International Journal of Advanced Multidisciplinary Research (IJAMR)
ISSN: 2393-8870
www.ijarm.com

Research Article
Bioethanol Production Using Marine Microbes

S. Jayalakshmi[1*], Pingal kumari [2] and S. Sankar[2]

[1]Associate Professor, CAS in Marine Biology, Faculty of marine sciences, Annamalai University, Parangipettai – 608502, Tamilnadu, India.
[2]Research Scholar, CAS in Marine Biology

*Corresponding Author : *jayacas@gmail.com*

Keywords

Anaerobe,
Cellulase,
Pachysolen tannophilus,
Desulfovibrio sp,
Ethanol production.

Abstract

The present study is on ethanol production using cheaper biomass based natural substrates using marine anaerobes. In Vellar estuary, sediment samples were collected and anaerobic cellulolytic bacteria were isolated. Cellulolytic anaerobic bacterial density was found in the range of 1.0 x 10^4 CFU/g - 1.8 x 10^5 CFU/g. The potential cellulolytic anaerobic bacterium was identified as *Desulfovibrio sp*. and designated as *Desulfovibrio* SJ 31. The potential ethanol producing yeast was identified as *Pachysolen tannophilus.* The optimization experiments regarding growth of *Desulfovibrio* SJ 31 strain showed that pH – 6, temperature - 40^{o}C, NaCl - 2%, cellulose - 1.5%, yeast extract - 0.5% and incubation period 48 hrs were ideal. When these optimum conditions were kept *Desulfovibrio* strain showed maximum cellulase activity (10.0 U/ml/min) at 48 hrs of incubation period. Cellulase producing strain in combination with yeast produced 1.0% of ethanol at the end of 72 hrs. Instead of cellulose when cheaper substrated were used in coconut husk 1.7% ethanol was produced.

Introduction

Biofuels that are currently in use, known as first generation biofuels, are mainly produced from sugarcane, maize or soy. An increased usage of first generation biofuels leads to an increased need of high-quality agricultural land and fertilizer, which has been associated with global warming and can contaminate groundwater. The most critical point is probably the food vs. fuel problem (Walter and Rosillo-Calle, 2008). As first generation biofuels have some drawbacks, bioethanol that is generated from cellulose, so called second generation biofuel can reduce the emission of greenhouse gases. Bioethanol from cellulose has a much better eco-balance, it does not compete with food production and cellulose is readily available at low costs.

Cellulose, the main component of plant cell walls is the most abundant polymer in the world which makes a large fraction of the plant dry weight (30-35%). Lignocellulosic biomass makes about 50% of the total biomass in the world with an estimated annual production of 10-50 billion tons (Sanchez and Cardona, 2008). Since cellulose is the most abundant organic material on Earth, the microbes involved in its breakdown are of interest both in ecological terms, for their importance in the global carbon cycle, and in economic terms, for their role in processes involving the conversion of cellulosic wastes to valuable products such as ethanol and organic acids (Ljungdahl and Eriksson, 1985 and Leschine, 1995).

To make the sugar monomers available for fermentation, the cellulose and hemicellulose chains have to be further hydrolyzed. After the hydrolysis, the sugars have to be fermented to ethanol. There are a lot of microorganisms that produce a variety of enzymes that are able to do that, known as the cellulase systems. There exist both anaerobic and aerobic bacteria and fungi that produce cellulase systems.

Anaerobic microorganisms are from extreme environments and, in particular, their fermentation products and thermostable enzymes, have been the subject of much research over the past 15-20 years. Although most of the cellulose

produced globally each year is broken down aerobically, there are many anaerobic environments in which cellulose decomposition occurs.

Anaerobic cellulolytic bacteria have been isolated from such diverse habitats as soils, sediments and estuarine muds (Madden *et al*., 1982; Murray *et al*., 1986; He *et al*., 1991 and Monserrate *et al*., 2001), anaerobic digestors and the rumens or intestines of various mammals. Many of the reliably described anaerobic, thermophilic, cellulose-degrading bacteria form spores and are placed in the genus *Clostridium* (Wiegel, 1992). Common anaerobic cellulolytic bacteria are various *Clostridium* species. Cellulose decomposing bacteria and fungi are widely distributed in the marine environment and they play an important role in mineralizing organic matter (Kadota, 1956). The present study aimed to use the marine *Desulfovibrio sp*. in cellulose biomass conversion for bioethanol production.

