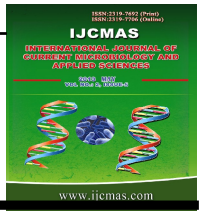
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 5 (2013) pp. 67-74**
http://www.ijcmas.com



Original Research Article

Biofuel Production using Marine Microbes

V.Kalaiselvi*, S.Jayalakshmi and R. Lakshmi narayanan

CAS in Marine Biology, Annamalai University, Parangipettai-608 502, Tamilnadu, India.

**Corresponding author e-mail: micro_kalai@yahoo.co.in*

Keywords

Biofuel production; *Klebsiella ozeanae;* *Pseudomonas aeruginosa;* SDS- PAGE; wood and agricultural residues.

A B S T R A C T

In the present study Biofuel production, carried out from wood powder and agricultural residue as substrates using marine microbes and marine yeast. Among 35 cellulose degradation bacteria isolated from water and sediment samples; *Klebsiella ozeanae*, and *Pseudomonas aeruginosa* created largest zone of lyses. Hence, selected for further production, optimization; the optimized parameters for *Klebsiella ozeanae* (incubation period 30hr, temperature 35°C, pH 7, salt 3%, nitrogen source: yeast extract and carbon source: cellulose) and for the incubation period of 36hr, temperature 35°C, pH 8, salt 2%,nitrogen sources yeast extract carbon source cellulose were found to be ideal for *Pseudomonas aeruginosa*. The enzyme thus produced, partially purified with ammonium sulphate. The SDS PAGE results were of 32 kDa protein. The biofuel (ethanol) production projected using cheaper sources and agricultural residues such as wood powder and corn stalk, paddy straw, ragi stalk, millet stalk and sugarcane stalk. The ideal strains were tried as a consortium, 11.99 % of ethanol content observed. When Wood and agricultural residues used with the consortium of bacteria, the yeast yielded 11.0% of ethanol.

Introduction

One of the greatest challenges, world in the 21st century is to meet the growing demand of energy for transportation, heating and industrial processes and to provide raw material for the industry in a sustainable way. Ethanol satisfies the requirement ever since its production and ignition do not contribute significantly to the total amount of carbon dioxide in the atmosphere (Nigam, 2000). Ethanol can be blended with petrol or used as efficient alcohol in dedicated engines, taking advantage of the higher octane value and higher heat of vaporization. Furthermore, an excellent fuel for advanced flex fuel hybrid vehicles of the future (Hagerdal *et al.*, 2006). Hence, bacteria are used were

ethanol production could be cheaper (Dienet *et al.,* 2003). The fermentation reaction, represented by the simple equation with the predictable depletion of the world's energy supply, resulted in an increasing worldwide interest on alternative source of energy (Aristides and Penttila, 2000; Jeffries and Jin, 2000; Zaldivaret *et al.,* 2001). The main reason, bioenergy can contribute to sustainable development (Van den Broek, 2000; Monique *et al.,* 2003). Lignocellulosic materials such as crop residues, grasses, sawdust, wood chips and solid animal wastes are potential sources for low cost ethanol production (Sun and Cheng, 2002). They are mainly of starch, arabinoxylans, cellulose, glucan, protein and lignin (Maes and Delcour, 2001). Cellulase, class of enzymes produced primarily by fungi, bacteria, and protozoa that catalyze cellulose. The enzyme cellulase is useful in converting the plant biomass in to fuels and basic chemicals and many other useful products (Fan *et al.,* 1987; Wu and Lee, 1997; Solomon *et al.,* 1999). Cellulose decomposing bacteria and fungi are widely distributed in the marine environment and they play an important role in mineralizing organic matter and also influencing the productivity of the sea (Kadota, 1956). In the present agricultural residue such as wood powder, corn stalk, paddy straw, ragi stalk, millet stalk and sugarcane stalk are used in ethanol production.

