trichoderma sp.* and *Aspergillus sp.* (Duff et al., 1985). Hence, the mixed cultivation of *Trichoderma sp.* and *Aspergillus sp.* grown in MFEX-treated RS under solid state fermentation for the production of cellulolytic enzymes was studied.

3.5 Laboratory Prepared Cellulases and hemicellulasescocktail

The major bottleneck in biomass to bioethanol conversion is the cost ofcellulase enzymes and any strategy, which can bring down theproduction cost of cellulases can significantly reduce the cost ofbioethanol.Shin, Lee, Lee, & Park, 2000 showed that cellulaseproduction, was the most expensive step during bioethanol production from lignocellulosic biomass, in that it accounted for approximately 40% of the cost. Significant cost reduction is required in order to enhance the commercial viability of cellulase production technology. Solid state fermentation (SSF) is a well established technology for enzyme production and provides several advantages likelower cost of operation, lesser infrastructure requirements, abilityto operate with less skilled manpower and above all the ability touse cheap agricultural residues and biomass as raw materials.

Cellulolytic and hemicellulolytic enzymes for biomass hydrolysis were produced using solid-state fermentation on MFEX-treated RS as substrate. In house crude enzymes were produced using *Trichodermareesei* BTS 1990,*Aspergillusniger* BTS 2004, and *Phanerochaetechrysosporium*BTS 44 . The most crucial enzymes involved in the enzymatic degradation of cellulose and hemicelluloses require the synergistic activity of: Endoglucanase (EC 3. 2. 1. 4), which cleaves the molecule in a random fashion to produce free chain ends. Exoglucanase (EC 3.2. 1. 91), which cleaves cellobiose units from the non reducing chain ends, β – glucosidase(EC 3. 2. 1. 21), which hydrolyses the cellobiose to glucose (Eriksson, 1979).The enzymatic degradation of cellulose and hemicelluloses requires the synergistic activity of at least five crucial enzymes: Endoglucanase (EC 3.2.1.4), Exoglucanase(EC 3.2.1.91), β-glucosidase(EC 3.2.1.21), Xylanase (EC 3.2.1.8), and β-xylosidase (EC 3.2.1.37) (Eriksson, 1979).

MFEX-treated RS was utilized as sole source of carbon by*Trichodermareesei*BTS 1990, *Aspergillusniger* BTS 2004, and *Phanerochaetechrysosporium*BTS 44 for production of enzymes such as (CMC-ase, FP-ase, ß-glucosidase, Xylanase, ß-xylosidase) in solid-state fermentation (SSF) , reached maximum with incubation period (6 days), inoculum size ($3x10^7$ spores/mL), pH (4.8), temperature (30 ^oC).. It was obvious from Tables 1&2that strain *Trichodermareesei* BTS 1990 gave the highest activity of CMC-ase and FP-ase reaching 6.40, 3.60 IU/m L, respectively after 6 days. *Aspergillusniger* BTS 2004 showed maximum yield for β-glucosidase(BGL) production, reaching 7.50 IU/mL after 6 days.

*Phanerochaetechrysosporium*BTS 44 grown under the same culture conditionsgave the highest activity of xylanase and β-xylosidase reaching 39.60, 0.70 IU/m L, respectively after the same period. It was concluded that the cellulolytic and hemicellulolytic activities were greatly affected by the strain specificity. From the results presented in Tables 3 & 4, it can be seen that the production of enzymes were further increased by 2-3 folds on using MFEX-treated RS fortified with 1% wheat bran as carbon source and 0.1 % yeast extract as nitrogen source.Results indicated that favorable fermentation conditions and the selection of a suitable growth substrate played a key role in the production of cellulases from newly isolated microorganisms. Consequently, it was concluded that the cellulolytic and hemicellulolytic activities were greatly affected by the strain specificity as well as the culturing conditions. Data were analyzed by two-way ANOVA. All theenzymes activitywere significantly affected by thestrain specificity as well as the culturing conditions.In particular, the effect ofthe variables' interactions produced significant changes inthe activities of all enzymes.

Higher amounts and different types of enzymes are required to achieve high sugar yields from both cellulose and hemicellulose fractions, thus the enzymatic saccharification of cellulose incur high cost. In this context, development of cellulases and hemicellulases enzymes needed for complete degradation of lignocellulose wastes is an important issue. New balanced enzymatic complexes containing optimal combinations to effectively modify the complex structure of lignocellulosic materials are to be developed (Shawky, Kasulke, Philipp, Schulz & Hirte 1984, and Bahaa, Manal, Eman, Mohsen & Ghada 2011).