Materials and Methods

Collection of samples

Sediment samples were collected from Vellar estuary (Lat 11^0 29' N, Long 76^0 46'E) using PVC corer and taken to the laboratory for analysis. All procedures were carried out inside an Anaerobic Glove Bag as the obligate anaerobes should not be expose to air or oxygen even at lower concentration**.**

Fig. 1 – Anaerobic Glove Bag

Isolation and Screening of cellulase producing anaerobic bacteria

Properly diluted sediment samples were plated on cellulose agar (1% corboxy methyl cellulose and 2% agar) with cellulose as the sole source of carbon and incubated at 30°C for 2-3 days. The broth cultures of isolated bacterial strains were inoculated into cellulose broth. After incubation cell free were added in 6 mm wells. After an appropriate incubation done at 28 ± 2^0C for 48 hrs, the agar medium was flooded with an aqueous solution of Congo red (1mg/ml for media containing CMC) for 15 min. 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 potential organism was selected, biochemical identification was done and used for further study.

Determination of cellulase activity

Cellulase activity was assayed by the determination of reducing sugar released from carboxymethyl cellulose (CMC). 0.5ml of culture supernatant fluid was incubated with 0.5ml of 1% CMC in 0.05M sodium acetate buffer, pH 4.8 at 40ºC for 1hr. The reducing sugar product was assayed by the dinitrosalicylic (DNS) method (Miller, 1959). The amount of glucose released per ml was estimated from a standard curve prepared with known glucose concentration. One unit of cellulase activity was expressed as the amount of that liberated reducing sugar equivalent to 1 µg of glucose per minute under assay conditions.

Determination of protein concentration

The protein concentration of the crude as well as that of the purified enzyme was determined by the method of Lowry *et al*., 1951 using bovine serum albumin (BSA) as a standard.

Optimization of culture conditions for cellulase enzyme production

The culture conditions viz., pH. temperature, salinity, incubation period were optimized for maximum enzyme production using minimal medium containing 1% cellulose. Cellulase production was studied at different temperature (25-50^0C with 5^0C interval), pH (6 to 10 with 1 interval),

Salinity (0.5 to 2.5% with 0.5% interval), incubation period (24-96 hrs with 24 hrs interval), carbon source (cellulose - 1 to 3% with 0.5% interval) and Nitrogen source (yeast extract - 0.1 to 0.9% with 0.2% interval).

Preparation of cell free supernatant

The culture was harvested at 48hrs and centrifuged at 3000rpm under 4^0C for 60min. The cell free filtrate containing cellulase enzyme were collected and recovered through precipitation.

Ammonium sulphate precipitation

The optimum percentage saturation of ammonium sulphate giving maximum cellulase activity was checked using varying concentration such as 40%, 60%, 80% saturation. Total culture filtrate was precipitated with solid Ammonium sulphate of 60%. Saturation of solutions was carried out under 4^0C for over night. The precipitated proteins were collected through centrifugation at 3000rpm for 30 min. The non residue matters were discarded and resulted pellets were dissolved in 5ml of phosphate buffer of pH 7.0. Enzyme activity was determined.

Dialysis using membrane

The obtained residues, collected from the ammonium sulphate precipitation were introduced into a regenerated cellulose-dialysis tube for dialysis under 4^0C against phosphate buffer at pH 7.0. The dialysis was done for 24 hrs and then the samples were centrifuged. Finally the resulting pellets were lyophilized. The lyophilized, powdered samples were preserved under 4^0C for further analysis.

Isolation, Screening and Identification of Yeast

For isolation of yeast, sediment samples collected from Vellar estuary. The samples were kept in ice box and transported to the laboratory. After serial dilution the sample were spread plated on the surface of YMA (Yeast Malt Agar) agar and incubated for 3 days at room temperature.

Distinctly apparent yeast colonies were isolated and pure cultures were stored in the same medium. The potential strain was identified based on colony color, shape, texture, microscopic morphology, physiological, biochemical tests and various sugar assimilations (Sanni and Lonner, 1993; Yarrow, 1998 and Barnett *et al*., 2000).