Materials and Methods

Isolation of cellulase Producing Bacteria

Isolation of cellulase producing organisms, from water, sediment and decayed mangrove twigs were collected from the Uppanar estuary. 1g/1ml of sample was suspended in 99 ml sterile 50% aged sea water, agitated for 45min in a shaker at 50^0C and 0.1 ml was spread on cellulase agar plates ($MgSO_4$ - 0.01g, $(NH_4)_2SO_4$ - 0.02g KH_2PO_4,- 0.7g, K_2HPO_4- 0.05g, Cellulose- 0.1g). 50% aged seawater - 100ml and incubated at 30^0C for 48hrs (Ekperigin, 2007).

Screening for cellulase producing bacteria (well diffusion assay)

The isolated bacterial strains were inoculated into cellulose agar (1% carboxy methyl cellulose and 2% agar) with cellulose as the sole source of carbon. After an appropriate incubation was done at 28 ± 2^0C for 48 hours, the agar medium was flooded with a aqueous solution of Congo red (1mg/ml for media containing CMC) for 15 minutes. The Congo red solution was poured off, and plates were further treated by flooding with 1M NaCl for 15min. Based on the diameter of zone of clearance, the organism was selected for identification and further study.

Sabouraud Glucose Agar with Chloramphenical and Cycloheximide (Yeast)

Glucose-40g,Agar-15g,Pancreatic digest of Casein-5g,Peptic Digest of Animal Tissue-5g pH-.6 $\pm$ 0.2 at 25^oC 10 ml of Cycloheximide and Chloramphenical solution were mixed well with above media after sterilization.

Screening for cellulase producing bacteria (well diffusion assay)

The isolated bacterial strains were inoculated into cellulose agar (1% cellulose and 2% agar) with cellulose as the sole source of carbon. After an appropriate incubation was done at 28 ±

2^0C for 48 hours, the agar medium was flooded with a aqueous solution of Congo red (1mg/ml for media containing CMC) for 15 minutes. The Congo red solution was poured off, and plates were further treated by flooding with 1M NaCl for 15min. Based on the diameter of zone of clearance, the organism was selected for identification and further study.

Optimization of culture conditions for enzyme production

The culture conditions (incubation period, pH, temperature, salt concentration and different sources of C, N and Agriculture residues) were optimized for maximum enzyme production using cellulose producing medium. Cellulase production was studied at different pH (5-9 with 1 interval), temperature (30-50^{0}C with 5^{0}C interval), incubation period (24hrs -72 hrs), salt concentration (1% to 5%), different carbon sources 1g each (cellulose, glucose, xylose, fructose and sucrose), nitrogen sources 1g each (yeast extract, beef extract, peptone, Ammonium sulphate, Ammonium chloride) and different Agricultural residues 1g each (paddy stalk, ragi stalk, millet stalk, sugarcane stalk and cornstalk) were also assessed.

Mass Scale Culture Using Optimized Parameters for Enzyme production

Based on the results obtained from the optimization, the mass scale culture of the cellulase producing organism was carried out. 1000ml of production media were inoculated with 1% of inoculum. The fermentation was carried out in 1000ml Erlenmeyer flasks on a rotary shaker (300rpm). The biomass and the enzyme activity were tested at every 6hrs interval. At the end of the 30thhr the culture was harvested for the recovery of cellulase enzyme.

Ethanol estimation

Ethanol was analyzed by gas chromatography (Chemito GC 8610, poropack Q - SS, 3 meter length. ⅛ Diameter) using isopropanol as an internal standard. A flame ionization detector and integrator were used for detection and quantitative determination respectively (Holdeman, 1977).

