Utilization of Colocasia as Substrate for Cellulase Production

Gayatree Jadeja
Assistant Professor, Krishi Vigyan Kendra, Arnej, A.A.U., Gujarat, India

Dhwanika Patel
Assistant Professor, Smt. K.P.Patel College of Home Science, S.P.U., Anand, Gujarat, India

Apurva Goswami
Scholar, Smt. K.P.Patel College of Home Science, S.P.U., Anand, Gujarat, India

Abstract - The study was planned to fulfill the objectives like Isolate the cellulolytic bacteria and fungi, determination of the cellulase production from the colocasia as a substrate using the isolated cellulolytic fungi and determination of the optimal condition for the cellulase production condition such as Temperature (30˚c, 35˚c, 40˚c, 45˚c ana 50˚c), pH (Alkaline and Acidic), Time (24, 48, 72 hrs) and substrate (Colocasia Leaf, Root, Stem) and estimate CMCase activity, reducing sugar and protein. The cellulase biomass was also measured on dry weight base. This study revealed that colocasia leaf, stem and root which are very economic as well as produce large amount of cellulase enzyme when hydrolysed by cellulytic fungi in acidic pH. The suitable conditions for cellulase production are 50°C temperature and 24 hrs, 48 hrs and 72 hrs are suitable for stem, root and leaf, respectively. So instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase.

Keywords: Cellulase, Colocasia, Cellulolytic Fungi, Submerge fermentation

I. INTRODUCTION

The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component [1]. Plant biomass comprises an average of 23% lignin, 40% cellulose and 33% hemicellulose by dry weight [2]. Cellulose, a linear polymer of d-anhydroglucopyranase units linked by β-1,4-glucosidic bonds, with a degree of polymerization ranging from 15 to 10,000-14000, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature [3, 4]. Cellulose is the principal constituent of the cell wall of most terrestrial plants. The source of cellulose is in plants and it is found as micro-fibrils (2-20nm in diameter and 100 – 40,000nm long). These form the structurally strong frame work in the cell walls [5].

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis (i.e. the hydrolysis of cellulose). However, there are also cellulases produced by a few other types of organisms, such some termites and the microbial intestinal symbionts of other termites [6]. several different kinds of cellulases are known, which differ structurally and mechanistically [7].

Cellulases are of substantial industrial interest. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting, brewing and extraction of fruit and vegetable juices and pulp and paper industries [8]. In recent years, the interest in cell has increased due to many potential applications, for example, in the production of bio-energy and bio-fuel, in the textile industry and pulp and paper industry [9]. The most promising technology for conversion of lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulose enzyme [10].

Cellulase were initially investigated several decades back for the bioconversion of biomass which gave way to research in the industrial applications of the enzymes in animal feed , food industry, with the shortage of fossile fuels and the arising need to find alternative source for renewable energy and fuels,there is a renewal of interest in the bioconversion of lignocellulosic biomass using celluloses and other enzymes.

Colocasia is a genus of 25 or more species of flowering plants in the family Araceae, native to tropical polynesia and southeastern Asia. Its scientific name is colocasia esculenta. They are herbaceous perennial plants with a large rhizome on or just below the ground surface. The leaves are large to very large, 20–150 cm (7.9–59 in) long, with a sagittate shape. The elephant's-ear plant gets its name from the leaves, which are shaped like a large ear or shield [11]. In addition, the Colocasia has anti-cancer, Anti-inflammatory, Antioxidant / flavonoid glycosides effect.[12]. The main object of present work is to utilize low cost substrate colocasia which is produced in large scale in this country and use for the production of cellulases.

II. METHODOLOGY

The Present study was planned to fulfil the objectives like Isolate the cellulolytic bacteria and fungi. To Determine the cellulase production from the colocasia substrate using the isolated cellulolytic fungi ,To determine the optimal condition for the cellulase production condition such as Temperature (30˚C, 35˚C, 40˚C, 45˚C, 50˚c), pH (Alkaline and Acidic), Time (24, 48, 72 hrs) and substrate (Colocasia Leaf, Root, Stem) and estimate CMCase activity by Mandel"s and Andreoti, reducing sugar by DNS method and protein by Lowry method. Determine the dry weight of cellulase biomass Measurement of molecular weight of the sample which contains highest cellulase production was also perform using SDS PAGE gel electrophoresis.

