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OPTIMIZATION OF CELLULASE PRODUCTION FROM A NOVEL BACTERIAL ISOLATE *Mesorhizobium sp.* FROM MARINE SOURCE

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Abstract- Cellulases are hydrolytic enzymes capable of degrading Lignocellulosic materials which are used to break up cellulose into glucose or other oligosaccharide compounds. The application of Lignocelluloses for ethanol production from agricultural and industrial wastes has led to extensive studies on Cellulase producing fungi and bacteria. Chemical pre-treatments were more effective than physical pre-treatments for better Cellulase production and activity. The ideal pre-treatment would accomplish reduction in crystallinity, reduction in lignin content and increase in surface area. This work focuses on optimization of physical and chemical parameters for improvement of enzymatic hydrolysis of paper, Eucalyptus, Rice straw and Ragi straw by novel *Mesorhizobium sp.* isolated from marine source. In this study several parameters such as acid and alkali pre-treatment of substrate, different pH, temperature and time duration of incubation and nitrogen sources at varied concentration were optimized and the efficiency of the pre-treatment was checked by determining Cellulase activity. Eucalyptus was the better substrate and showed maximum Cellulase activity followed by rice straw. Cellulase activity was lowest when paper is used as substrate. From the results it can be conclude that pre-treatment is an essential prerequisite to enhance the susceptibility of Lignocellulosic residue for Cellulase activity.

Keywords- *Mesorhizobium,* Cellulase, Optimization**,** pre-treatment**,** Lignocellulosic waste

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Introduction

Lignocellulosic biomass such as agricultural residues has been considered as possible raw material for ethanol production since they have advantages such as renewability, large quantities, low prices, and environmental benefits [1-4]. Plants have cellulose-based rigid cell walls composed of cellulose, hemicelluloses, xylan, and mannan [5-7]. Hydrolysis of Lignocellulosic is slow process because of resistant crystalline structure of cellulose, physical barrier of Lignin surrounding cellulose and limited sites available for enzymatic attack. The purpose of the pre-treatment is to alter the lignocelluloses structure and increase the rate of enzymatic hydrolysis of primarily the cellulose [8,9]. In spite of significant progress in advancing conversion of lignocelluloses to ethanol major barrier to achieve commercialization is the lack of cost effective processes for ethanol production [10,11,12]. Both fungi and bacteria have been exploited for their abilities to produce a wide variety of cellulases and hemicellulases. Most emphasis has been placed on the use of fungi because of their capability to produce abundant amounts of Cellulases; however, the isolation and characterization of novel bacteria capable of producing Cellulase are now becoming widely exploited [13,14]. For economical Lignocellulosic ethanol process requirements include efficient pre-treatment methods of lignocelluloses, availability of low-cost hydrolytic enzymes [15,16]. This study is focused on novel Cellulase producing marine bacteria *Mesorhizobium*. Paper, eucalyptus, rice straw and ragi straw were subjected to various physical and chemical pre-treatment's and evaluated the effect of this pre-treatment's on the Cellulase enzyme activity. This study will help to identify effective pre-treatment and the relative importance of individual pre-treatment on the rate of enzymatic hydrolysis of the Lignocellulosic. Moreover, it will examine how this rare Cellulase can help to overcome some of the major hurdles in the bio-fuel industry.

Materials and Methods

Sample Collection

Different samples from marine environment were collected from the coastal areas of Cuddalore, Pondicherry, Tamilnadu and Mangalore, Karnataka. The samples collected for the present work included sea water samples, wood scrap, rock scrap, algae/ sea weeds and sediment samples. The samples were collected in sterile polythene bags and were preserved in refrigerator for further studies.

Microorganism and Media

Standard microbiological methods were followed for the purpose of

isolation. (Brown, 1985) One milliliter of the desired dilution was transferred aseptically into Nutrient Agar (supplemented with sea water)/Marine agar plates. Plates were incubated at room temperature 28ºC for 24-48 hrs. The bacterial colonies which were seen after incubation were individually cultured on sterile Nutrient Agar prepared in sea water and maintained as pure cultures.

The isolated organisms were subject to screening for cellulase activity on CMC agar to check for degradation of cellulose. The isolates which exhibited maximal zone of degradation were chosen as the organisms for the further study.

Identification

The Cellulase producing experimental organism isolated from marine source was identified based on the morphological, biochemical characterization and were subjected to molecular analysis for genus and species level identification. The isolated organism was sent to Bioserve Hyderabad, India for 16SrDNA sequencing.

Substrate Preparation

5 gm of the different substrate paper, Eucalyptus, Rice straw and Ragi straw were cut into small pieces and added into conical flask containing 100 ml of water. The flasks were autoclaved at 121^0C for 15 minutes at 15 lbs. Once the flask had cooled, they were inoculated with *Mesorhizobium*. The readings were taken at start and after every 7^{th} day for 8 consecutive weeks for reducing sugar estimation using DNS.

Optimization of pH

Optimization was carried out by adjusting the pH ranges from 4, 5, 6, 7, 8 and 9 of the substrate. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. After inoculation with *Mesorhizobium* the flasks were kept for incubation at room temp. The readings were taken at start and after every 7^{th} day for 8 consecutive weeks for reducing sugar estimation using DNS.

Optimization of Temperature for Growth

Optimization of temperature was carried out by incubating the substrate medium inoculated with *Mesorhizobium* at 25°C, 30°C, 37°C and 40°C. The readings were taken at the start and after every 7 days for 8 consecutive weeks for reducing sugar estimation using DNS.