Results and Discussion

When the three strains were optimized, growth pH 7, 3% salinity, temperature 35°C, and 30 hrs as incubation period were found to be ideal parameters for *Pseudomonas aeruginosa* (Figs. 1, 2, 3 and 4). With these growth parameters 1% carbon source like cellulose, glucose, xylose, fructose and sucrose were and 1% nitrogen source like yeast extract, beef extract, peptone, ammonium sulphate, ammonium chloride and peptone were tried for a strain for both growth (Figs.5,7) and enzyme production (Figs. 6,8). The strains preferred cellulose as the most preferable substrate for the cellulase production at which 263 Unit/ml/min were the enzyme activity observed in *Pseudomonas aeruginosa* respectively. Among nitrogen sources, yeast extract showed maximum enzyme activity in the above strain were respectively, 275 Unit/ml/min. Mass scale culture was done keeping the ideal parameters and substrates where *Pseudomonas aeruginosa* showed enzyme activity 280 Unit/ml/min respectively. Wood powder and Agricultural residues such as paddy stalk, ragi stalk, millet stalk, sugarcane stalk and cornstalk were estimated for their cellulose content which was in the range of 56.3 to 78.5% (Fig.9).

Figure .1 Optimization of pH for growth in *Pseudomonas aeruginosa*

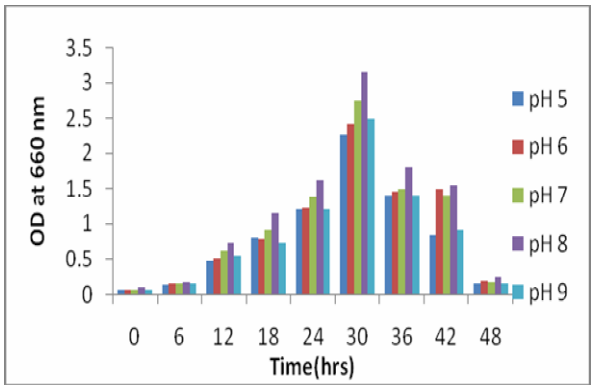


Figure2. Optimization of salinity for growth in *Pseudomonas aeruginosa*

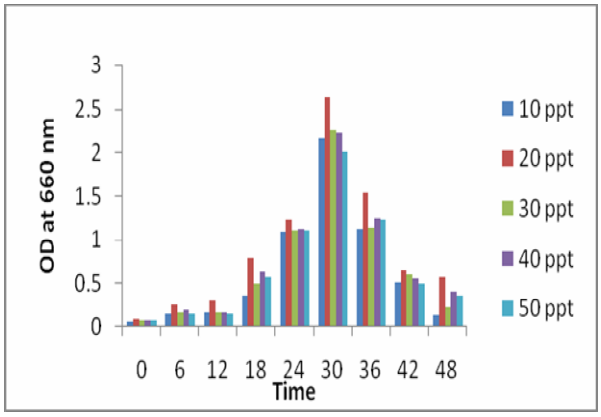


Figure 3.Optimization of Temperature for growth in *Pseudomonas aeruginosa*

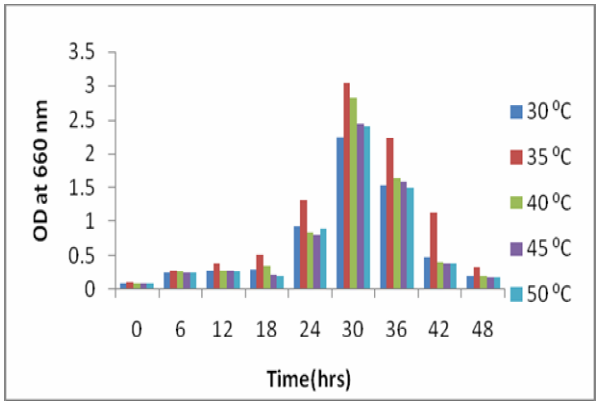


Figure 4. Optimization of Incubation period for growth in *Pseudomonas aeruginosa*