Mass culture for production of enzymes and ethanol

Based on the results obtained in optimization studies, for mass scale culture, 500 ml of cellulase production media was prepared. After sterilization, 1ml of *Desulfovibrio sp.* SJ 31 was added to cellulase production medium (pH-6, temperature - 40^0C, NaCl - 2%, cellulose - 1.5% and yeast extract - 0.5 %). After the incubation period, to cell free supernatant, the yeast culture *Pachysolen tannophilus* isolated from Vellar estuary was added (2 ml/ 100 ml) and incubated in anaerobic condition for 72 hrs for ethanol production. Ethanol production was estimated in Gas Chromatography.

Ethanol estimation

Ethanol was analysed by gas chromatography (Shimadzu, model 14 B: Japan, equipped with a porapack Q column) using isopropanol as an internal standard. A frame ionization detector and integrator were used for detection and quantitative determination respectively (Holdeman., 1977)

Results

Sediment samples were found to harbour cellulolytic anaerobic bacteria in the range of 1.0 x 10^4 CFU/g - 1.8 x 10^5 CFU/g.

The potential cellulolytic anaerobic bacterium was identified as *Desulfovibrio sp*. and designated as *Desulfovibrio sp*. SJ 31 (Table 1). The potential ethanol producing yeast was identified as *Pachysolen tannophilus* (Table 2) and designated as *P. tannophilus* SJ 101.

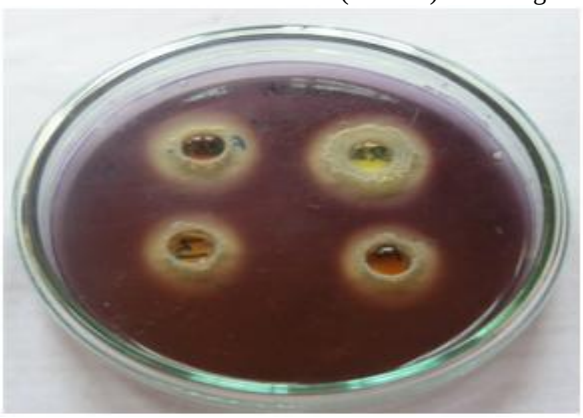

Fig 2: Screening of potential strain (cellulase producer)

Table 1: Biochemical identification of *Desulfovibrio sp.*

Biochemical characters	Results
Gram staining	Negative
Shape	Curved Rod
Spore	Negative
Pigment	Negative
Vancomycin	Resistant
Kanamycin	Sensitive
Co-Trimoxazole	Resistant
H_2S production	Positive
Motility	Positive
Citrate Utilization	Negative

Table 2: Biochemical identification of yeast *Pachysolen tannophilus*

Aerobic Utilization and Growth	
Glucose	Positive
Galactose	Positive
Sucrose	Negative
Maltose	Negative
Cellobiose	Positive
Trehalose	Negative
Lactose	Negative
Melibiose	Negative
Raffinose	Negative
Melizitose	Negative
Inulin	Negative
Soluble Starch	Negative
Xylose	Positive
L-Arabinose	Negative
Rhamnose	Negative
Glycerol	Positive
Mannitol	Positive
Sorbitol	Positive
Salicin	Positive
Lactic acid	Negative
Succinic acid	Negative
Citric acid	Negative
Inositol	Negative

The optimization experiments regarding cellulase enzyme production by *Desulfovibrio* showed that pH-6, temperature - 40^{o}C, NaCl - 2%, cellulose - 1.5%, yeast extract - 0.5% and incubation period 48 hrs were ideal (Fig. 3 to 8). When these optimum conditions were kept *Desulfovibrio* strain showed maximum cellulase activity (10.0 U/ml/min) at 48 hrs of incubation period.

