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Research Article

Ethanol Production Using a Xylanolytic *Clostridium* SJ 110 and a Marine Yeast *Pachysolen tannophilus* SJ 101 strain

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Abstract

The study was on bioethanol production using a xylan hydrolyzing anaerobic bacterium ana a marine yeast. In Vellar estuary, sediment samples were collected and were anaerobic xylanolytic bacteria. xylanolytic anaerobic bacteria were found in the range of 1.9 x 10^6 CFU/g. The most potential xylanolytic anaerobic bacterium was identified as *Clostridium sp.* and named as *Clostridium* SJ 110. The potential ethanol producing yeast was identified as *Pachysolen tannophilus* and designated as *P. tannophilus* SJ 101. The optimization experiments regarding growth of *Clostridium sp.* showed that pH 9, temperature - 40°C, NaCl - 2.5%, xylan - 2%, yeast extract - 0.5% and incubation period 48 hrs were ideal for xylanase production. When these optimum conditions were kept, *Clostridium sp.* showed maximum xylanse activity (17.78 U/ml/min) at 48 hrs. Xylanase from anaerobic bacterium *Clostridium* SJ 110. obtained from the results of SDS PAGE revealed a kDa protein with hydrolyzing potential again xylan. For ethanol production the xylanase producing strain was used in combination with yeast produced 1.6% ethanol at the end of 72 hrs.

Keywords: Anaerobe, Xylanase, *Clostridium sp*, *Pachysolen tannophilus*, Ethanol production.

Introduction

There are two global bio-renewable transportation fuels that might replace oil derived gasoline and diesel fuel. These are bioethanol and biodiesel (Demirbas, 2006). Biofuels have gained increased interest in recent years due to environmental and economic reasons (Qureshi et al. 2006). Biofuels are renewable, can substitute fossil fuels, reduce fossil greenhouse gas emissions and they can be produced, where they are needed, to reduce the dependence on oil producing countries.

Ethanol has been trusted as an alternate fuel for the future (Smith, 2007). Ethanol production industry is considered one of the important commercial activities for many countries. There is a world-wide search for alternative methods of energy production from renewable sources.

Bioethanol as an alternative to fossil fuels has been extensively studied and its annual, worldwide production is about 51 million liters (Renewable Fuels Association, 2008). Bioethanol, produced from sugarcane and maize, and biodiesel, produced from soybeans, are presently the only biofuels that are produced on an industrial scale. Hence, of bacteria are used ethanol production could be cheaper (Dien *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 (Zaldivaret *et al.,* 2001).

Several microorganisms have been considered as ethanologenic microbes. The yeast *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* and the facultative bacterium *Zymomonas mobilis* are better candidates for industrial alcohol production (Mohammed *et al*., 2001).

Xylan is the most abundant polymer in plants next to cellulose. Xylanases have expanded their use in many processing industries, such as pulp, paper, food, and textile. Xylans are highly complex polysaccharides that are found in plant cell walls and some algae made from units of xylose. Xylanase, enzyme produced by microorganisms to break down xylans, the major constituents of lignocellulose which are enzymatically hydrolyzed to xylose by xylanase. Research into microbial xylanase production is a welcome development as the enzyme has several important industrial applications. Cellulase-free xylanases from anaerobes are quite rare (Rani and Nandha, 2000).

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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 (Fig. 1) as the obligate anaerobes should not be exposed to air or oxygen even at a lower concentration**.**

Fig. 1 – Anaerobic Glove Bag

Isolation and Screening of xylanase producing anaerobic bacteria

The medium used to screen xylanase producing anaerobic bacteria was mineral salt medium (Min-Jen *et al.*, 2002). Besides, 0.1% yeast extract, 1 % xylan as a source of carbon was added to the medium so that the colonies selected have the ability to grow in xylan, by virtue of xylanase production. The pH was adjusted to 7.0 using 1 N NaOH. The supernatant comprising crude enzyme was subjected to the estimation of xylanase activity by DNS method. The potential anaerobic bacteria for biochemically identified for further analysis.

Optimization of culture conditions for Xylanase enzyme production

The culture conditions (incubation period, pH and temperature) were optimized for maximum enzyme production using Mineral salt agar medium. Xylanase production was studied at different temperature (25-50^0C with 5^0C interval), pH (6 to 10 with 1 interval), Salinity (1 to 3% with 0.5% interval), incubation period (24-96 hrs with 24 hrs interval), carbon source (xylan - 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 48^{th}hrs and centrifuged at 3000rpm under 4^0C for 60min. The cell free culture filtrate containing xylanase enzyme were collected and recovered through precipitation.