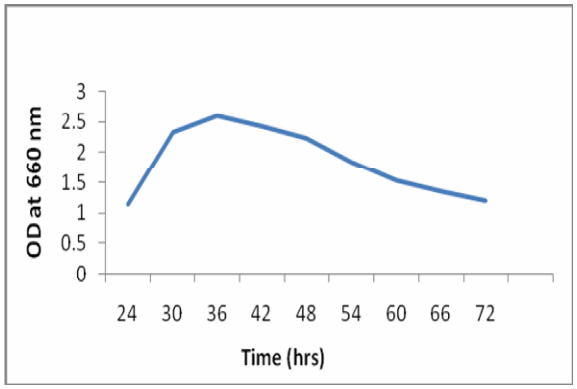


Figure 5.Optimization of carbon source for growth in *Pseudomonas aeruginosa*

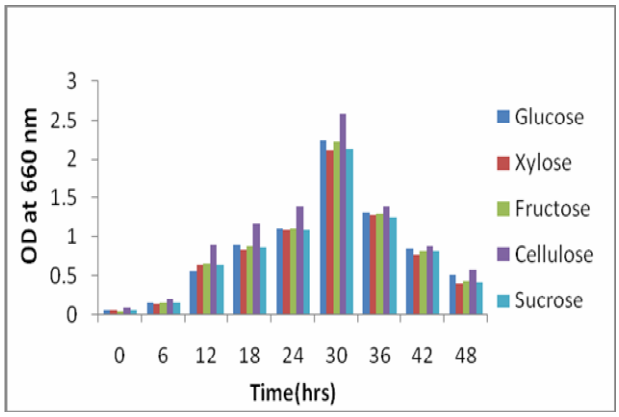


Figure 6. Effect of carbon source on Enzyme activity in *Pseudomonas aeruginosa*

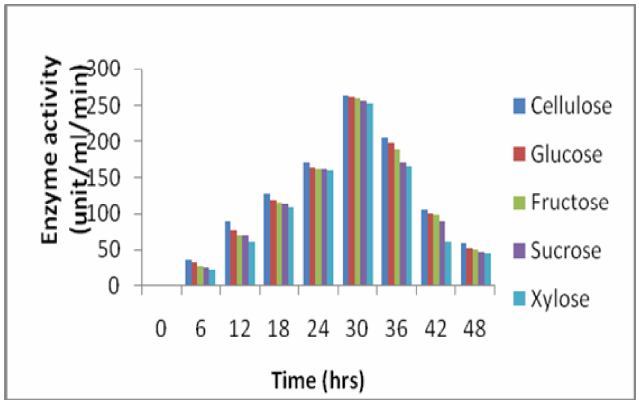


Figure.7 Optimization of nitrogen source for growth in *Pseudomonas aeruginosa*

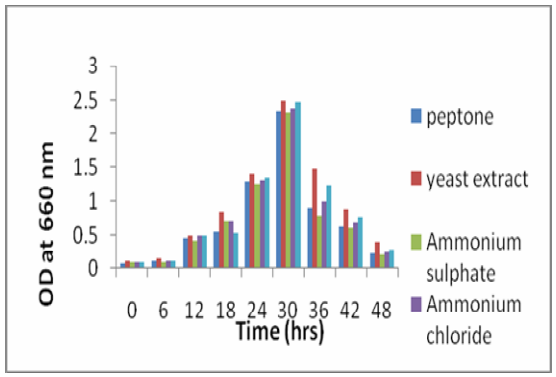


Figure. 8 Effect of Nitrogen source on Enzyme activity in *Pseudomonas aeruginosa*