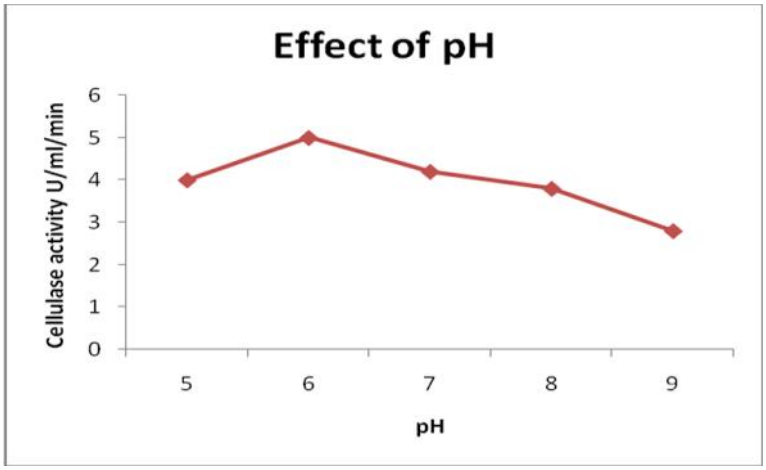

Fig. 3: Effect of pH on Cellulase production

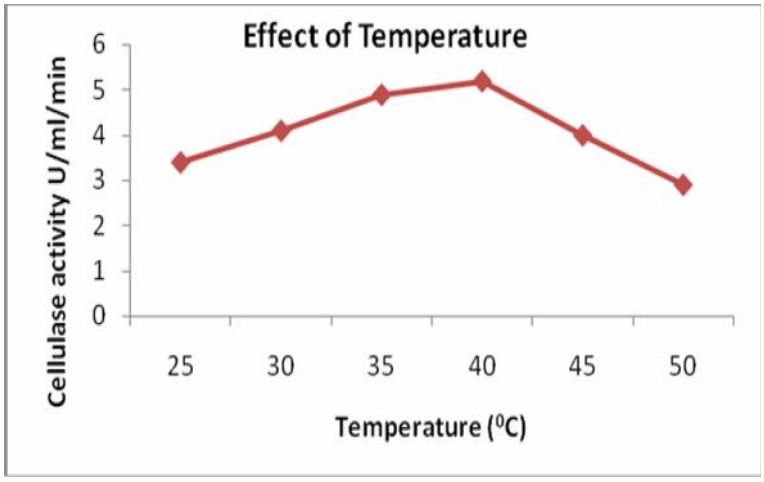

Fig. 4: Effect of Temperature on Cellulase production

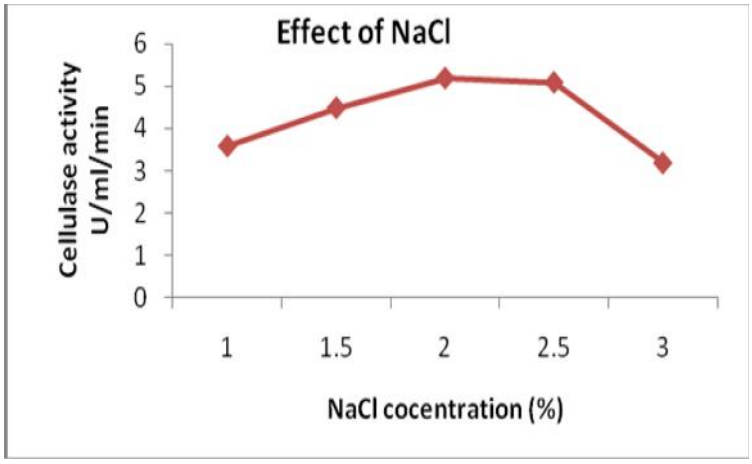

Fig. 5: Effect of Salinity on Cellulase production

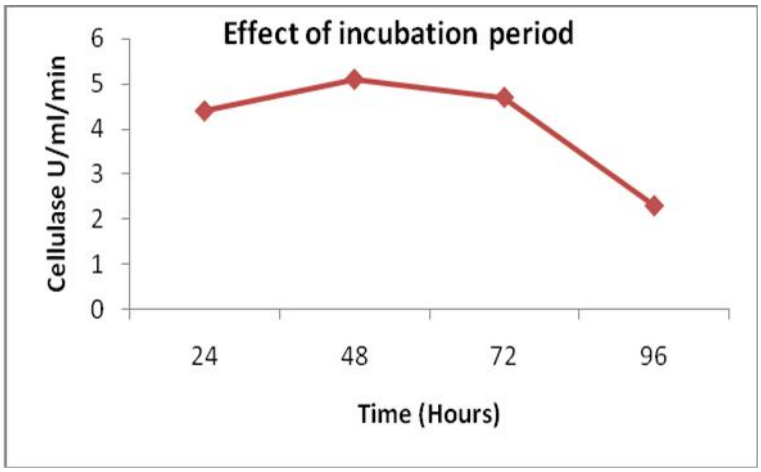

Fig. 6: Effect of incubation period on Cellulase production

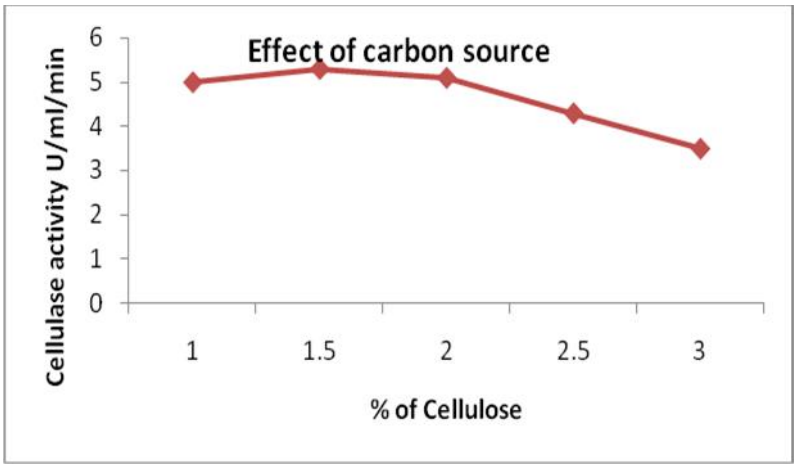

Fig. 7: Effect of Carbon source on Cellulase production

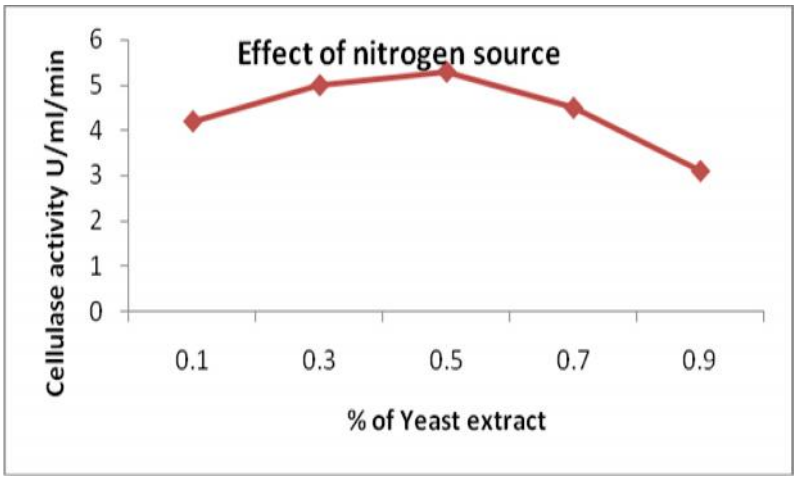

Fig. 8: Effect of nitrogen source on Cellulase production

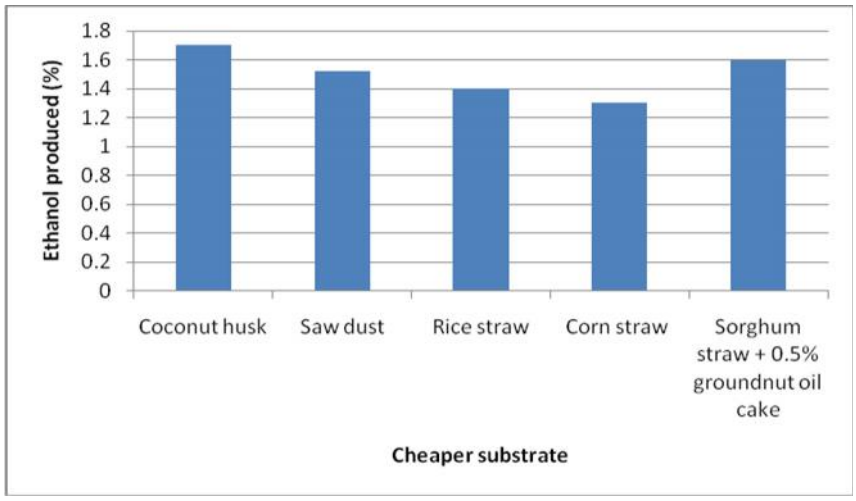

Fig. 9: Effect of cheaper substrates on ethanol production

At 48 hrs incubation period, cells were removed and yeast strain was added. At the end of 72 hrs ethanol produced in anaerobic condition was analyzed. The cellulase producing strain in combination with yeast produced 1% of ethanol at the end of 72 hrs.

When coconut husk, saw dust, rice straw, corn straw and sorghum straw along with 0.5% groundnut oil cake were used as cheaper substrates for ethanol production, they produced 1.7, 1.52, 1.4, 1.3, and 1.6% of ethanol respectively.

Discussion

The study deals with ethanol production using cellulolytic anaerobic marine bacteria isolated from Vellar estuary. The cellulolytic anaerobic bacteria were found in the range of 1.0×10^4 CFU/g - 1.8×10^5 CFU/g.