Isolation of cellulolytic bacteria and fungi: Collect the sample of rotten wood from the yogi park near Greed, Anand. Isolate the bacteria on Nutreint Agar medium and fungi on Potato Dextrose Agar medium using Agar plate method [13]. Study the morphology and cultural characteristics of bacteria and fungi by Gram staining [14]. Motility test, Colony Characteristics.

Screening of cellulolytic fungi for cellulose production: The cellulolytic nature of fungi confirm first through the screening test. To this 1% of CMC was amended with Czepek-Dox agar media and the pH was adjust to 5. The media pour into sterile petri dishes, after solidification of media a small hole was made on the center of petri dishes aseptically and culture spore were added to this center. The plates were incubated for 3 days at 30˚c and 2 days at 50˚c. After incubation the plates were stained with 1% congo red solution for 15 min,after that the congo red stain was neutralized with 1M Nacl solution. The yellow colour zone formation concern the ability of cellulose utilization and enzyme activity of fungal culture [15].

Substrate: The substrate colocasia collected from Kanjri near Anand.

Preperation of Substrate: Leaves, Root , Stem of colocasia chopped into bits then oven dried slowly 50˚c for 2 days.Pulverised into coarse partical size then used as substrate.

Preperation of fungal spore inoclums for cellulose production in liquid fermentation medium: Loopful of isolated fungi inoculated in the 100ml of Czepek-Dox Thom's liquid fermentation media. After inoculation incubate at 37˚c for 48 hours.

Preperation of cellulase production media: Add 1% substrate [Leaves, Root, Stem]in the marry mandel's mineral salt solution fermentation media and 5% fungal inoculums from Czepek Dox Thom's liquid medium into mary mandel's mineral salt solution and incubate at 37˚c temperature for 24,48,72 hours.

Table 1. Mary Mandel's Mineral salts Solution [16]

Composition	gm/lit	Control	Alkaline (A)	Alkaline + Substrate (B)	Acidic (C)	Acidic + substrate (D)
Substrate (Root/Stem/ Leaves)	10g	-	-	√	-	√
Cellulose	10g	√	√	-	√	-
Peptone	1g	√	√	√	√	√
(NH4)2SO	1.4g	√	√	√	√	√
KH2PO4	2g	√	√	√	√	√
CaCl2	0.3g	√	√	√	√	√
MgSO4.7H20	0.3g	√	√	√	√	√
Urea	0.3g	√	√	√	√	√
pH	-	7.5	9.5	9.5	3.5	3.5

Extraction of enzyme: Sample withdrawn from the grown culture at 24, 48,72 hours and then filter it using whatmen filter paper no 1 to remove solid then used for different assay.

Dry weight of cellulose biomass [17]: Dry in an oven an empty aluminum weighing pan or a sheet of cellulose acetate filter membrane, 47mm in diameter, 0.45µm in pore size. Weigh them and store them in a desiccator lined with Drierite (anhydrous $CaSO_4$). Stir the flask to suspend the culture evenly. Pour out 100 ml of the culture into a graduated cylinder. Separate the cells from the broth either by centrifugation at 10,000 g for 5 minutes or by filtration. In the case of centrifugation, carefully discard the clear broth and scrape the cell paste from the centrifuge tube into a weighing pan. Rinse the centrifuge tube with a few ml of water. Pour the rinse water into the weighing pan, as well. In the case of filtration, the culture is poured into the holding reservoir fitted on the filter membrane. A vacuum is applied to pull the liquid through the membrane. Rinse the reservoir with a few ml of water and scrape any paste adhering to the glassware. The wet weight of the culture is measured immediately after all the water has been pulled through. Dry the cell paste in an oven set at 100ºC. The cells will be charred and the filter membrane will be burned if the temperature of the oven is set too high. Measure the weight of the pan/filter plus the cell paste periodically until there is no further decrease in the dry weight. It will take 6-24 hours to dry the sample completely, depending on the oven temperature and the thickness of the paste. Calculate the difference in the weight, and express the dry weight in g/l.