Optimization of Pretreatment with Acid

Substrate was added into conical flask containing 100ml of different concentrations of acid (0. 1%, 0.3%, 0.5%, 0.7%, 0.9% and 1%). The flasks were left at room temperature for 24 hours. The substrates were then neutralized with NaOH and auto autoclaved at 121^0C for 15 minutes at 15 lbs. Once the flasks had cooled, they were inoculated with *Mesorhizobium*. The readings were taken at the beginning and after every 7 days for 8 consecutive weeks for reducing sugar estimation using DNS.

Optimization of Pretreatment with Alkali

Substrate was added into conical flasks containing 100ml of different concentrations of alkali (0. 1%, 0.3%, 0.5%, 0.7%, 0.9% and 1%).The flasks were left at room temperature for 24 hours. The substrates were then neutralized and autoclaved at 121^0C for 15 minutes at 15 lbs. Once the flasks had cooled, they were inoculated with *Mesorhizobium*. The readings were taken at beginning and after every 7 days for 8 consecutive weeks for reducing sugar estimation using DNS.

Optimization of Treatment Temperature

Substrate was added into conical flasks containing 100ml of water and kept at different temperatures (100^0C, 150^0C and 200^0C) for 1hr in hot air oven. Once the flasks had cooled, they were inoculated with *Mesorhizobium.* The readings were taken at start and after every 7 days for 8 consecutive weeks for reducing sugar estimation using DNS.

Optimization of Nitrogen Source

Different nitrogen source such as Ammonium Nitrate, Ammonium Sulphate, Ammonium Phosphate, Sodium Nitrate and Urea were used for optimization. Nitrogen source was added into conical flasks containing 100ml water with different concentrations (0.5%, 1.0%, 1.5% and 2.0%) of nitrogen source. The flasks were autoclaved at 121^0C for 15 minutes at 15 lbs. Once the flasks had cooled, they were inoculated with *Mesorhizobium*. The readings were taken at start and after every 7^{th} day for 8 consecutive weeks for reducing sugar estimation using DNS.

Cellulase Assay

Cellulase activity was determined by using carboxymethyl cellulose as a substrate. A reactive mixture contains 0.5 ml of 1% (w/v) substrate in 0.1 M citrate buffer (pH 4.8) and 0.5 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The reducing sugar released was measured using DNS. One unit of enzyme activity was expressed as the amount of enzyme required to release 1 µmol reducing sugars per ml under the above assay condition.

Results

Micro-organism was isolated from marine source and screened for Cellulase production using CMC assay. The isolated Cellulase producing organism was identified by both morphological and biochemical characterization as well as 16SrDNA sequencing. The organism was identified as *Mesorhizobium sp.* by sequencing and comparative sequence alignment analysis [Table-1], [Fig-1] and [Fig-2]. The identified *Mesorhizobium sp* was further studied for Cellulase production and activity at various physical and chemical parameters.

Table 1- 16S rDNA Sequence of sample

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1 acggctaccy tgttacgact tcaccccagt cgctgagcct accgtggtca gctgcctcct
61 tgcggttagc gcactgcctt cgggtaaacc caactcccat ggtgtgacgg gcggtgtgta
121 caaggcccgg gaacgtattc accgcggcat gctgatccgc gattactagc gattccaact
181 tcatggggtc gagttgcaga ccccaatccg aactgagatg gcttttggag attagctcga
241 cgttgccgtc tcgctgccca ctgtcaccac cattgtagca cgtgtgtagc ccagcccgta
301 agggccatga ggacttgacg tcatccccac cttcctctcg gcttatcacc ggcagtcccc
361 ttagagtgcc caacttaagg ctggcaacta agggcgaggg ttgcgctcgt tgcgggactt
421 aacccaacat ctcacgacac gagctgacga cagccatgca gcacctgtct tgggtccagc
481 ctaactgaag gttaccgtct ccggtaaccg cgacccagat gtcaagggct ggtaaggttc
541 tgcgcgttgc ttcgaattaa accacatgct ccaccgcttg tgcgggcccc cgtcaattcc
601 tttgagtttt aatcttgcga ccgtactccc caggcgggaa gcttaatgcg ttaactgcgc
661 caccgacagg taaacctgcc aacggctagc ttccatcgtt tacggcgtgg actaccaggg
721 tatctaatcc tgtttgctcc ccacgctttc gcacctcagc gtcagtatcg agccagtgag
781 ccgccttcgc cactggtgtt cctccgaata tctacgaatt tcacctctac actcggaatt
841 ccactcacct ctctcgaact ctagatcggc agtattagag gcagttccgg ggttgagccc
901 cgggwtttca cccctaactg accgatccgc ctacgcgcgc tttacgccca gtaattccga
961 acaacgctag ccccttcgt attaccgcgg ctgctggcac gaagttagcc ggggcttctt
1021 ctccggttac cgtcattatc ttcaccggtg aaagagcttt acaaccctag ggccttcatc
1081 actcacgcgg catggctgga tcagggttgc ccccattgtc caatattccc cactgctgcc
1141 tcccgtagga gtctgggccg tgtctcagtc ccagtgtggc tgrtcatcct ctcagaccag
1201 ctactgatcg tcgccttggt aggyctttac cccaccaact agctaatcag acatgggctc
1261 atccaactcc gataaatctt tcccccgaag ggcgcatacg gtattagttc aagtttccct
1321 gagttattcc gtagagctgg gtagattccc atgcattact cacccgtctg ccgctccccc
1381 gaaggggcgc tcgacttgca tgtgttaagc ctgccgccag cgttcgttct gagccawgat
1441 caaactctt
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Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU937747.1	Mesorhizobium sp. JG 04 16S ribosomal RNA gene, partial sequence	2594	2594	99%	0.0	99%
DQ416795.1	Mesorhizobium sp. G2DM-29 16S ribosomal RNA gene, partial sequen	2545	2545	99%	0.0	98%
DQ659444.1	Mesorhizobium sp. W6-20 16S ribosomal RNA gene, partial sequence	2542	2542	98%	0.0	99%
EU440986.1	Nitratireductor aquibiodomus strain PR57-9 16S ribosomal RNA gene,	2526	2526	99%	0.0	98%
AB098586.1	Mesorhizobium sp. TUT1018 gene for 16S rRNA, partial sequence	2516	2516	99%	0.0	98%
NR_025262.1	Nitratireductor aquibiodomus strain NL21 16S ribosomal RNA, partial s	2507	2507	99%	0.0	98%
JF263531.1	Alpha proteobacterium 44B9 16S ribosomal RNA gene, partial sequen	2502	2502	99%	0.0	98%
DQ659452.1	Pseudaminobacter sp. W11-4 16S ribosomal RNA gene, partial sequer	2443	2443	98%	0.0	97%
EU874874.1	Mesorhizobium sp. W17 16S ribosomal RNA gene, partial sequence	2432	2432	94%	0.0	99%
HQ176466.1	Nitratireductor sp. CL-SC22 16S ribosomal RNA gene, partial sequenc	2425	2425	97%	0.0	97%