Table 1: *Cellulolytic and Hemicellulolytic Activities of Some Local Wildfungi Grown in MFEX-Treated Rice Straw under Solid State Fermentation*

Organisms	Days	Substrate				
		MFEX-treated rice straw				
		CMC-ase (IU/mL)	FP-ase (IU/mL)	ß-Gl (IU/mL)	Xylanase (IU/mL)	ß-Xyl. (IU/mL)
Trichodermareesei BTS 1990	2	4.00^{d}	1.05^{ef}	2.05^{f}	31.20^{f}	0.25^{e}
	4	5.55^{b}	2.10^{c}	2.80^{e}	33.15^{e}	0.38^{d}
	6	6.40^{a}	3.60^{a}	2.90^{de}	36.02^{c}	0.40^{cd}
Aspergillusniger BTS 2004	2	2.05^{f}	0.80^{f}	5.05^{c}	10.80^{h}	0.09^{f}
	4	2.80^{e}	1.25^{de}	6.80^{b}	11.90^{g}	0.35^{d}
	6	3.01^{e}	1.50^{d}	7.50^{a}	12.30^{g}	0.49^{bc}
Phanerochaetechrysosporium BTS 44	2	3.90^{d}	1.17^{e}	2.40^{ef}	34.25^{d}	0.39^{d}
	4	4.88^{c}	2.60^{b}	3.00^{de}	36.80^{b}	0.52^{b}
	6	5.25^{bc}	2.80^{b}	3.86^{d}	39.60^{a}	0.70^{a}

Enzymes activity was replicated four times. Mean values within a column followed by the same letter are not significantly different at *P = 0.05* (least-significant-difference's test)

Table 2: *P-values of the two-way analysis of the activities of cellulolytic and hemicellulolytic enzymes (CMC-ase, FP-ase, ß-glucosidase, Xylanase, and ß-xylosidase) ofsome local wildfungi (Trichodermareesei BTS 1990, Aspergillusniger BTS 2004, and PhanerochaetechrysosporiumBTS 44) grown in MFEX-treated rice straw under solid state fermentation. P-values in bold are considered significant (<0.05, n = 4). 'O': effect of organisms; 'D': effect of days; O x D: effect of the variables' interaction.*

	Main-factor effects		Significant interaction
	O	D	O x D
CMC-ase (IU/mL)	<0.0001	<0.0001	0.0004
FP-ase (IU/mL)	<0.0001	<0.0001	<0.0001
ß-glucosidase (IU/mL)	<0.0001	<0.0001	0.0096
Xylanase (IU/mL)	<0.0001	<0.0001	<0.0001
ß-xylosidase (IU/mL)	<0.0001	<0.0001	0.0085

Table 3: *Cellulolytic and hemicellulolytic activities of some local wildfungi grown in MFEX-treated rice straw supplemented with1% Wheat bran and 0.1%Yeast extract under solid state fermentation*

Organisms	Days	Substrate				
		MFEX-treated rice straw +1% Wheat bran +0.1%Yeast extract				
		CMC-ase (IU/mL)	FP-ase (IU/mL)	ß-Gl (IU/mL)	Xylanase (IU/mL)	ß-Xyl. (IU/mL)
Trichodermareesei BTS 1990	2	12.02^{d}	3.68^{g}	6.34^{g}	52.56^{e}	0.62^{h}
	4	13.56^{b}	5.42^{e}	8.54^{f}	58.19^{d}	0.80^{g}
	6	13.98^{a}	6.01^{d}	8.98^{e}	60.48^{c}	1.10^{e}
Aspergillusniger BTS 2004	2	7.00^{i}	2.87^{h}	14.67^{c}	25.68^{h}	1.02^{f}
	4	7.79^{h}	4.34^{f}	16.79^{b}	32.27^{g}	1.43^{d}
	6	8.75^{g}	5.48^{e}	18.58^{a}	35.58^{f}	1.98^{b}
Phanerochaetechrysosporium BTS 44	2	9.54^{f}	7.5^{c}	5.69^{h}	60.46^{c}	1.78^{c}
	4	10.79^{e}	8.31^{b}	8.58^{f}	63.68^{b}	1.94^{b}
	6	13.24^{c}	9.65^{a}	9.46^{d}	72.19^{a}	2.13^{a}

Enzymes activity was replicated four times. Mean values within a column followed by the same letter are not significantly different at *P = 0.05* (least-significant-difference's test).