The most potential cellulolytic anaerobic bacterium was identified as *Desulfovibrio sp.* based on standard manuals and designated as *Desulfovibrio sp.* SJ 31. It produced 13 mm zone of clearance in the cellulose amended medium.

The optimization experiments regarding growth of *Desulfovibrio*, a cellulase producing strain showed that pH - 6, temperature - 40°C, NaCl - 2%, cellulose - 1.5%, yeast extract - 0.5% and incubation period 48 hrs were ideal. Ali *et al.*, 1991 reported the maximum yield of cellulase from the *Aspergillus terreus* at 40°C using water hyacinth after 6 days of fermentation. In the present study it was observed that pH- 6 was the optimum for the maximum yield of cellulase production which was supported by the work done by Akiba *et al.*, 1995. However Waksman, 1967 reported initial pH value of 7.0 as optimal for cellulase production.

The optimum incubation period for the present study was 48 hrs. However Ali *et al.*, 1991 and Vancoyk, 1998 got the maximum cellulase production at 96 hrs only. The present study showed that the anaerobic bacterial strain *Desulfovibrio sp.* SJ 31 strain produced higher cellulase production with a lower incubation period. When these optimum conditions were kept *Desulfovibrio* strain showed maximum cellulase activity (10.0 U/ml/min) at 48 hrs of incubation period.

In the present investigation the yeast strain *Pachysolen tannophilus* isolated from Vellar estuary was used for ethanol production. To the spent medium when yeast culture (2%) was added, 1% ethanol was produced.

