### INTRODUCTION

Fast depletion of conventional energy resources and increasing energy demands diverts the concentration of research towards an alternative energy sources which must be renewable and environmentally friendly. Among various processes, ethanol from lignocellulosic biomass is promising method of alternative energy generation. The lignocellulosic biomass include, trees, shrubs, yard waste, wood products, grasses and agricultural residues such as wheat straw, corn stover, rice straw, cotton stalk etc. (Silverstein et al., 2007). Ethanol production from this agricultural biomass requires series of treatment include pretreatment, hydrolysis and fermentation (Balat et al., 2008). Hydrolysis of biomass by sulfuric acid is well known method to obtain fermentable sugars. However, hydrolyzate obtained contains not only fermentable sugars but also some furans such as furfural and 5-hydroxymethyl furfural which are formed by degradation of sugars and various phenolic compounds. These compounds in the hydrolyzate inhibit the fermentation of sugars by microorganisms. Therefore, for achieving high fermentability detoxification of hydrolyzate is necessary before the fermentation to remove inhibitors (Palmqvist & Hahn-Hagerdal, 2000). Various methods have been studied for improving the fermentability of hydrolyzate including enzyme treatment, overliming, evaporation, extraction with organic solvents, steam stripping, ion exchange, activated carbon treatment etc. (Miyafuji et al., 2003).

In the present study optimization of over liming and charcoal treatment has been carried out on acid hydrolyzate of cotton stalk, which is one of the abundantly available crop residues in India for the purpose to remove maximum inhibitory compounds and to increase the fermentability of hydrolyzate.

### MATERIAL AND METHODS

**Collection of raw material**  
The cotton stalk was collected from farmer’s field in Marathwada region of Maharashtra state, India

**Physical pre-treatment of biomass**  
The cotton stalk was shredded and bailed in the field and was debarked, chopped, dried and ground to pass 1-2 mm sieve in laboratory. Dried sample was stored in sealed plastic bags at room temperature until further use.

**Compositional analysis**  
A major portion of biomass feedstock is made up of carbohydrates, which are polysaccharide in nature and primarily composed of glucose, xylose and arabinose subunits while another major portion is lignin. These sub units (glucose, xylose and arabinose) were quantified by HPLC (Zodiac Ltd) as per NREL protocol of NREL (Ruiz and Ehrman, 1994). The lignin was also determined as per NREL procedure.

### ACID HYDROLYSIS

Acid hydrolysis was carried out in two stages including decrystallization of biomass with 75% H₂SO₄ (Merk Sp. Gr 1.84) at fixed sample acid ratio of 1:2 (by weight) till the color of the paste turned brown without resulting into the oxidation by acid in the first stage followed by dilution of hydrolyzate up to 1 N with distilled water. Finally the hydrolyzate was treated with steam at 121°C for 30 minutes and heated up to four hour at 90°C in water bath (Baig and Dharmadhikari, 2012).

### NEUTRALIZATION AND DETOXIFICATION

After acid hydrolysis, the hydrolyzate was detoxified by over liming and activated charcoal treatment (Sriekha Yadav et al., 2011).

### OPTIMIZATION OF OVER LIMING

The optimization of over liming was carried out by increasing pH from 7.0 to 12.0 separately using calcium oxide (CaO) and keeping it for an hour. The slurry was then filtered to remove precipitation followed by centrifugation (3000g, 20minutes) to remove traces of salt precipitation. Later the pH of hydrolyzate was brought back to pH 6 using dilute H₂SO₄ (Martinez et al., 2000).

### OPTIMIZATION OF ACTIVATED CHARCOAL TREATMENT

In order to obtain the optimization of charcoal treatment, increasing concentration of activated charcoal was added to hydrolyzate from 1% to 5% (w/v) separately along with stirring for half an hour followed by filtration through vacuum filter (Geet et al., 2011).

### ANALYTICAL METHODS

**Total reducing sugars**  
After appropriate dilution the solubilisation of fermentable sugars were determined by DNS (3, 5-dinitrosalicyclic acid) method of Miller (1959).
D- Glucose
Glucose concentration, obtained after every treatment was determined by enzymatic method of glucose oxidase and peroxidase based on Bergmeyer's methods (1972) of enzymatic analysis.

Phenolic compounds
Total phenolic estimation of hydrolyzate was carried out by Folin-Ciocalteu methods (Singleton and Rossi, 1965).

Furans
The bi-product of sugars i.e. furfural and 5-hydroxy methyl furfural was determined the method given by Martinez et al. (2000).

Statistical analysis
Statistical analysis were carried out in factorial completely randomized design (CRD) by software MAUSTAT developed by department of statistics ofVasantrao NaikMarathwada Agriculture University, Parbhani, Maharashtra, India.