Table 4: *P-values of the two-way analysis of the activities of cellulolytic and hemicellulolytic enzymes (CMC-ase, FP-ase, ß-glucosidase, Xylanase, and ß-xylosidase) ofsome local wildfungi (Trichodermareesei BTS 1990, Aspergillusniger BTS 2004, and PhanerochaetechrysosporiumBTS 44) grown in MFEX-treated rice straw supplemented with1% wheat bran and 0.1% yeast extract under solid state fermentation. P-values in bold are considered significant (<0.05, n = 4). 'O': effect of organisms; 'D': effect of days; O x D: effect of the variables' interaction.*

	Main-factor effects		Significant interaction
	O	D	O x D
CMC-ase (IU/mL)	<0.0001	<0.0001	<0.0001
FP-ase (IU/mL)	<0.0001	<0.0001	<0.0001
ß-glucosidase (IU/mL)	<0.0001	<0.0001	<0.0001
Xylanase (IU/mL)	<0.0001	<0.0001	<0.0001
ß-xylosidase (IU/mL)	<0.0001	<0.0001	<0.0001

3.6 Enzymatic Hydrolysis of MFEX-Treated Rice Straw to produce Fermentablesugars

The main hydrolysis product of cellulose is glucose, whereas the hemicellulose gives rise to several pentoses and hexoses (Taherzadeh&Niklasson, 2004). Various factors influencing the yields of the lignocellulose to the monomeric sugars are, e.g. particle size, liquid to solid ratio, temperature, and reaction time. Thesteam–added CO_2 expansion treated RS washydrolyzed using in house prepared enzyme cocktail [CMC-ase, FP-ase, β-glucosidase (BGL), Xylanase, and ß-xylosidase] produced from locally potent cellulolytic organisms, namely *Trichodermareesei* BTS 44, *Aspergillusphoenicis* BTS 90, and *Phanerochaetechrysosporium*BTS 44. It was recognized that the synergistic interaction of endoglucanase, exoglucanase, β-glucosidase, xylanase, and β-xylosidase resulted in efficient hydrolysis of theMFEX-treated RS. A total reducing sugars of 645 mg/g dry MFEX- treated RS was achieved.The results have significant implications regarding to production of fermentable sugars that could be used for bioethanol production as biofuel. Besides thelocally potent cellulolytic organisms could be a potent candidates for the enzyme cocktail preparation for biomass saccharification in lignocellulosic bioethanol program.

3.7 Fermentation

There are many reports stating that the simultaneous saccharification andfermentation (SSF) is superior to the traditional saccharificationand subsequent fermentation in the production of bioethanol fromRS because the SSF process can improve bioethanol yields byremoving end-product inhibition of saccharification process andeliminate the need for separate reactors forsaccharification and fermentation (Chadha,Kanwar, & Garcha, 1995). Hence, the glucose and xylose released during hydrolysis of MFEX- treated

RS can be converted to bioethanol by Simultaneous Saccharification and Fermentation (SSF) processe.

The Simultaneous Saccharification and Fermentation experiments were carried out in pH-controlled, stirred fermenters, using 500 mL fleakers under anaerobic conditions with working volumes of 300 mL. The hydrolysates of the solid fraction resulting of the pretreatment of RS at the optimal conditions were used as substrates, amended with fermentation medium (Wood & Ingram, 1992). The fermentation was carried out using *Klebsiellaoxytoca* P_2 at pH 4.7 and 37 ˚C for 72 h. Samples were taken in sterile vials at various times, placed immediately in an ice bath, and then centrifuged at 10.000g and 4 ºC for 20 min. Supernatants were stored at -20 ºC until required for analysis. Estimation of bioethanol was done by gas chromatography as outlined in NREL Laboratory Analytical Procedures # 011 (David, 1994).

Fermentation efficiency was calculated by the relationship:

$$\text{Practical yield} \;/\; \text{Theoretical yield} \times 100 \qquad (3)$$

A total reducing sugars of 645 mg/g dry MFEX-treated RS was achieved within 24 hours hydrolysis with in-house cellulases. Of this total, 400 mg/g was glucose, which was rapidly fermented within 24 hours by the recombinant *Klebsiellaoxytoca*P2 leading to an ethanol yield of 375 mg/g dry MFEX-treated RS. In fermentation, glucose utilization was rapid, whereas xylose utilization was slow and incomplete.An ethanol yield of 93 % was achieved via simultaneous saccharification and fermentation (Fig. 3).

