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RESEARCH ARTICLE

INFLUENCE OF CARBON SOURCES ON PLANT CELL-WALL HYDROLYSING ENZYME PRODUCTION BY NEWLY ISOLATED *STREPTOMYCES* SP.

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Abstract

The underexplored microbial biodiversity of southern forest ranges in Kerala may have potent microorganism producing enzymes of industrial importance. Keeping this in mind, search for bacteria from forest regions near Agasthyamala in Kerala has been attempted in the present study. The newly isolated 18 cultures were showing clear zones near the bacterial growth on the media screened. Of the different cultures the UC A 31 showed the highest xylanase, CMCase and FPase activities. The 16 S rRNA sequencing and subsequent sequence alignment and phylogenetic tree building studies showed the possibility of the bacterium belonging to the genus *Streptomyces*. Maximum xylanase production occurred in cost effective method of using wheat bran as carbon source which showed three times higher production of that in xylan and xylose containing media.

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Introduction:-

Measures to regulate the problems caused by lignocellulosic waste is a difficult task facing most of the countries in the world. Enzyme such as cellulases and xylanases are highly valued enzymes in the treatment of lignocellulosics (Puentes-Téllez and Salles 2018). Treatment of waste at any level is highly needed in most of the cities all over world (Zuin, and Ramin 2018). Isolation and screening of microbes solubilising lignocellulosic substrates was complicated due to the fact that the degradation of each of the main components in the lignocellulosic substrates such as cellulose, hemicellulose and lignin require two or more enzymes. More over these enzymes are under the control of intricate pathways of biochemical and genetic regulation (Kluepfel, 1988). Microorganisms like bacteria are the potent resources for various enzymes that are of industrial importance. With this in mind the present study aimed at isolation of plant cell wall hydrolysing bacteria from forest soil samples

Methodology:-

Screening and strain improvement:-

Bacteria were isolated from various soil samples collected from different forest regions near Agasthyamala in Kerala. These newly isolated bacteria were spread out in wheat bran agar plates. The colonies that showed areas of clear zones with a minimum radius of 0.5 – 1.0 cm were selected. Microorganism was cultured on agar plates having the composition based on modified Horikoshi Basal medium (Subramaniyan et. al., 1997) as below:
 Wheat bran (1gm); Yeast extract (0.125gm); Peptone (0.125gm); MgSO₄ (0.02gm); K₂HPO₄ (0.1gm); Agar (2gm) and Distilled water (100ml).

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Growth studies:-

Aliquots taken from the growth medium was subjected to optical density measurement at 600nm against water blank. The pH of the growth medium taken in every 24 hours was measured using a pH meter.

From the culture broth periodically taken from the growth medium, about 5 ml was subjected to centrifugation at 10000 rpm for 10 minutes. Cell free culture supernatant was decanted from the centrifuge tubes and subjected to xylanase and cellulase enzyme studies and the sediment was dissolved in 3ml of 0.1M N NaOH. This was taken for cell protein studies.

Soluble protein:-

Soluble protein of the cell free culture supernatant was periodically estimated by Lowry's method (1951). Solution of 0.2ml was taken and the final volume was made up to 1 ml by adding 0.8ml distilled water. This was taken in a test tube added 5.0 ml of reagent C and kept for 10 minutes at room temperature.

Xylanase assay:-

Xylanase assay was conducted using birch wood xylan. The reaction mixture containing 1.8 ml of pre incubated oat spelt xylan suspension and 0.2 ml of enzyme preparation was incubated at 50°C for 10 minutes. The reaction was terminated by adding 3 ml of dinitro salicylic acid reagent. The concentration of reducing sugars was estimated against xylose standard by noting the absorbance at 540nm. The xylose (Xylose, Merk) stock solution (10 ml) and its different dilutions served as standards, absorbance of which was used to plot the standard curves. (Bailey et. al., 1992).