Ammonium sulphate precipitation

A small portion of the culture filtrate was added with 40%, 60%, 80% saturation with solid ammonium sulphate. Saturation of solutions was carried out under 4^0C for over night. The precipitated proteins were collected through centrifugation at 3000 rpm for 30 min. The non residual matters were discarded and resulted pellets were dissolved in 5ml of phosphate buffer of pH 7.0. Based on the enzyme activity the ammonium sulphate concentration was selected and used to precipitate the crude enzyme of mass scale culture.

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.

Determination of molecular weight of the enzyme by SDS-PAGE

Molecular weight of the crude enzyme was determined according to the method of Laemmli, 1970. Proteins were separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein was determined by comparing its electrophoresis mobility in SDS-PAGE gel with standard marker proteins of known molecular weight.
The samples were solubilized in reducing sample buffer and equal amount of protein was loaded into 12% SDS-polyacrylamide gel and electrophoresis was carried out at constant current (30mA).

Isolation, Screening and Identification of Yeast

For isolation of yeast, sediment samples from Vellar estuary were collected in sterile polythene bags. The samples were kept in ice box and transported to the laboratory. After serial dilution, the samples 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 enzyme and ethanol

Based on the results obtained in optimization studies for mass scale cultures, 500 ml of each xylanase production media were prepared. After sterilization, 1ml of *Clostridium sp.* (pH 9, temperature 40^0C, Nacl - 2.5%, Xylan - 2% and Yeast extract - 0.5 %) containing approximately 10^6 cells /ml were inoculated and incubated for 48 hrs. After the incubation period to cell free supernatant, the yeast culture *Pachysolen tannophilus* isolated from Vellar estuary was added (1 ml/ 100 ml) and incubated 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).

Ethanol Production using cheaper substrate

Dried corn straw (4%) with groundnut oil cake (0.5%) and coconut husk (4%) with coconut oil cake (0.5%) were used as cheaper sources for ethanol production. To the minaeral medium these substrate were added separately to which 1ml of xylanase producing organism (10^6 cells / ml) was added and incubated for 48 hrs with optimized parameters as in the previous experiment. After 48 hrs particles were removed using whatman No.1 filter paper and centrifuged the culture at 10,000rpm for 15 min. to remove the bacterial cells. The yeast strain was added (2%) and incubated at

room temperature for 72 hrs in both aerobic and anaerobic conditions. Ethanol was estimated in gas chromatography.

Results

Sediment samples were found to harbour xylanolytic anaerobic forms around 1.9x 10^6 CFU/g. The most potential xylanolytic anaerobic bacterium was identified as *Clostridium sp*. based on biochemical tests (Table 1). The potential ethanol producing yeast was identified as *Pachysolen tannophilus* (Table 2)

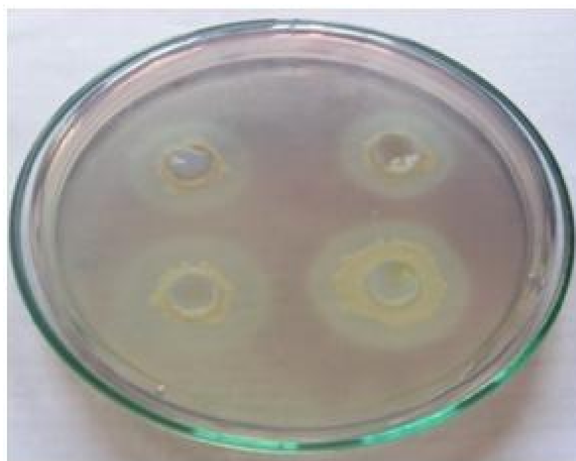


Fig 1: Screening of potential strain (xylanase) in mineral salt medium at pH 7

Table 1: Biochemical identification of *Clostridium* SJ 110 strain

Biochemical characters	Results
Gram staining	Negative
Spore	Positive
Shape	Rod
Pigment	Negative
Vancomycin	Sensitive
Kanamycin	Sensitive
Co-Trimoxazole	Sensitive
H_2S production	Positive
Nitrate reduction	Negative
Indole test	Negative
Urease production	Negative

Table 2: Biochemical identification of yeast *Pachysolen tannophilus* SJ 101 strain

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 study regarding xylanase enzyme production by *Clostridium sp.* showed that pH - 9, temperature - 40°C, NaCl - 2.5%, xylan - 2%, yeast extract - 0.5% and incubation period 48 hrs were found to be ideal (Fig. 2- 7). When these optimum conditions were kept *Clostridium sp.* showed the maximum xylanse activity (17.78 U/ml/min) at 48 hrs.