In addition to glucose, lignocellulosichydrolysates include xylose, which consists of up to 40% of total sugars in xylan-rich biomass such as hardwood (Casey, Sedlak, Ho, & Mosier, 2010). A majority of previous studies on strain co-cultures reported that, while the fermentation of glucose in the sugar mixture proceeded efficiently, the fermentation of xylose was of low efficiency due to the conflicting oxygen requirements between the two strains and/or the catabolite repression on the xylose assimilation caused by the glucose (Ezeonu, 2016). Therefore, many attempts have been devoted to the introduction and optimization of heterologous metabolic pathways for xylose utilization by these organisms. The efficient co-fermentation of glucose and xylose is necessary for the economically feasible bioethanol production from lignocellulosic biomass. In order to develop single strain or di-culture as a more robust bioethanolproducer, this will be improved by further engineering.

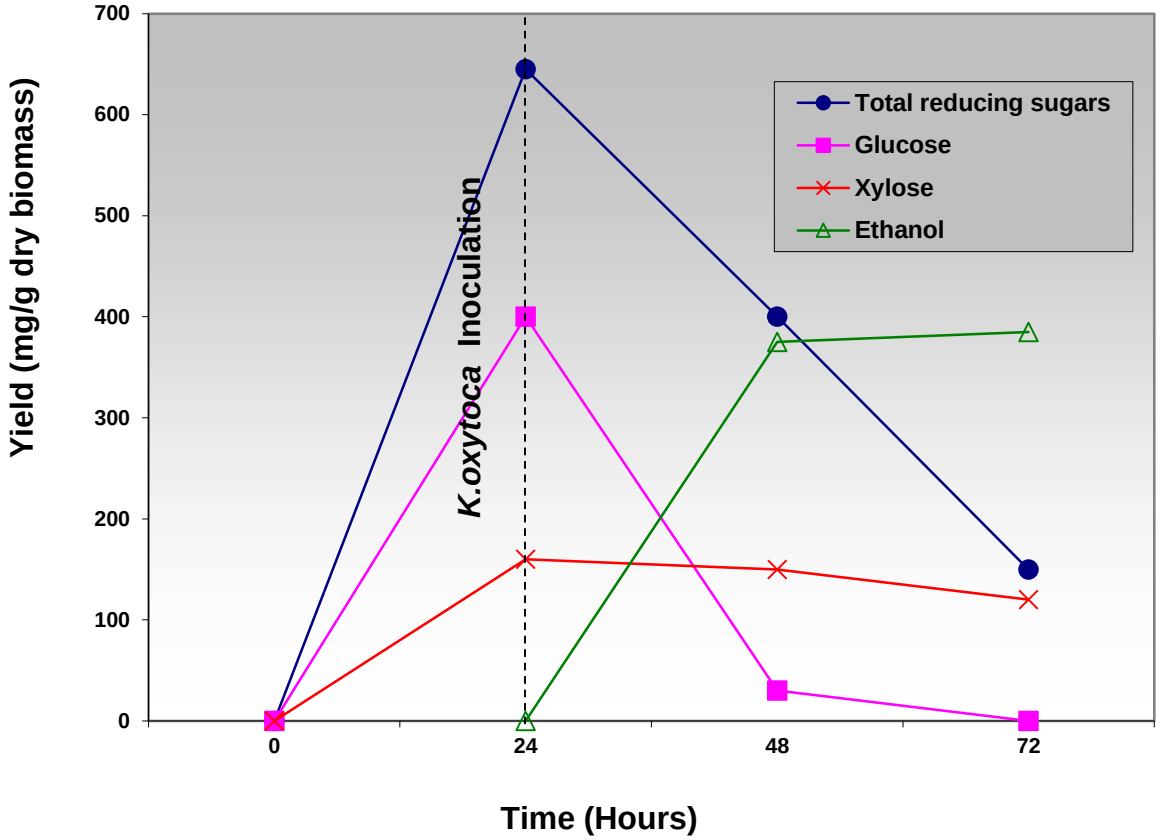

Figure 3: *Total fermentable sugars, glucose, xylose, and bioethanol profiles of MFEX - treated rice straw hydrolyzed by laboratory prepared cellulases cocktail and fermented by Klebsiella oxytoca P2*