Panesar *et al.*, 2001 reported 3.24% of ethanol production by *Zymomonas mobilis* at 30°C, whereas at 40°C, 1.92% (v/v) was obtained. Jyothi *et al.*, (2005) reported the ethanol production from *Candida intermedia.* Benschoter and Ingram (1986) reported that *Zymomonas mobilis* produced maximum ethanol production at 30°C. In the present study incubation for ethanol production was done at 28°C.

The proportion of cellulose in plant tissues ranges from 20 to 45% of dry weight and over 90% in cotton fiber (Stephens and Hatchel, 1995). Waste paper is an important source of cellulose (Crueger and Crueger, 1990). In the present study when cheaper sources viz. coconut husk, saw dust, rice straw, corn straw, sorghum straw each at 4% were tried supplementing with 0.5% ground nut oil cake respectively 1.7%, 1.52%, 1.4%, 1.3%, 1.6% were the ethanol quantity produced. However the substrates were not pretreated. If pretreated the percentage of alcohol would be still higher.

Wiselogel *et al.,* 1996 reported high ethanol yield using strains that produce ethanol with few byproducts which may contain all major sugars like glucose, arabinose, xylose, galactose and mannose etc. According to Vidyasagar *et al.,* 2005. Most of the yeast isolates from mulberry leaf produced ethanol around 1.2%.

Strain improvement using genetic manipulation may further improve the prospective and the researcher is aiming that in future.