At 48 hrs incubation period, cells were removed and yeast inoculum was added. At the end of 72 hrs ethanol produced in anaerobic condition was analyzed. The xylanase producing strain was used in combination with yeast produced 1.6% ethanol at the end of 72 hrs.

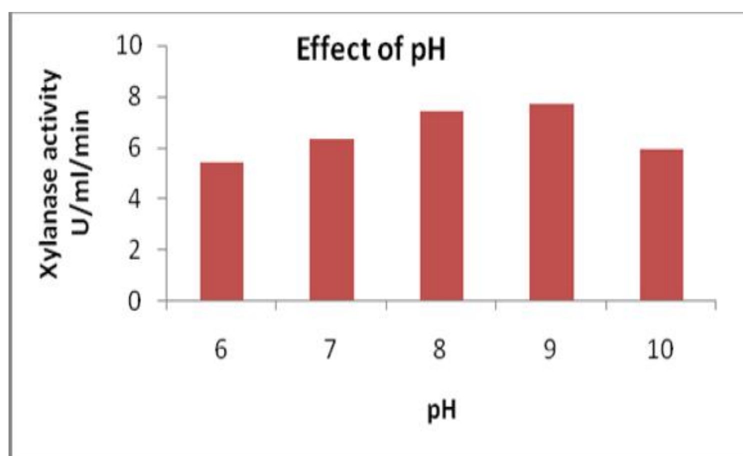


Fig. 2: Effect of pH on Xylanase production

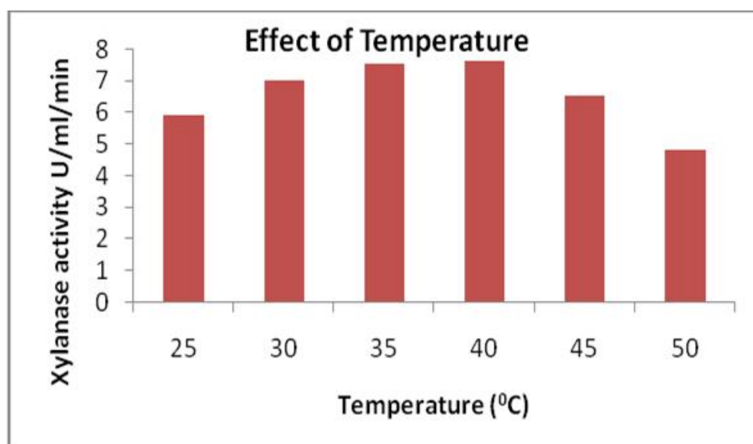


Fig. 3: Effect of Temperature on Xylanase production

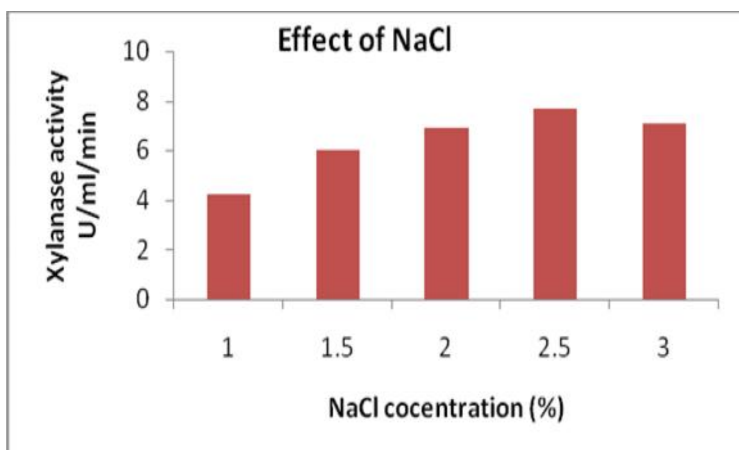


Fig. 4: Effect of Salinity on Xylanase production

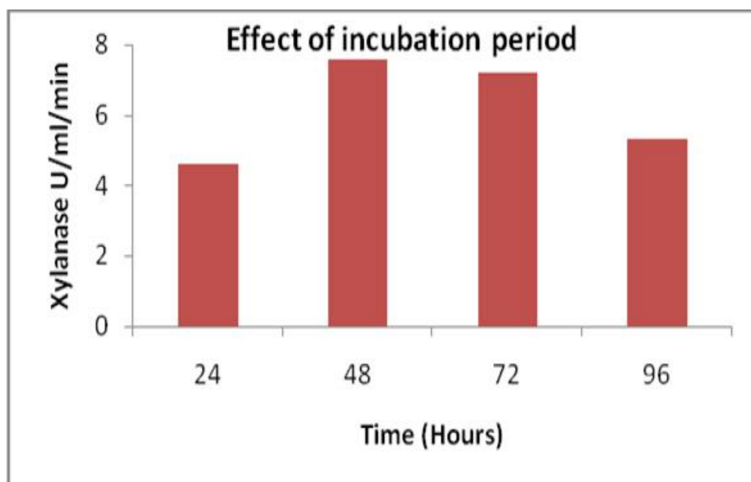


Fig. 5: Effect of incubation period on Xylanase production

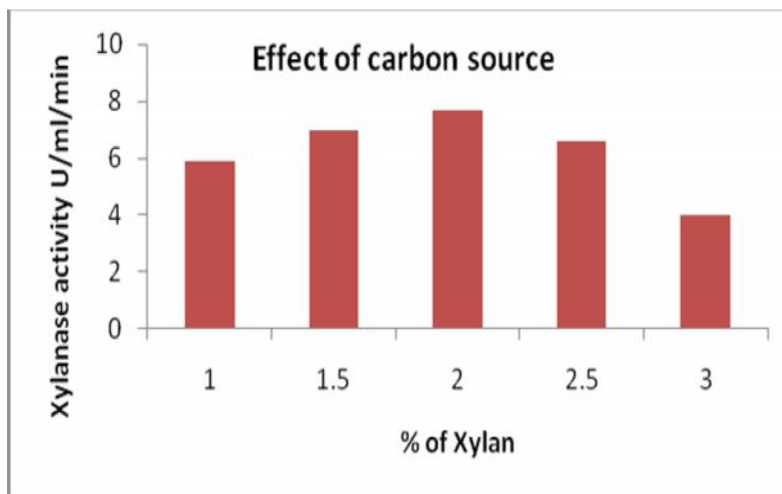


Fig. 6: Effect of Carbon source on Xylanase production

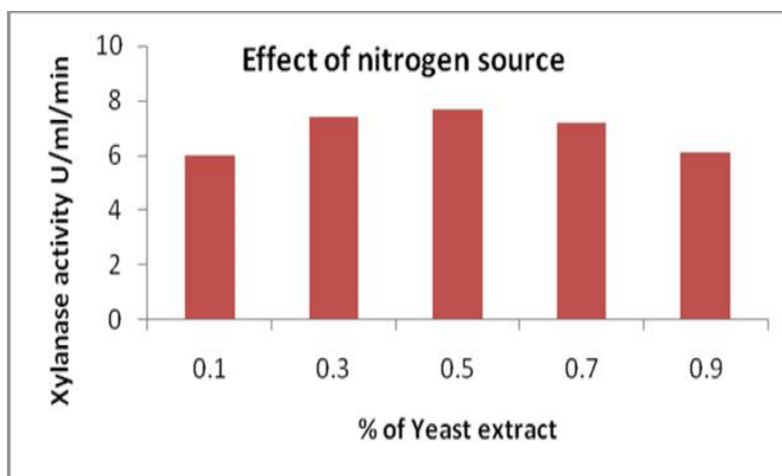


Fig. 7: Effect of nitrogen source on Xylanase production

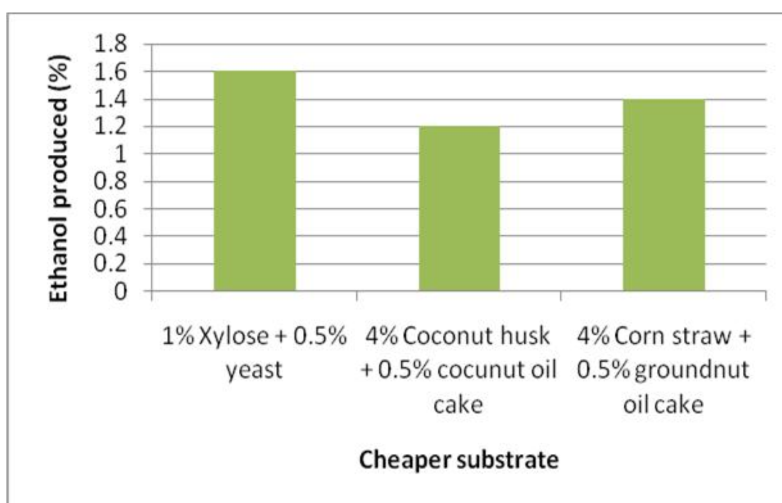
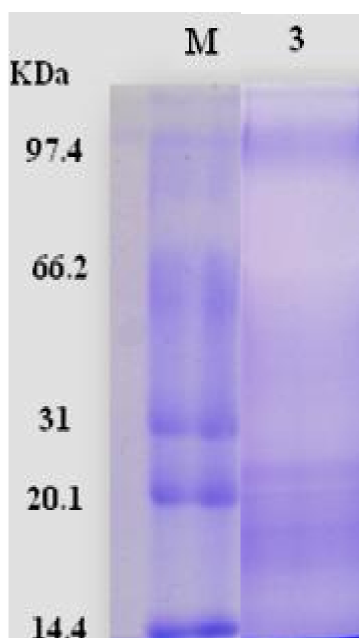


Fig. 8: Effect of cheaper substrates on Xylanase production



M: Molecular weight markers, 2: Partially purified xylanase.

Fig. 9: SDS-PAGE of partially purified Xylanase from *Clostridium sp.*

Xylanase from the anaerobic bacteria *Clostridium sp.* showed total of 3 bands. The molecular weight of different enzyme bands was compared with standard protein markers and the results showed that the molecular weight of protein ranged as 22, 31and 91 kDa (Fig.8). Only 22 kDa protein hydrolysed xylose.