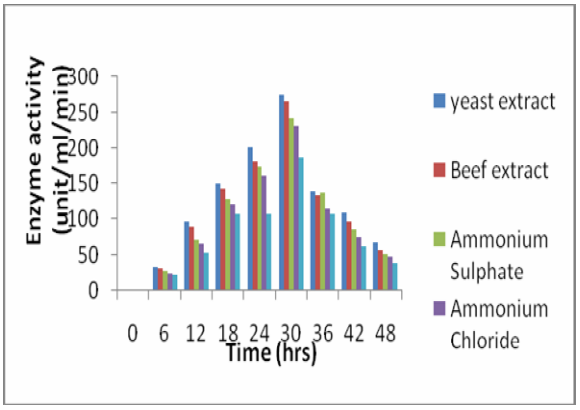


Figure.9 Estimation of cellulose in Wood and agricultural residues

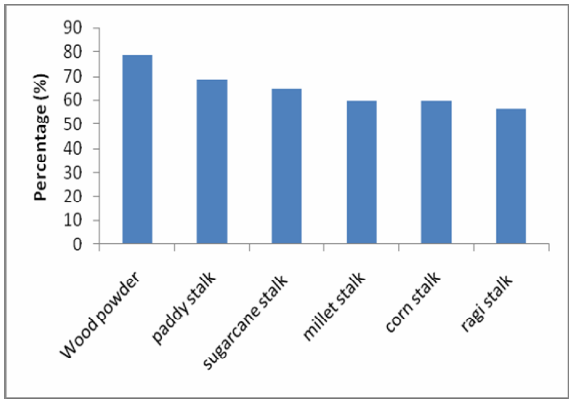


Figure 10. Estimation of enzyme activity with Wood and Agricultural residues in *Pseudomonas aeruginosa*

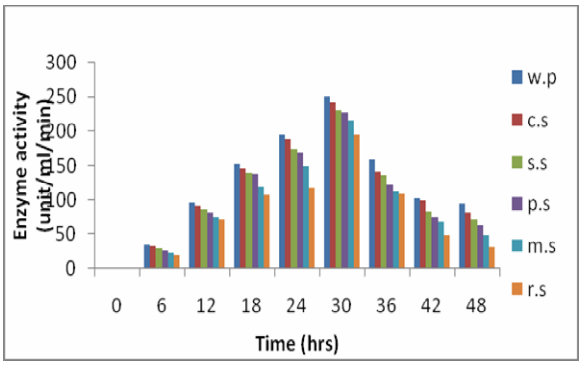
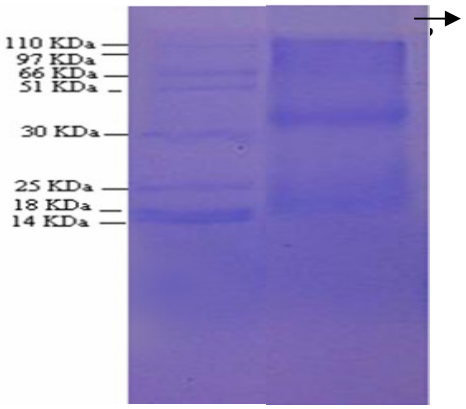
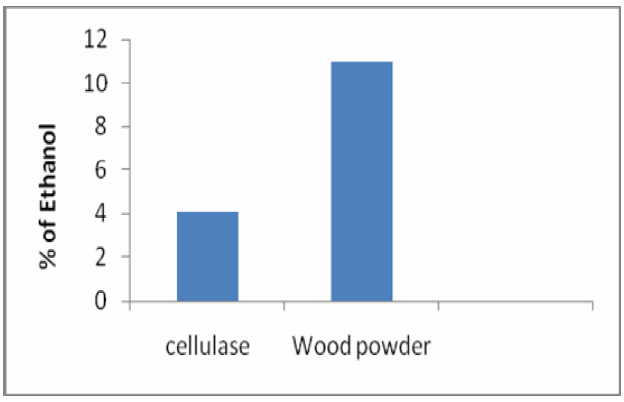


Figure. 11 SDS-PAGE of partially Purified Cellulase



M – Protein marker,
Lane 1 - *Pseudomonas aeruginosa*

Figure. 12 Percentage ethanol production



The highest cellulose content was found in wood powder (78.5%) followed by paddy stalk (68.8%), sugarcane stalk (64.6%), millet stalk and corn stalk (59.6%) and ragi stalk (56.3%). the strain preferred cellulose as the most preferable wood powder as a substrate for the cellulase production at which 251 Unit/ml/min and were the enzyme activity observed in *Pseudomonas aeruginosa* respectively (Fig. 10).The enzyme produced by these strain was partially purified with ammonium sulphate. The SDS PAGE showed all of them were of 32 KDaproteins (Fig. 11).