RESULTS AND DISCUSSION

Compositional analysis of cotton stalk
The major chemical composition of cotton stalk is cellulose, hemicellulose and lignin but their concentration varied depending on growing location, harvesting methods as well as analysis procedure (Agblevor et al., 2003). Silvertineit al. (2007) from United States found 30% cellulose, 13% hemicellulose and 31% lignin while Ververis et al. (2004) (from Greece) found 40% s-cellulose and 17% lignin. The studies conducted by Binod et al. (2012) showed that the cotton stalk (Gossypium hirsutum) collected from India (Andhra Pradesh) contains 33.3% glycan and 14.8% xylan along with very small proportion of arabinan and manan.

Cotton stalk (Gossypium hirsutum) used in this study collected from Marathwada region (India) was composed of 42% glycan and 22% xylan while other ingredient of hemicellulose was in very small proportion. The lignin content was 24.18%. Comparatively high amount of sugars and less amount of proteins and to increase the fermentability of hydrolysate.

Acid hydrolysis
Acid hydrolysis of biomass was carried out by using H₂SO₄ in two stages including concentrated acid decrystallization followed by dilution up to 1N along with steam at 121°C and heating at 90°C for four hours as was done in previous studies. Hydrolysis process releases 0.494g of sugar per gram of biomass and specifically dextrose concentration was 0.363g/g of biomass along with fermentation inhibitors such as furans (1.971mg/l) and phenolic (4.9g/l). Presence of these inhibitors, thereby becoming more suitable for fermentation.

Effect of activated charcoal
Over liming was not dynamically affected on phenolics as compare to furans there for after over liming, efforts were taken to remove phenolic compounds by exposing it with activated charcoal. Being good adsorbent, activated charcoal treatment can effectively remove phonic compounds from hydrolysate. The amount of phenolic compound in present hydrolysate was higher than expected (4.9 g/L) as compared to previous reports in literature, which might be due to avoiding separate chemical pretreatment for delignification. Thus for optimization of charcoal treatment on cotton stalk hydrolysate after over liming and neutralization were carried out by varying their concentration from 1% to 5% as shown in Table 2 and Fig.2.

From the data it was demonstrated that 4% charcoal treatment was an efficient concentration for maximum reduction in inhibitors including 92.69% furans and 88.89% phenolics while 19.84% total fermentable sugar (in which 15.49% glucose was present) losses were also being reported to 5.48%, which were also reported by Miyafugiet al. (2003) and Martinzet al. (2001).

CONCLUSION
Conclusively, the optimized detoxification studies of cotton stalk hydrolysate (1 N strength) was achieved by overliming up to pH 10 and keep it an hour followed by filtration and brought back to pH 6 using H₂SO₄, later on 4% charcoal treatment for half an hour followed by filtration gives maximum reduction in inhibitors including 92.69% furans and 88.89% phenolics while 19.84% total fermentable sugar (in which 15.49% glucose was present) losses were also be re-
Table 1. The concentration of sugars, furans and phenolics treated with various liming strength.

| Liming with different pH | Fermentable sugar (g/g of biomass) | Glucose (g/g of biomass) | Total furans (mg/lit) | Total phenolics (g/lit) |
|--------------------------|-----------------------------------|-------------------------|----------------------|------------------------|
| Acid hydrolyzate          | 0.494                             | 0.368                   | 1.971                | 4.900                  |
| pH-07                    | 0.461                             | 0.353                   | 1.105                | 4.772                  |
| pH-08                    | 0.453                             | 0.351                   | 0.889                | 4.636                  |
| pH-09                    | 0.435                             | 0.340                   | 0.576                | 4.318                  |
| pH-10                    | 0.422                             | 0.329                   | 0.312                | 4.181                  |
| pH-11                    | 0.404                             | 0.312                   | 0.312                | 4.090                  |
| pH-12                    | 0.369                             | 0.273                   | 0.288                | 4.090                  |
| SEM±                     | 0.013                             | 0.007                   | 0.257                | 0.297                  |
| C.D. at 5%               | 0.041                             | 0.023                   | 0.781                | 0.900                  |

Table 2. Concentration of sugars, furans and phenolics treated with various charcoal concentrations.