Discussion

In the present study production of ethanol was carried out using a xylan hydrolyzing bacterium and an estuarine yeaset isolated from Vellar estuary. In the present investigation, xylanolytic anaerobic bacteria were around 1.9 x 10^6 CFU/g in sediment samples.

In the present investigation the most potential xylanase producing potential anaerobic bacterium was identified as *Clostridium sp*. Monisha *et al.,* (2009) reported *Bacillus pumilus* as a potential xylanase producing strain from soil sources of Bangalore, India.

Optimum conditions for xylanase producing *Clostridium sp.* were pH - 9, temperature - 40°C, NaCl - 2.5%, xylan - 2%, yeast extract - 0.5% and incubation period 48 hrs. When these optimum conditions were kept *Clostridium sp.* showed maximum xylanse activity (17.78 U/ml/min) at 48 hrs.

The molecular weight of xylanase from anaerobic bacteria *Clostridium sp.* ranged as 22, 31and 91 kDa (Fig. 8). However when plate assay was done only 22 kDa resulted in hydrolysis of xylan. Hence it was assumed to be the mol. Wt of xylanase. Further purification is going on Silas *et al.*, 2008 also reported 22 kDa as a molecular weight of xylanase isolated from *Aspergillus terreus*, UL 4209. The xylanase enzyme from a fungus *Plectosphaerella cucumerina* had a molecular weight of 19 kDa (Gui *et al*., 2007) and that obtained from alkaliphilic *Bacillus sp*. strain K-8 was of 24 kDa (Chakrit *et al.*, 2006). Xylanase produced by *B. circulans* was reported to be 38 kDa (Julio *et al.*, 2006). A second xylanase from *B. firmus* had a molecular weight of 45 kDa (Jen *et al.*, 2002). Thus xylanase obtained from varied sources had different molecular masses.

In the present investigation, the yeast strain *Pachysolen tannophilus* isolated from Vellar estuary was used for ethanol production. Yeast culture (i.e) *Pachysolen tannophilus* was added in separate flask containing cell free spent medium to produce ethanol. After 48 hrs from the mass scale culture, *Clostridium sp.* cells were centrifuged and removed. To this spent medium yeast culture was added, so that ethanol can be produced using the glucose present. Ethanol concentration in the fermentation broth was estimated by using gas chromatographic technique.

When the cell free extract was heavily inoculated with log phase culture of yeast (2%) and kept under anaerobic condition, at 28°C, 1.6% ethanol was produced at the end of 72 hrs. Panesar *et al.,* 2001 reported 1.92% ethanol at 40°C. Karnawat (2009) reported 1.8% using different bacteria and yeast.

Acknowledgments

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