In Vellar estuary, yeast was isolated at the level of 2.5×10^2 CFU/ml in water samples and 1.8×10^2 CFU/g in sediment samples. Based on the aroma developed in sugar solutions, one yeast strain was selected for ethanol production. Using this yeast ethanol production was tried on the sugar obtained using the enzyme hydrolysis of cellulose using the above three bacterial strains. When the strains were used *Pseudomonas* was in the range of 4.032 % ethanol obtained with cellulose. When Wood and agricultural was used bacteria and yeast yielded 11.0% of ethanol (Fig. 12) Tai *et al*., (2004) reported cellulose degradation at an optimum growth range of pH 7.5.

Zhang et al., 2009 observed the optimum pH range 6.0 to 6.5 and 35^0C as the optimum temperature for maximum growth. However Jahangeer et al., (2005) reported the maximum cellulase growth at 37^0C. But Kathiresan and Manivanan (2006) reported to the maximum cellulase growth at 30^0C. Shanmughapriya et al., (2009) reported the optimum growth for cellulose degradation at 2.5% NaCl. Among the carbon source cellulose was a good substrate which was supported by Liming and Xyeliang (2004); Narasimha et al., (2006); Haq et al., (2005). Among nitrogen sources yeast extract showed maximum enzyme activity Pourramezan et al., (2009) reported a higher yield of cellulase in the presence of yeast extract as the nitrogen source.

Liang et al., (2009) reported peptone as the nitrogen source for maximum cellulase production. Daniel et al., (2003) reported that sulfuric acid treatment of corn Stover resulted in the cellulose conversion of 80-87%. Kurabi et al., (2005) observed a cellulose hydrolysis from steam-exploded method resulted in conversion of 76%.Absar and Allam (2009) observed the molecular weight of an extracellular cellulase protein as 48KDa. Andong et al., (2007) observed the ethanol production of 15.8% from corn stalk by Pacysolentannophilus with pretreatment. Reddy et al., (2006) observed the ethanol production of 4.6% at 30^0C using Saccharomyces cerevisiae.

References

Absar, N., and Alam, S.2009. Purification and characterization of extracellular cellulase from *A. oryzae*. Appl. Sci. Res. 5(10): 1645-1651.

Ali, S., A, Sayed, R.T, Sarker and Alau, R. 1991. Factors affecting cellulase production by *Aspergillus niger* and *Aspergillus terrus* using water Hyacinth. Microb. Biotechnol. 7: 62-66.

Andong, S., W.U. Kun X.I.E, Hui and Bailiang, Z. 2007. Biodegradation of straw stalk and experiment of ethanol fermentation. Nat.sci. 12(2): 343-348.

Aristides, A., and Penttila, M. 2000. Metabolic engineering applications to renewable resource utilization. Curr.Opin.Biotechnol. 11: 187-198.

Danie, L..J., J. Farmer, M. Newman, and Mcmillan, J.D. 2003. Dilute–Sulfuric Acid Pretreatment of Corn Stover in Pilot-Scale Reactor. Biochem. Biotechno. 85*:* 105–108.

Dien, B.S., M.A. Cotta and Jeffries, T.W. 2003. Bacteria engineered for fuel ethanol production. Appl. Microb. Biotechnol. 63:258–266.

Fan, L.T., M.M, Gharpurayn and Lee,Y.H. 1987. Cellulose hydrolysis biotechnology monographs. Springer-Berlin. 3: 1-68.

Hagerdal, B.H., M. Galbe M. Gorwa-Grausiund, F.G. Liden and Zacchi, G. 2006. Bioethanol the fuel of tomorrow from the residues of today. *Biotechnol*.24: 549-556.

Haq, I.U., H. Ashraf, J. Iqbal and Qadeer, M.A. 2002. Biosynthesis of α-amylase by chemically treated mutant of Bacillus*subtilis*. Pak. J. Biol. Sci. 2: 73-75.

Jahangeer, S., N. Kha , S. Jahangeer, M. Sohail, S.Shahzad, A. Ahmadand, Ahmed and Khan, S. 2005. Screening and Characterization of Fungal Cellulases Isolated from the Native Environmental Source. Pak. J. Bot. 37(3): 739-748.