Fig. 1- Top 10 sequencing producing Significant Alignments

Fig. 2- Phylogenetic tree for Sample ACT

Optimization was carried out for pH ranges from 4, 5, 6, 7, 8 and 9. The highest Cellulase activity (3.633 units /ml) was observed for eucalyptus substrate at pH 9 [Fig-3], [Fig-4], [Fig-5] and [Fig-5]. Cellulase activity was calculated at different temperatures ranging from 25°C to 40°C. Maximum Cellulase activity (3.670 units /ml) at 40°C was observed for eucalyptus substrate [Fig-7], [Fig-8], [Fig-9] and [Fig-10]. Cellulase activity was calculated after treating substrate with acid (0. 1%, 0.3%, 0.5%, 0.7%, 0.9% and 1%). Maximum Cellulase activity (2.351 units /ml) was observed at 1 % acid treatment for eucalyptus substrate [Fig-11], [Fig-12], [Fig-13] and [Fig-14]. Cellulase activity was calculated after treating substrate with alkali (0. 1%, 0.3%, 0.5%, 0.7%, 0.9% and 1%). Maximum Cellulase activity (2.381 units /ml) was observed at 0.9% alkali treatment for eucalyptus substrate [Fig-15], [Fig-16], [Fig-17] and [Fig-18]. Cellulase activity was calculated at different temperatures treatment of substrate at 100°C, 150°C and 200°C. Maximum Cellulase activity (2.277 units /ml) was observed when eucalyptus was treated at 200°C for 1 hr. [Fig-19], [Fig-20], [Fig-21] and [Fig-22].

Different nitrogen sources at different concentration were used and Cellulase activity was determined. Maximum Cellulase activity (2.247 units /ml) was observed with 1.5 % ammonium nitrate for eucalyptus substrate [Fig-23], [Fig-24], [Fig-25] and [Fig-26]. Maximum Cellulase activity (1.403 units /ml) was observed with 2 % ammonium sulphate for eucalyptus substrate [Fig-27], [Fig-28], [Fig-29] and [Fig-30]. Maximum Cellulase activity (0.560 units /ml) was observed with 2% urea for rice straw substrate [Fig-31], [Fig-32], [Fig-33] and [Fig-34]. Maximum Cellulase activity (0.973 units /ml) was observed with 2% ammonium phosphate for eucalyptus substrate [Fig-35], [Fig-36], [Fig-37] and [Fig-38]. Maximum Cellulase activity (0.820 units /ml) was observed with 0.5 % sodium nitrate for rice straw substrate [Fig-39], [Fig-40], [Fig-41] and [Fig-42].