Acknowledgments

The authors are thankful to University Grants Commission, Govt. of India, New Delhi for funding this project.

References

Ali, S., A. Sayed, R. I. Sarkar and R. Alam, 1991. Factors affecting cellulase production by *Aspergillus terreus* using water hyacinth. *World J. Microbiol and Biotechnol.*, 7:62-66.

Benchoter, A. S. and L.O. Ingram, 1996. Thermal tolerance of *Zymomonas mobilis,* temperature induced changes in membrane composition. *Appl. Environ. Microbiol.*, 51:1278-1284.

Ekperigin, M. M., 2007. Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella sp*. *Afr. J. Biotechnol.*, 6(1): 028-033.

He, Y., Y. Ding and Y. Long, 1991. Two cellulolytic *Clostridium* species : *Clostridium cellulosi* sp. nov. and *Clostridium cellulofermentans* sp. nov. *Int. J. Syst. Bacteriol.*, 41: 306 - 309.

Holdmen, L.V., E.P. Cato, and W.E.C. Moore, 1977. Anaerobe laboratory manual .4th(eds). Virginia: Virginia Polytechnic Institute, Blacksburg. p: 115-118.

Jyothi, C. H., R. Sreenivas Rao, S. Rajesham and L. Venkateswar Rao, 2005. Production of ethanol yield by *Candida intermedia*. *Asian J. Microbiol. Biotechnol.*, 7(4): 679-684.

Kadota, H., 1956. A study on the marine aerobic cellulose decomposing bacteria. Memories of the college of agriculture Kyoto University. *Appl. Microbiol. Biotechnol*., 74: 1-178.

Leschine, S. B., 1995. Cellulose degradation in anaerobic environments. *Annu. Rev. Microbiol.*, 49: 399-426.

Ljungdahl, L. G. and K.E. Eriksson, 1985. Ecology of microbial cellulose degradation. *Adv. Microb. Ecol.*, 8: 237-299.

Lowry, O.H., N.J. Rosebrough and R.J. Randall, 1951. Protein measurement with Folin's phenol reagent. *J .Biochem.*, 198: 265-275.

Madden, R. H., M. J. Bryder and N. J. Poole, 1982. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium papyrosolvens* sp. nov. *Int. J. Syst. Bacteriol*., 32:87-91.

Miller, G. L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31. p: 426-428.

Monserrate, E., S.B. Leschine and E.Canale-Parola, 2001. *Clostridium hungatei* sp. nov., a mesophilic, cellulolytic bacterium isolated from soil. *Int. J. Syst. Evol. Microbiol.*, 51: 123-132.

Murray, W. D., L. Hofmann, N.L. Campbell and R.H. Madden, 1986. *Clostridium lentocellum* sp. nov., a cellulolytic species from river sediment containing paper-mill waste. *Syst. Appl. Microbiol.*, 8: 181-184.

Panesar, P.S. and S. S. Marwaha, 2001. High productivity ethanol fermentations with *Zymomonas mobilis*. *Asian J. Microbiol. Biotech. Env. Sci.*, 3(4):283-285.

Sanchez, O. J. and C. A. Cardona, 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour. Technol*., 99(13): 5270-5295.

Sanni, A. I. and C. Lonner, 1993. Identification of yeasts isolated from Nigirian traditional alcoholic beverages. *Food Microbiol.*, 10: 517-523.

Walter, A. and F. Rosillo-Calle, 2008. Perspectives on fuel ethanol consumption and trade. *Biomass. Bioenergy*, 32(8): 730-748

Wiegel, J., 1992. The anaerobic thermophilic bacteria. In: *Thermophilic Bacteria*, J. K. Kristjansson (edt). London : CRC Press. 105-184.

Wiselogel, A., S. Tyson and D. Johnson, 1996**.** Biomass feedstock resources and composition. In: Hand book on bioethanol Production and utilization Wyman, C.E. (Eds)., *Appl. Energy. Technol.* Series. Washington, D.C., p: 105-118.

Yarrow, D., 1998. Methods for the isolation, maintanence and identification of yeasts. In : The- Yeast A Taxonomic study. Kurtzman, C. P. and Fell, J. W., (Eds). 4th edition. p: 77-100.