CMCase assay:-

The endoculcanase property of cellulases - CMCase assay was conducted by using carboxymethyl cellulose. The reaction mixture containing 1.0 ml of pre incubated 1% CMC solution in phosphate buffer ($\text{NaHPO}_4/\text{Na}_2\text{HPO}_4$) and 0.5 ml of enzyme preparation was incubated at 50°C for 10 minutes. The reaction was terminated by the addition of dinitro salicylic acid reagent which was then kept in boiling water bath for 5 minutes. The absorbance was measured at 540nm (Mandels et. al., 1976).

FPase assay:-

FPase is often regarded as representative of total cellulase activity. The substrate used here is filter paper (Whatman No. 1) strips of size 6 cm length and 1 cm breadth in 1ml phosphate buffer at pH-7. The reducing sugars released was detected using DNS reagent with absorbance at 540 nm (Mandels et. al., 1976).

Isolation of 16 S rRNA Gene - DNA Amplification

The selected bacterium was identified according to the following procedure. Genomic DNA was extracted through CTAB method (Wilson et al., 1987). 16S rRNA gene was amplified with forward and reverse primers - EUB 341f (5'- CCT ACG GGA GGC AGC AG -3') and EUB 907r (5' CCG TCA ATT CMT TTG AGT TT-3') (IDT, USA) under standard PCR conditions using a thermo cycler. The 16 S rRNA gene sequence obtained was compared with corresponding sequences of related organisms retrieved from GenBank database with BLAST algorithm for identifying the isolated strain using bioinformatics tools.

Effect of different carbon sources on enzyme production:-

Bacteria were cultured on liquid medium and 10ml of each of the sample was taken from it and added to different carbon sources in 13 conical flasks. The different carbon sources used were

1.	Sucrose	-	0.5gm
2.	Glucose	-	0.5gm
3.	Xylose	-	0.5gm
4.	Xylan	-	0.5gm
5.	CMC	-	0.5gm
6.	Wheat bran	-	0.5gm
7.	Rice husk	-	0.5gm
8.	Rice straw	-	0.5gm
9.	Rice bran	-	0.5gm
10.	Vegetable waste	-	0.5gm
11.	Bagasse	-	0.5gm
12.	Saw dust	-	0.5gm

All the 13 substances were taken separately in 13 flasks as carbon sources. Then the following chemicals were added

Yeast extract	-	0.25gm
Peptone	-	0.25gm
K ₂ HPO ₄	-	0.1gm
MgSO ₄	-	0.02gm
Distilled water	-	100 ml

The 13 flasks with the given medium were subjected to shaking for a period of 168 hours on a shaker at 120rpm at ambient temperature. Samples (5ml) were taken every 24 hours and after 168 hours all media were decontaminated and discarded. Cells were separated from each sample by centrifugation (10,000rpm, 10 minutes) and samples were used for measuring pH and optical density. The cells free supernatant was used as the extracellular crude enzyme preparation. The enzyme was used for estimating soluble protein, xylanase activity, CMCase activity and FPase activity.

Results and Discussion:-

Soil samples were collected from forest regions near Agasthyamala region in Kerala. Soils samples were transported in air tight containers and subjected to serial dilution. Isolation studies were conducted using wheat bran extract medium. All the isolated bacteria were grown and maintained in agar plates containing lignocellulosic materials such as CMC and wheat bran. Colonies that were showing good growth were selected and sub-cultured. These were then subjected to secondary screening. Selected cultures were then grown in liquid medium to assess the enzyme production capabilities.

Even though wheat bran contains several nutrients including protein, it also contains sufficient quantity of plant cell wall materials like cellulose and xylan. Use of wheat bran and carboxy methyl cellulose as the carbon source can give a clear idea regarding xylanase and cellulase production pattern by the selected bacterial strains. 18 cultures were obtained from the soil samples from different forest areas. The 18 cultures were tentatively given code names as UCWI(2), UCWI(3), UCWI(1), UCWII(3), UCWII(4), UC Slimy, UC White, UC Yellow, UC Orange, UC Y2, UC A11, UC A21, UC A22, UC A31, UC T1, UC T3, UC T4, UC T5.