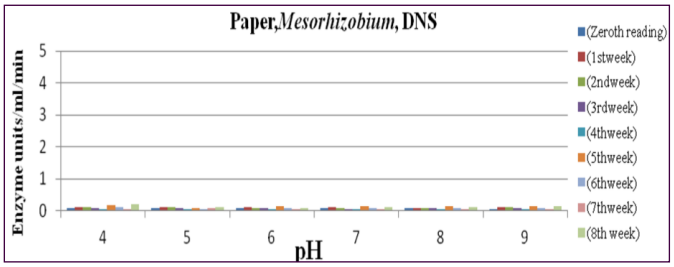

Fig. 3- Optimization for pH for paper substrate

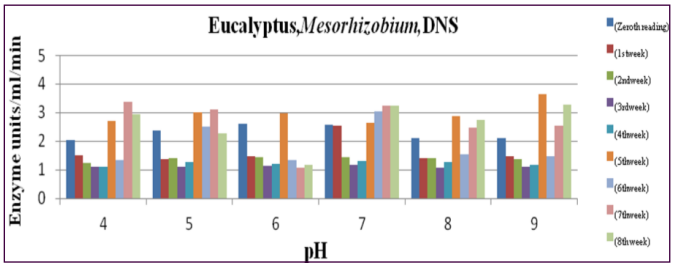

Fig. 4- Optimization for pH for Eucalyptus substrate

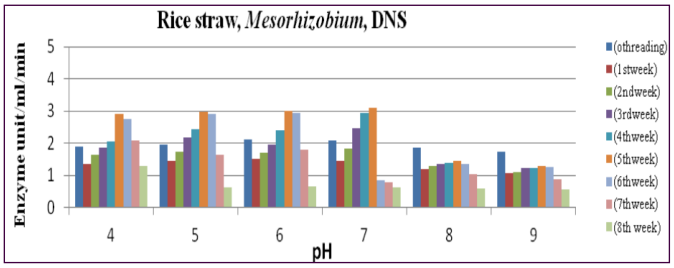

Fig. 5- Optimization for pH for Rice straw substrate

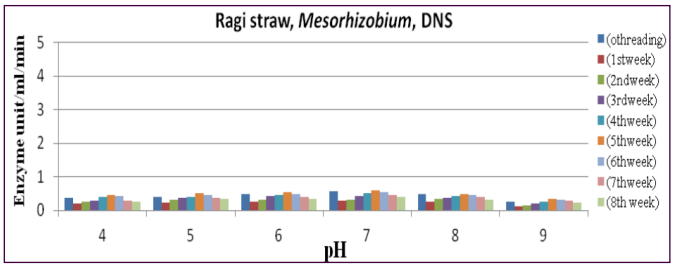

Fig. 6- Optimization for pH for Ragi straw substrate

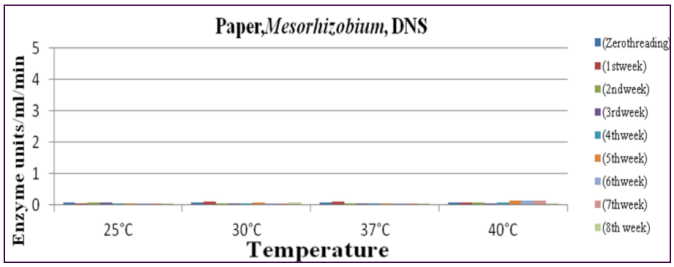

Fig. 7- Optimization for temperature for paper substrate

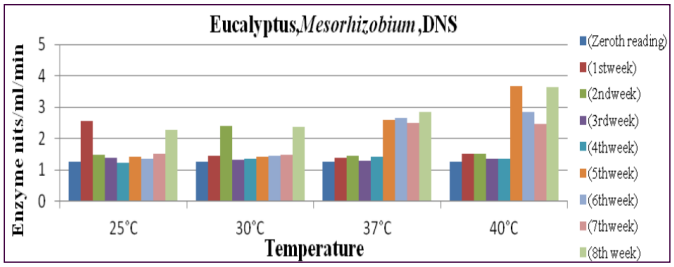

Fig. 8- Optimization for temperature for Eucalyptus substrate

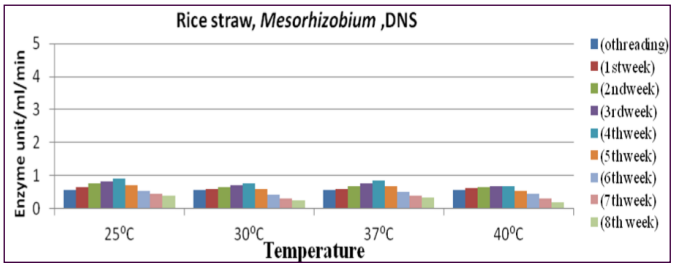

Fig. 9- Optimization for temperature for Rice straw substrate

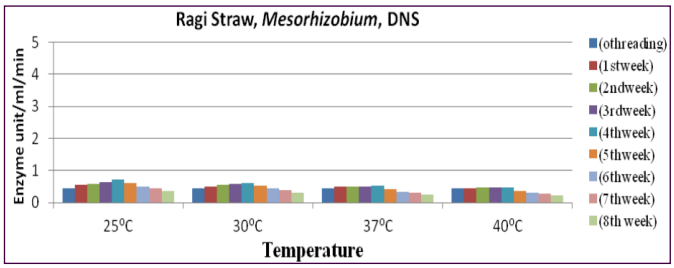

Fig. 10- Optimization for temperature for Ragi straw substrate

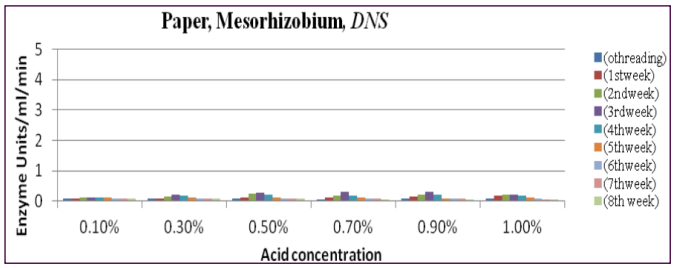

Fig. 11- Optimization for acid treatment for paper substrate

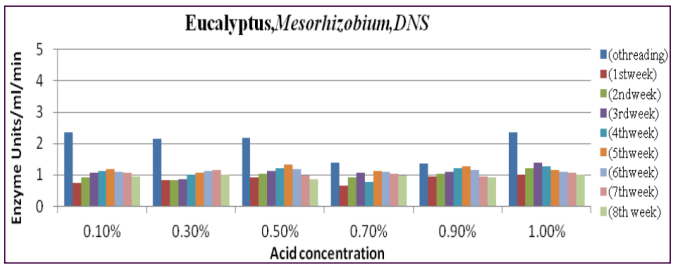

Fig. 12- Optimization for acid treatment for Eucalyptus substrate