| Charcoal treatment      | Fermentable sugar (g/g of biomass) | Glucose (g/g of biomass) | Total furans (mg/lit) | Total phenolics (g/lit) |
|-------------------------|-----------------------------------|-------------------------|----------------------|------------------------|
| Lime treated            | 0.422                             | 0.329                   | 0.312                | 4.181                  |
| 1% charcoal             | 0.416                             | 0.325                   | 0.288                | 2.909                  |
| 2% charcoal             | 0.411                             | 0.316                   | 0.240                | 1.409                  |
| 3% charcoal             | 0.402                             | 0.313                   | 0.168                | 0.863                  |
| 4% charcoal             | 0.396                             | 0.311                   | 0.144                | 0.545                  |
| 5% charcoal             | 0.369                             | 0.281                   | 0.144                | 0.500                  |
| SEM±                    | 0.015                             | 0.010                   | 0.021                | 0.257                  |
| C.D. at 5%              | 0.046                             | 0.032                   | 0.067                | 0.793                  |

Table 3. Concentration of sugars, furans and phenolics after optimized detoxification treatment.

| Analytical Solution     | Total reducing sugars | Glucose | Furans | Phenolics |
|-------------------------|-----------------------|---------|--------|----------|
| Acid hydrolyzate        | 0.494g/g of biomass   | 0.363g/g of biomass | 1.971mg/lit | 4.909g/lit |
| Over limed solution     | 0.422g/g of biomass   | 0.32g/g of biomass  | 0.312mg/lit | 4.181g/lit |
| Charcoal treated        | 0.396g/g of biomass   | 0.311g/g of biomass | 0.144mg/lit | 0.545g/lit |

REFERENCE
1. Agblevor F A, Batz S, Trumbo J, (2003). Composition and ethanol production potential of cotton gin residues. AppBiochemBiotechnol, 105: 219-230.
2. Baig M Z &Dharmadhikari S M, (2012). Biphasic acid treatment of debarked cotton stalk: one novel approach towards bioethanol production. International J Chem Res 04: 83-87.
3. Balat M, Balat, H., Oz C, (2008). Progress in bioethanol processing, Prog Energy Combust Sci, 34: 551-573.
4. Bergmeyer H U, Bernt E, (1969). In Determination with Glucose Oxidase and Peroxidase, Ed.; Methods of Enzymatic Analysis, 2nd Ed. 1206-1212.
5. Binod P, Kuttiraja M, Archana M, Janu K U, Sridhi R, Sukumar R K, Pandey A, (2012). High temperature pre-treatment and hydrolysis of cotton stalk for producing sugars for bioethanol production, Fuel, 92: 340-345.
6. Singleton V L, Rossi J A, (1965). Colorimetry of total phenols with phosphomolybdic-phosphotungstic acid reagents, Am J EnolVitic, 16: 144-158.
7. SrilekhaYadav K, Naseeruddin S, Prashanthi G S, Sateesh L, Rao L, (2011). Bioethanol fermentation of concentrated rice straw hydrolyzate using co-culture of Saccharomyces cerevisiae and Pichia stipites. Bioresour Technol. 102:6473-6478.
8. John, M. Harkin, John W. Rowe, (1971). Bark and its possible uses. U.S.
9. Miller GL, (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar, Anal Chem, 426-429.
10. Muller J E, Winterborn J, York S W, Preston J F, Ingram L O, (2001). Detoxification of dilute acid hydrolyzates of lignocellulose with lime, BiotechnolProg, 17: 287-293.
11. Myers T J, Monier M, Neureiter M, Thomasser C, (2007). A Comparison of chemical pretreatment methods for improving saccharification of cotton stalks, BioresourTechnol, 98: 3000-3011.
12. Palme G, Danner H, Neureiter M, Thomasser C, (2000). Tetrahydrobioethanol fermentation of sugarcane bagasses hydrolyzate improves ethanol production by Candida shehatae NCIM 3501, BioreosrTechnol 98: 1947-1950.
13. Palmqvist E & Hahn-Hagerkal B, (2000). Fermentation of lignocellulosic hydrolyzates. 2: Inhibition and detoxification, BioresourTechnol, 74: 17-24.
14. Ruiz R, Ehrman T, Determination of carbohydrates in biomass by high performance liquid chromatography, In: Laboratory analytical procedure#002, Revision 12/08/1996, National Renewable Energy Laboratory, (1994).
15. Silverstein R A, Sharma – Shivappa R R, Boyette M D & Osborne J, (2007). Colorimetry of total phenols with phosphomolybdic-phosphotungstic acid reagents, Am J EnolVitic, 16: 144-158.
16. Sridh K, Naseeruddin S, Prashanthi G S, Sateesh L, Rao L, (2011). Bioethanol fermentation of concentrated rice straw hydrolyzate using co-culture of Saccharomyces cerevisiae and Pichia stipites. Bioresour Technol. 102:6473-6478.
17. Ververis C, Georgiou K, Christodoulakis N, Santos P, Santos R, (2004). Fiber dimensions, lignin and cellulose content of various plant materials and their suitability for paper production. Ind Crop Prod 192:45-54.