Most of the cultures were showing clear zones near the bacterial growth. This was indicative of the extracellular production of lignocellulosic degrading enzyme production by different bacteria. However, this alone cannot quantify enzyme production by different bacteria.

This is only a qualitative analysis. The level of enzyme production could only be compared using liquid culture media. Liquid culture studies were carried out using modified Horikoshi medium. The components used are 0.25% each of Yeast extract and Peptone, 0.1% of K₂HPO₄, 0.02% of MgSO₄. Experiments were designed to incorporate lignocellulosics like wheat bran in order to obtain robust cultures that could grow well on lignocellulosics. Carboxy methyl cellulose was also used to isolate bacteria with cellulase producing capabilities.

The 18 cultures were grown in liquid medium for a period of 168 hours. Culture growth, pH and biomass were monitored at every 24 hours and the cell free supernatant was used for the estimations of xylanase activity (Bailey et. al., 1992), FPase and CMCase activities (Mandels et. al., 1976). Soluble protein (Lowry et. al., 1951) and reducing sugar (Miller, 1959) were also monitored.

Fixed quantities of samples were collected from the bacterial spent medium which was under batch fermentation. Sample collection was carried out at 24 hours interval. Cell free culture supernatant was obtained by centrifuging the bacterial liquid culture medium. The centrifugation was conducted at lower temperature so as to avoid damage due to heat generation at the time of centrifugation.

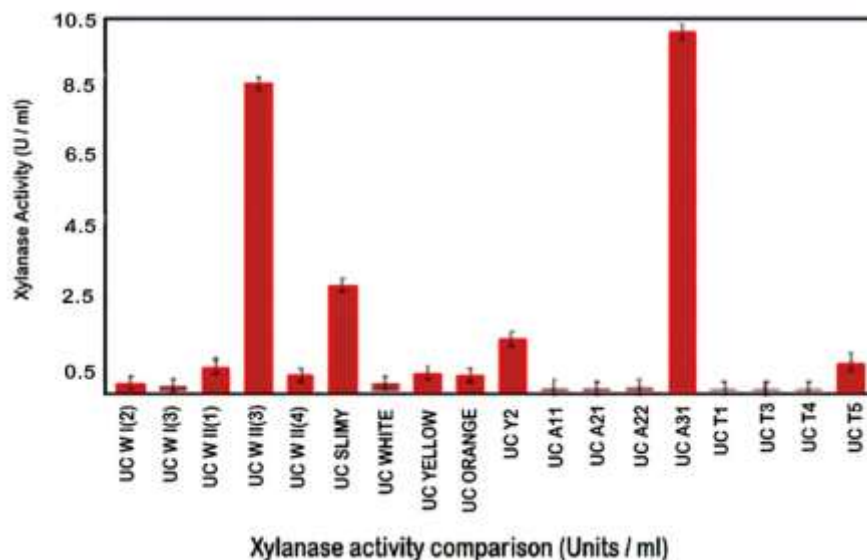


Figure 1:- Comparison of xylanase production in the selected 18 bacterial cultures .

Xylanase production estimated using Birch wood xylan (Sigma Co) showed a varying result. All the cultures were producing xylanases. The culture UC A31 showed the maximum activity of 10.07 U/ml. This was followed by the isolate UCWII (3) with an activity of 8.187U/ml (Figure. 1). Cellulase activities were also measured using CMCase assay and FPase assay. Cellulase activities were comparatively lower than xylanase activity. Maximum FPase production was effected by UCA31 with 0.698 an activity of U/ml. Maximum CMCase production was effected by UCWI (1) with an activity of 0.21 U/ml.