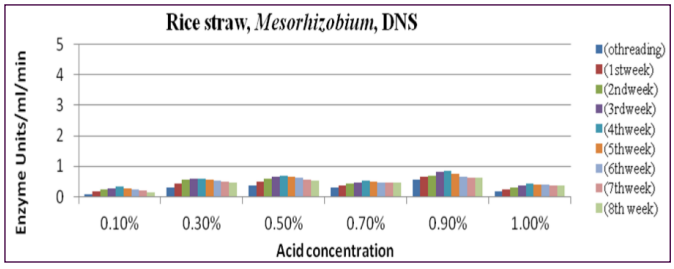

Fig. 13- Optimization for acid treatment for rice straw substrate

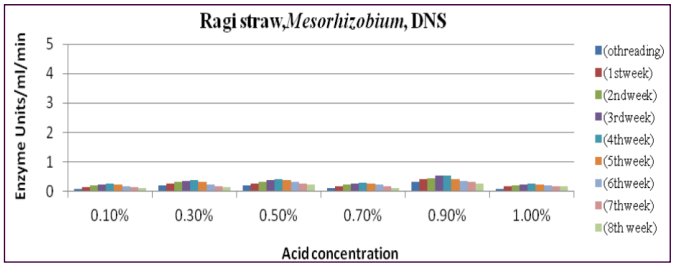

Fig. 14- Optimization for acid treatment for ragi straw substrate

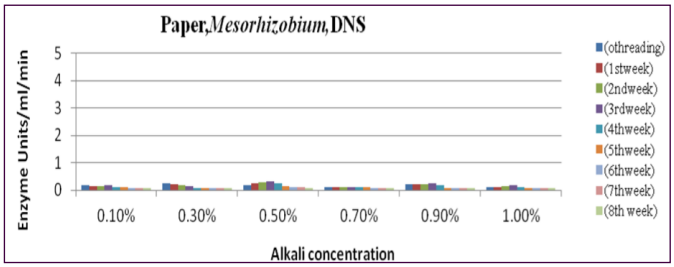

Fig. 15- Optimization for alkali treatment for paper substrate

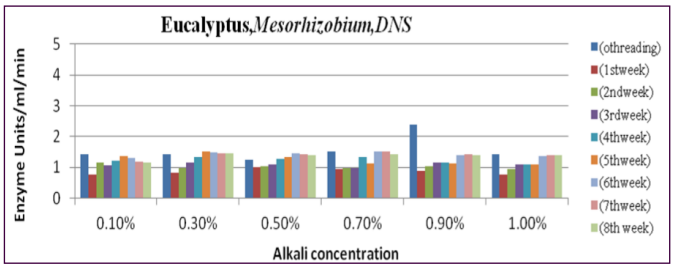

Fig. 16- Optimization for alkali treatment for eucalyptus substrate

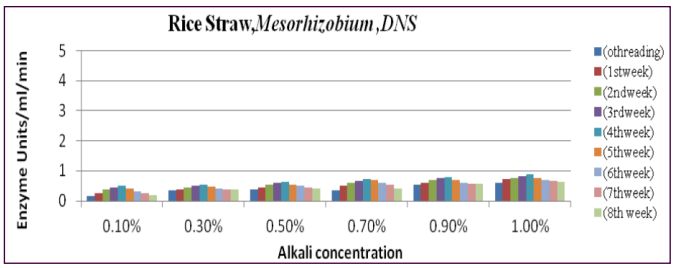

Fig. 17- Optimization for alkali treatment for rice straw substrate

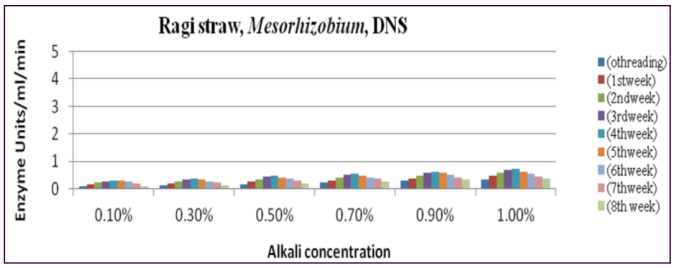

Fig. 18- Optimization for alkali treatment for ragi straw substrate

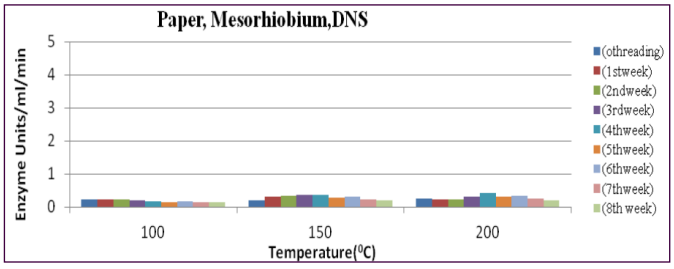

Fig. 19- Optimization for temp. treatment for paper substrate

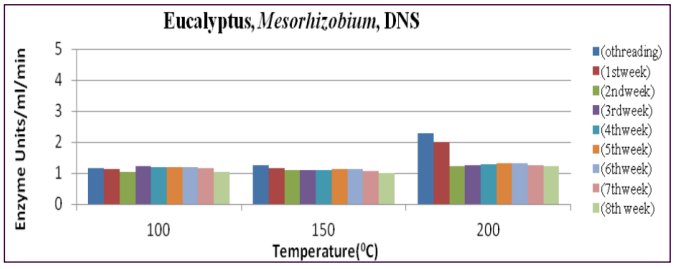

Fig. 20- Optimization for temp. treatment for eucalyptus substrate

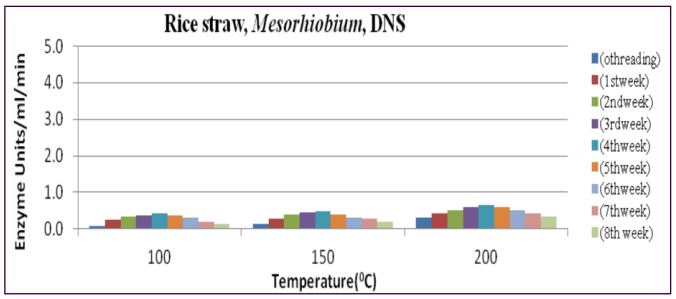

Fig. 21- Optimization for temperature treatment for rice straw substrate