This study forms the basic trials conducted to test the feasibility of using laboratory prepared enzymes cocktail for biomass hydrolysis and subsequent bioethanol fermentation. Efforts to optimize the conditions of saccharification and fermentation, as well as attempts will be made to increase the sugar content of the hydrolysate so as to obtain a better yield of bioethanol.Utilization of rice straw for bioethanol not only provides solution for its disposal but, also enhances the socioeconomic status of rural people. The feasibility of technology for convertingbiowaste to bioenergy is promising. The results have significant future applications regarding to provide a sustainable energy system

3. Conclusions

The utilization of lignocellulosic biomass for bioethanol productionnecessitates the production technology to be cost-effective andenvironmentally sustainable. Considering the evolution and need of second generation biofuels, rice straw appears a promising and potent candidate for production of bioethanol due to its abundant, availability and attractive

composition. It can be concluded that rice straw presents a great potential as a biomass for bioethanol production.

Novelpretreatment equipment andpromissing technique calledMultipurpose Fiber Explosion [MFEX][Bahaa T. Shawky, Patent ASRT No. 24507/2009], has been developed as an effective and efficient pretreatment for increasing cellulose hydrolysis. MFEXwas designed, fabricated, and applied toinvestigate a novel two-step RS pretreatment processto improve its bioethanol production economy. In the first step steam added, and in the second step CO_2 loading was employed. This model was verified by enzymatic saccharification, resulting in the glucose yield 90 % recovery from steam-CO_2 expansion treated rice straw.This process can be an example of RS biorefinery forbiofuel production.

In-housecellulases and hemicellulases enzyme cocktail production wereproduced by solid state fermentation (SSF) ontwo different combinations of substrate media were tried using MFEX-treated RSand MFEX-treated RSsupplemented with 1% wheat bran and0.1% yeast extract as a model substrate. In an effort toreduce the cost of bioethanol production. Solid sate fermentation needs lesser infrastructure and relatively less skilled manpower besides being able to use cheaper raw materials for enzyme production. All these add to the economic advantage of this mode of fermentation for enzyme/metabolite production. SSF also produces a more concentrated product, which in this case is very much advantageous.

CMC-ase, FP-ase, ß-glucosidase, Xylanase, ß-xylosidase productionreached maximum, IU/mL (6.40, 3.60, 2.90, 36.02, 0.40) , (3.01, 1.50, 7.50. 12.30, 0.49), and (5.25, 2.80, 3.86, 39.60, 0.70) in case of *Trichodermareesei* BTS 1990 , *Aspergillusniger* BTS 2004, and *Phanerochaetechrysosporium*BTS 44, respectively after incubation period (6 days), inoculum size (3 x 10^7 spores/mL), pH (4.8), temperature (30^{o}C). This in case of MFEX-treated RS as substrate. These figures increased 2-3 fold in case of MFEX-treated RS supplemented with 1% wheat bran and 0.1% yeast extract as fortified substrate. The cocktail containing the five enzymes resulted a maximum of645 mg/g dry treated RS oftotal reducing sugars in biomass enzymatic saccharification step.These results proved that the crude in-house cellulolytic and hemicellulolytic preparation from *Trichodermareesei* BTS 1990 ,*Aspergillusniger* BTS 2004, and *Phanerochaetechrysosporium* BTS 44 could be a potent candidates for the enzyme cocktail preparation for biomass hydrolysis in lignocellulosic bioethanol program.It will be demonstrated that the laboratory enzyme prepared, which containscrude cellulases and BGLpreparations produced through SSF by locally isolated fungi can be used forhydrolysis of biomass with considerable efficiency. The yield ofreducing sugars from pretreated biomass is more than 90% recovery;moreover

thehydrolysate did not contain anyinhibitory compounds as evidenced by the growth and bioethanolproduction on this by *Klebsiellaoxytoca* P_2.

Approaches in both process engineering and strain engineering still have to be carried out to circumvent the difficulties of xylose and glucose co-fermentation and to improve the system efficiency. A very balanced and intelligent combination of pretreatment, hydrolysis and fermentation processes have to be selected for maximum efficacy of the process. With the advent of genetically modified organism, synthetic hydrolyzing enzymes, other sophisticated technologies and their efficient combination, the process of bioethanol production employing rice straw will prove to be a feasible technology.

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