From among the newly isolated strains of bacteria the isolate no UC A31 was the most promising bacteria hence it was selected for further studies. Based on the screening study, it was observed that bacterium A31 could easily utilize lignocellulosic materials. This confirmed the effectiveness of the strategies used for the isolation of bacteria with plant cell wall hydrolysing enzymes. The bacterium A31 showed good clear zone around its colonies in a petri plate containing medium. The bacterium was rod shaped in morphology and answered positive to Gram stain (Figure 2).



Figure 2:- The culture isolate A31 isolated from Agasthyamala region was showing good growth in lignocellulosic substances and xylan

Identification of the bacteria was conducted by direct comparison. 16 S rRNA gene sequences of different species of the closely related bacteria resulting from BLAST were used for this study. The selected bacterial 16S rRNA sequences were loaded to the multiple sequence alignment bioinformatics tool CLUSTAL X version 2. After the alignment the tree output was saved in .phb format. The tree output was viewed in the tree viewing software Dendroscope 3. The results were given as Figure 3. The 16 S rRNA sequence of the potent strain A31 was clearly forming a prominent clade with good phylogenetic relation to the *Streptomyces* spp. Thus the systematic position at genus level of the newly isolated bacterium could be deduced as *Streptomyces*. The 16 S rRNA sequence from *Streptomyces* A31 was annotated and submitted to GENBANK with accession number KT379977.1.

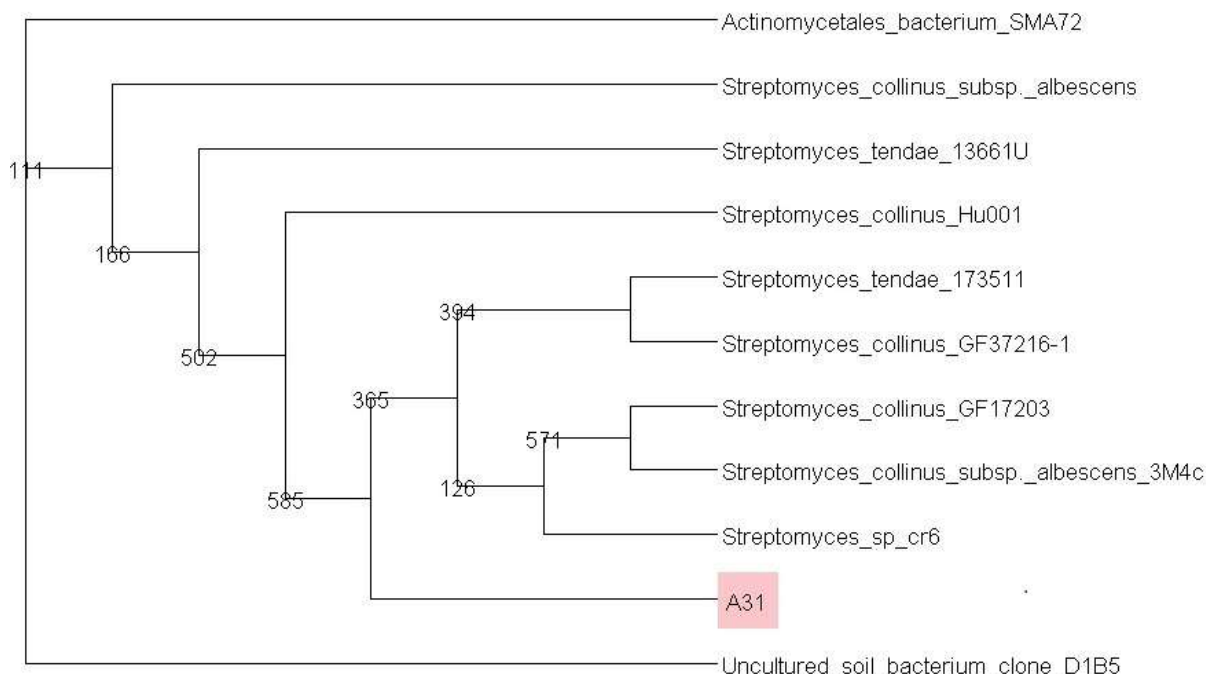


Figure 3:- Phylogenetic relationship of newly isolated bacteria A31 along with closely associated 16 S rRNA gene sequences of other *Streptomyces* sp. as resulting from BLAST analysis.