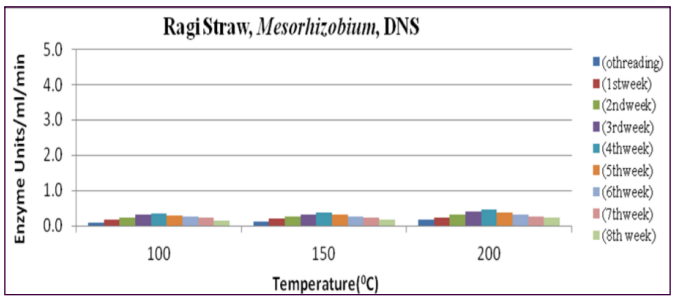

Fig. 22- Optimization for temperature treatment for ragi straw substrate

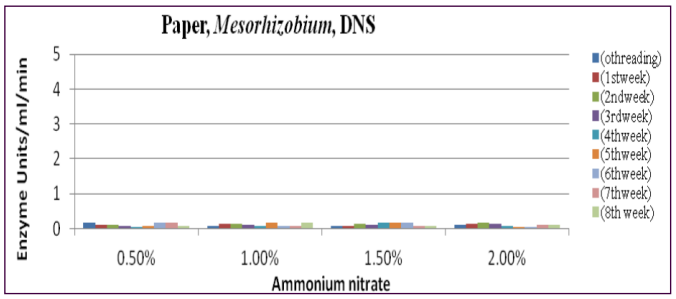

Fig. 23- Optimization for nitrogen source (Ammonium Nitrate) for paper substrate

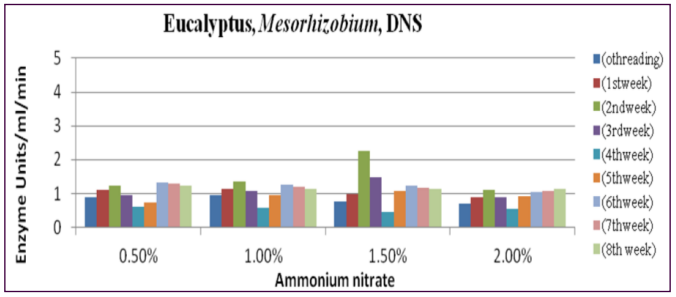

Fig. 24- Optimization for nitrogen source (Ammonium Nitrate) for eucalyptus substrate

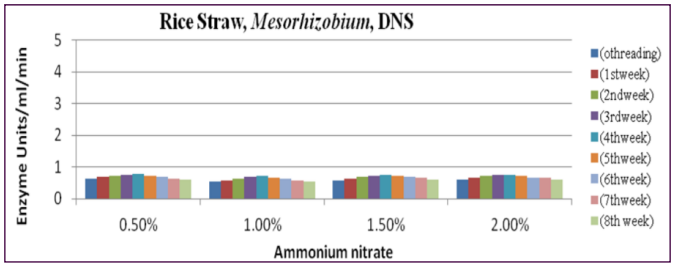

Fig. 25- Optimization for nitrogen source (Ammonium Nitrate) for rice straw substrate

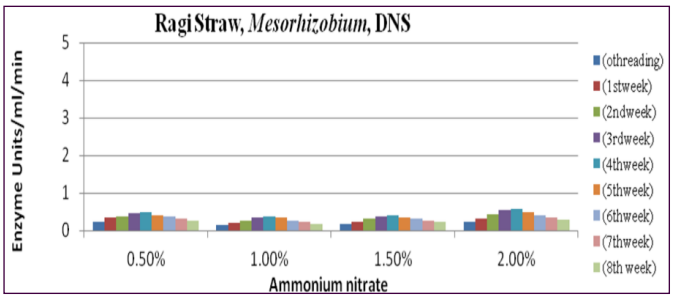

Fig. 26- Optimization for nitrogen source (Ammonium Nitrate) for ragi straw substrate

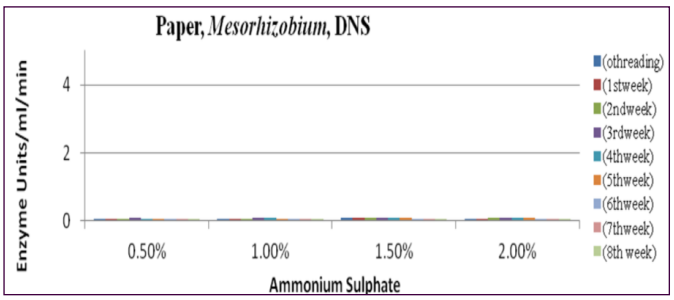

Fig. 27- Optimization for nitrogen source (Ammonium Sulphate) for paper substrate

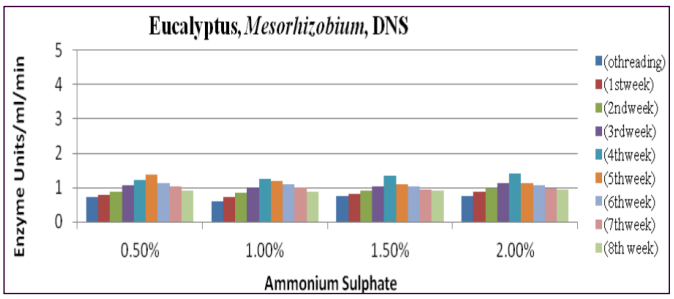

Fig. 28- Optimization for nitrogen source (Ammonium Sulphate) for eucalyptus substrate

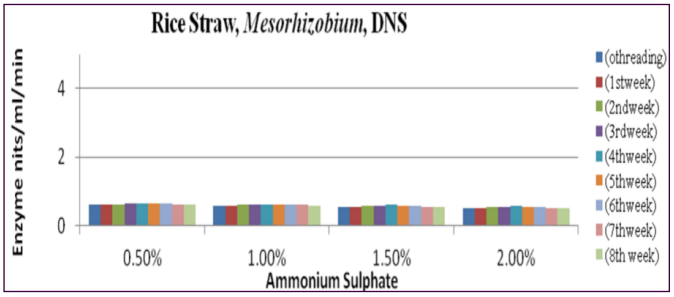

Fig. 29- Optimization for nitrogen source (Ammonium Sulphate) for rice straw substrate