Jeffries, T.W., and Jin, Y. S. 2000. Ethanol and thermotolerance in the bioconversion of xylose by yeasts. Adv. Appl. Microbiol. 47: 221–268.

Kadota, H., 1956. A study on the marine aerobic cellulose – decomposing bacteria memories of the college of Agric, Kyotouniv. Appl. Microb. Biotechnol.74:1-178.

Kathiresan, K., and Manivannan, S. 2006. Cellulase production by Penicilliumfellutanum Isolated from Costal Mangrove Rhizophere soil. Microbl. 1(5):438-442.

Kurabi, A., A. Berlin, N. Gilkes, D. Kilburn, R. Bura, L. Robinson, A. Markov, A.Skomarovsk, A.Gusako, O. Okune, D. Sinitsyn, D. Gregg and Saddler, J. 2005. Enzymatic Hydrolysis of Steam-Exploded and Ethanol Organosolv-Pretreated Douglas-Fir by Novel and Commercial Fungal Cellulases. Bioche.Biotechnol. 5:121-124.

Liang, Y., Z. Feng, J. Yesuf and Blackburn, J.W. 2009. Optimization of growth medium and enzyme assay conditions for crude cellulases produced by a novel thermophilic and cellulolytic bacterium *Anoxybacillus* sp. Appl. Biotechnol. 38:75-80.

Liming, X., and Xueliang, S. 2004. High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. Bioresours.Technol. 91: 259–262.

Maes, C., and Delcour, J.A. 2001. Alkaline hydrogen peroxide extraction of wheat bran non-starch polysaccharides. Cereal Sci. 34:29–35.

Monique, H., A. Faaij, R. Van den Broek, G. Berndes, D. Gielen and Turkenburg, W. 2003. Exploration of the ranges of the global potential of biomass for energy. Biomass. Bioeng. 25: 119 – 133.

Narasimha, G., A. Sridevi, B. Viswanath, S.Chandra and Reddy, R. 2006. Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. J. Biotechnol. 5(5): 472-476.

Nigam, J.N., 2001. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichiastipitis.* J. Biotechnol. 87: 17–27.

Pourrmezan, G.Z., A.M. Roayaei and Qezeibash, Q.R. 2009. Optimization of culture condition for bacterial cellulose production by *Acetobacter* sp. 4B-2. Biotechnol. 8 (1):150-154.

Shanmugha priya, S., G. SeghalKiran, J. Selvin, T. Anto Thomas and Rani, C.

2009. Optimization, purification, and characterization of extracellular mesophilic alkaline cellulase from sponge-associated *Marinobacter* sp. MSI032. Appl. Biochem. Biotehchnol. 14: 67–75.

Solomon, B.O., B. Amigun, E. Betiku, T.V. Ojumu and Layokun, S.K. 1999. Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on Bagasse. J. Chem. 16: 61-68.

Sun, Y., and Cheng, J. 2002. Hydrolysis of lignocellulosic material for ethanol production: A review. Bioresours. Technol. 83:1–11.

Tai, SK., H.P.P. Lin and Kuo, J. 2004.. Isolation and characterization of a cellulolytic *Geobacillus thermoleovorans* T4 strain from sugar refinery wastewater. Extremophil 8:345–349.

Van den Broek, R., 2000. Sustainability of biomass electricity systems-an assessment of costs, macro-economic and environmental impacts in Nicaragua, Ireland and the Netherlands. Utrecht University.Energy – Policy. 30: 167–169.

Wu, Z., and Lee,Y.Y. 1997. Inhibition of the enzymatic hydrolysis of cellulose by ethanol. Biotechnol.Lett. 19: 977-979.

Zaldivar, J., J. Nielsen and Olsson, L. 2001. Fuel ethanol production from lignocellulose a challenge for metabolic engineering and process integration. Appl. Microbiol. Biotechnol.56: 17–34.

Zhang, Y.M., X.B, Lu, H.B, Dan and Sun,Y.K. 2009. Screening and enzymatic study of a composite microbial system FH3.Biotechnol. Adv. 24:452–81.