Influence of nutrients on the production of enzymes from newly isolated *Streptomyces* A31 was carried out using different carbon sources. The rate of assimilation of a carbon source could often influence the formation of biomass and production of primary and/or secondary metabolites. Rapid growth resulted from high concentrations of readily metabolized sugars were often associated with low productivity of metabolites (Inamine *et. al.*, 1969). Another important point to be considered during the selection is that a single carbohydrate material may act as both carbon and energy source. Considering this, studies were initiated for understanding the influence of carbon sources on the cellulase production by the potent isolate. For enzyme production submerged fermentation was carried out for a period of 168 hours with analysis of the cell free supernatant every 24 hours.

Carboxymethyl cellulase production varied in different carbon sources. Glucose medium was unique in expressing carbon catabolite repression. Sucrose resulted in slight increase in CMCase production. Similar to the present study there are previous reports of sucrose acting as inducer for CMCase induction as evidenced in the study with *Bacillus subtilis* A-53 which also grow well in medium containing yeast extract and sucrose (Lee *et. al.*, 2010).

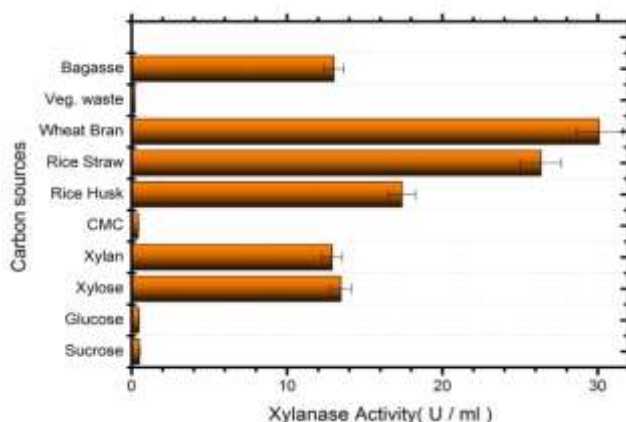


Figure 4:- Production of xylanase by the new isolate A31 in various media containing different carbon sources.

Filter paper assay was conducted using Whatman No. 1 filter paper. This enzyme is a measure of total cellulase activity. Cell free culture supernatant from all the media containing different carbon sources were analysed for FPase production. CMC resulted in the lower most induction for the FPase. Activity levels of xylanase are generally higher than that of both CMCase and FPases.

Of the different carbon sources tried, the xylanase activity was maximum in wheat bran (30 U/ml). In rice straw the xylanase activity was 27 U/ml (Figure 4). There are other reports regarding the elevated levels of xylanase and cellulase production by *Bacillus* spp. (Nakamura *et. al.*, 1993, Lee *et. al* 2008, 2010). Similar to the present study there are ample reports regarding the influence of wheat bran on elevated production of xylanases as in *Bacillus thermoalkalophilus* (Rajaram and Varma 1990) and *Streptomyces* (Vyas *et. al.*, 1990)

Conclusion:-

By careful manipulation of the growth environments, overproduction of metabolites by microorganisms could be elicited. Comparable level of xylanase activity was produced in Bagasse, Xylan and Xylose media with an activity near to 12 U/ml. The highest production in wheat bran is a promising result. The inclusion of wheat bran in the culture medium of newly isolated *Streptomyces* A31 resulted in the maximum level of xylanase production in wheat bran medium measuring 30 U/ml which is higher than that of both xylose and xylan which had an original activity of 10-12 U/ml). The cost effectiveness of wheat bran as substrate is evident as the cost of 1 gm Xylan is several times higher than the price of 1Kg of Wheat bran. Thus, the rate of assimilation of carbon source could often influence the formation of biomass and production of enzymes.

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