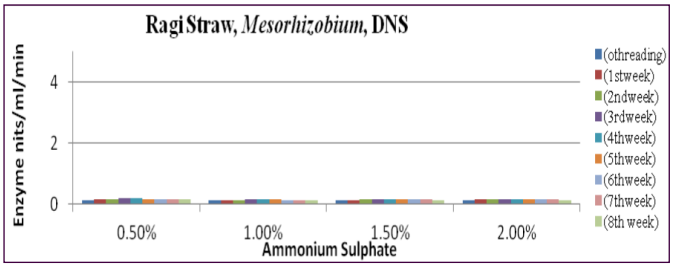

Fig. 30- Optimization for nitrogen source (Ammonium Sulphate) for ragi straw substrate

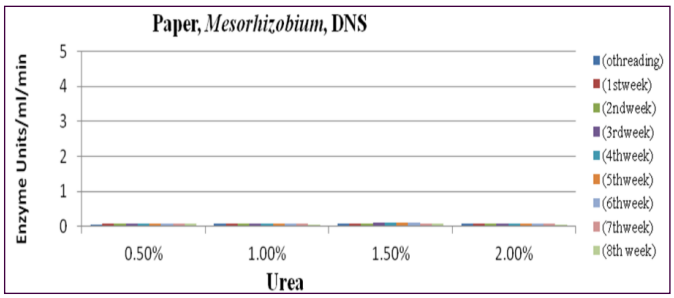

Fig. 31- Optimization for nitrogen source (Urea) for paper substrate

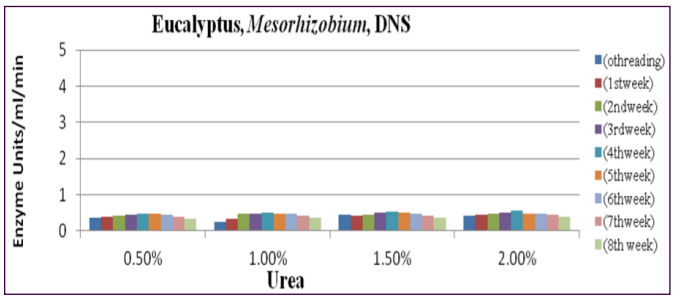

Fig. 32- Optimization for nitrogen source (Urea) for eucalyptus substrate

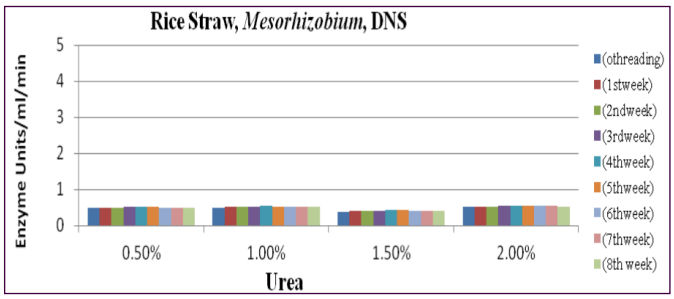

Fig. 33- Optimization for nitrogen source (Urea) for rice straw substrate

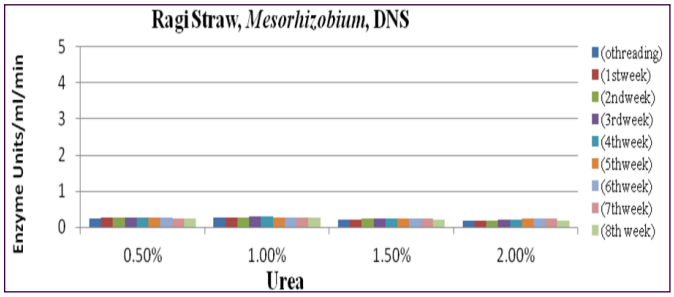

Fig. 34- Optimization for nitrogen source (Urea) for ragi straw substrate

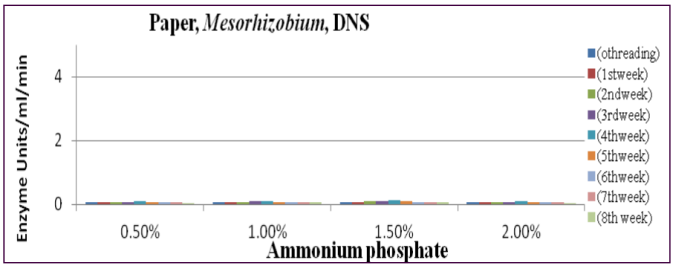

Fig. 35- Optimization for nitrogen source (Ammonium Phosphate) for paper substrate

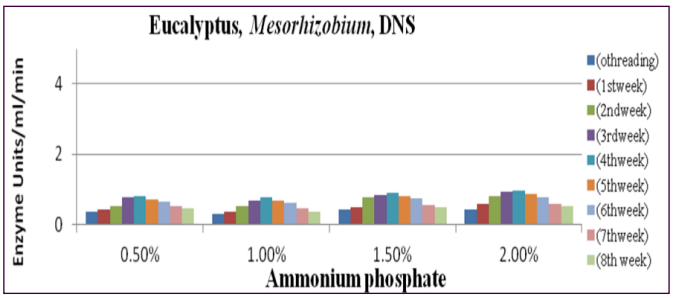

Fig. 36- Optimization for nitrogen source (Ammonium Phosphate) for eucalyptus substrate

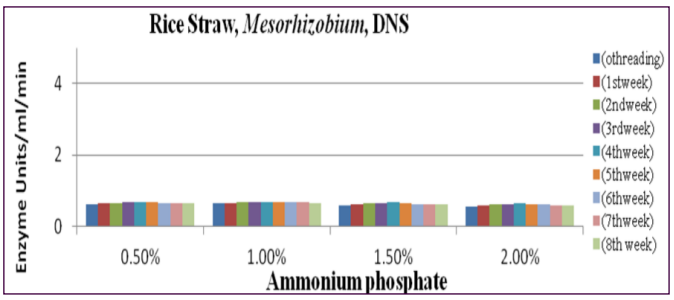

Fig. 37- Optimization for nitrogen source (Ammonium Phosphate) for rice straw substrate

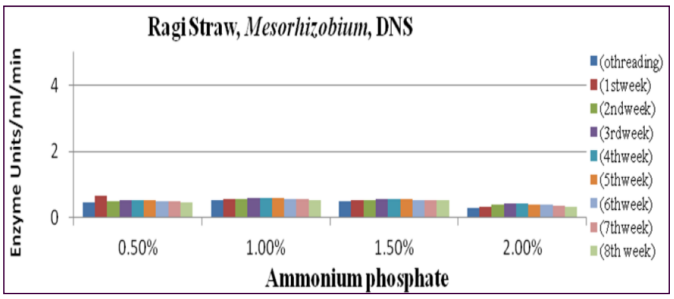

Fig. 38- Optimization for nitrogen source (Ammonium Phosphate) for ragi straw substrate

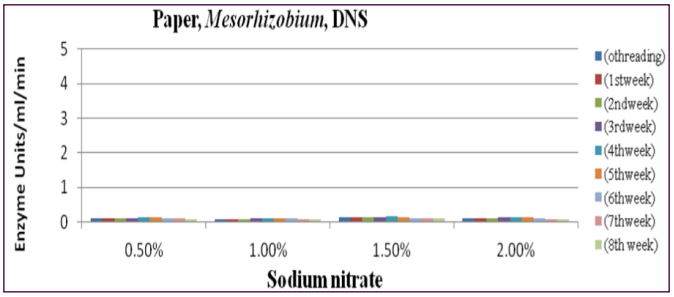

Fig. 39- Optimization for nitrogen source (Sodium Nitrate) for paper substrate

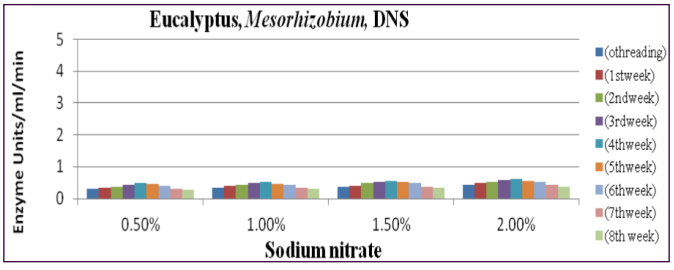

Fig. 40- Optimization for nitrogen source (Sodium Nitrate) for eucalyptus substrate

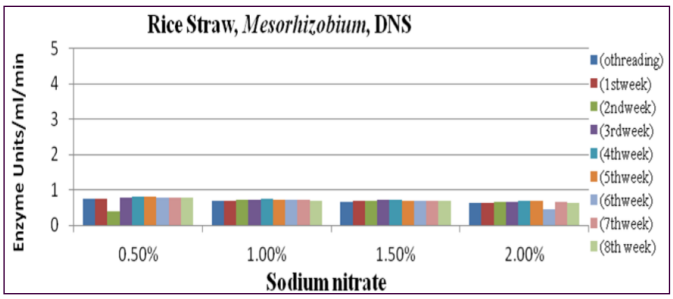

Fig. 41- Optimization for nitrogen source (Sodium Nitrate) for rice straw substrate

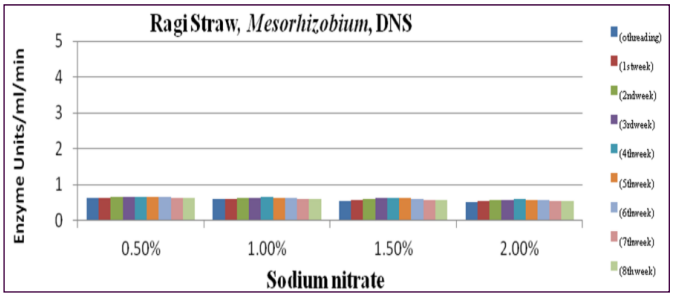

Fig. 42- Optimization for nitrogen source (Sodium Nitrate) for ragi straw substrate

Discussion

In this study optimization was done for Cellulase production by *Mesorhizobium* isolated from marine source. Among the substrates used, Eucalyptus was found to be the better substrate followed by rice straw, whereas Cellulase activity was lowest when paper was used as substrate. The difference in the Cellulase enzymes activity on a variety of lignocelluloses depends on various factors such as variable cellulose content in lignocellulose derived from different plant sources, heterogeneity of structure and cellulolytic abilities of the organisms. Better Cellulase production was observed in the range of pH 5-9 for Eucalyptus and pH 4-7 for Rice straw substrate. Cellulase activity was highest at 40^0C and similar pattern of enzyme production and productivity profiles were exhibited for all the different incubation temperatures used ranges from 25-40°C. Acid and alkali treatment increased the Cellulase activity and maximum activity was observed when 0.9 %- 1 % acid or alkali was added. Maximum Cellulase activity was observed when substrate was treated at 200 °C for 1 hr. Ammonium nitrate was found to be the better nitrogen source for Cellulase activity compared to other nitrogen sources. Thus, pre-treatment is an essential prerequisite to enhance the Cellulase production and activity. The data obtained in this study can be used to model the process parameters to make economical evaluations of the ethanol production process.

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