Carboxy methyl cellulose assay for cellulose [18] Endoglucanase assay: Endoglucanase activity of fungal culture was quantified by carboxy methyl cellulose method. According to this method, 0.5ml of 1% carboxy methyl cellulose as a substrate was added to the 0.5ml of enzyme .This mixture was incubated at 50˚c in a water bath for 30 min. The reducing sugar produced in the reaction was estimated by DNS method. After incubation, 3ml of DNS reagent was added to each test tube and boiled for 5 min in a boiling waterbath. After boiling transfer to a cold water bath and added 10 ml of distlled water.Mixed completely inverting the tube several time so that the solution seperates from the bottom of the tubes at each inversion. The color developed in the test tubes was read at 540nm in a spectrophotometer. The enzyme activity was expressed in terms of CMC units. CMC units were defined as the amount of enzyme releasing IU/ml (µ mole of reducing sugar from the substrate per minute per ml).

Glucose Estimation [19]: Enzyme removed from the fermentation media were assayed for total reducing sugars using DNSA method. The sample, control and glucose standards were prepare using the different aliquot of sample and standard in test tube and add 3 ml DNS reagent.The mixture was boil for 5 minutes after that cool it and add 10ml d/w in each test tube then absorbance were read at 540 nm using a spectrophotometer.plot the standards on semilog paper(log percent T versus concentration).

Protein Estimation [20]: The culture was mixed with 0.1ml 2N NaOH and hydrolyzed for 10 minute at 100˚c then treated with 1ml complex forming reagent (freshly prepared) incubated 10 minutes at room temperature then 0.5ml Folin-ciocalteau reagent was added.After 30 minutes of incubation in a dark place the colour developed was measured by spectrophotometer at 660nm.

Ammonium sulphate precipitation: It is very important to use fresh, desiccated ammonium sulfate. This ensures uniform and rapid dissolution.The day before use, place ammonium sulfate over night in ca. 120°C drying oven in a large beaker or drying dish (ammonium sulfate decomposes at 220°C).Clean grinder carefully and grind dry ammonium sulphate to a fine powder. Wear a dust mask (if you don't need a mask, the powder is not dry enough).Select the Amount of ammonium sulphate powder From Ammonium Sulphate Concentration table.Use ground powder immediately.Add the powder slowly but steadily with thorough mixing. Do not allow clumps to form.Allow precipitate to form for 30 minutes at 4°C with stirring.Recover precipitate by centrifugation. Solutions highly saturated in ammonium sulfate are quite dense and it can be difficult to pellet the precipitate. Remove supernatant, re spin briefly to clear remaining ammonium sulphate.Re suspend pellets in a volume of buffer equal to the volume of the extract.

SDS-PAGE gel electrophorsis: It was carried out for determination of the protein contain of the highest cellulose producing producing media.

Determination of optimal condition for cellulose production

A. Effect of varying Time: Cellulase activity was measured at regular intervals like 24, 48, 72 hours.
B. Effect of varying pH: The pH of the fermentation media were adjust to acidic(3.5),alkaline(9.5) and control (7.5) with 0.1 N Hcl or 0.1 N NaOH.

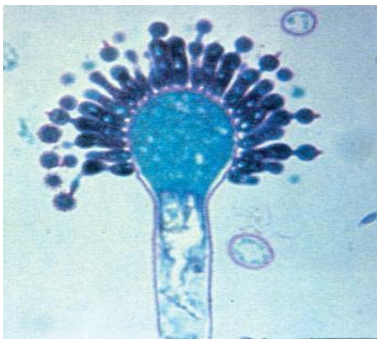
C. Effect of varying Temperature: The fermentation was carried out at different temperature like 30°C, 35°C, 40°C, 45°C and 50°C.
D. Effect of varying Substrate: The fermentation was carried out using three different part of colocasia like Root, Stem, Leaf.

III. RESULT AND DISCUSSION

Isolation of cellulolytic bacteria and fungi: Prepared suspension with dilution and used for Spreading on Agar plate and Potato Dextrose agar medium .In Agar plate different type of bacterial colonies observed and in potato Dextrose Agar different type of fungal growth observed .Colony transferred From that bacterial colony and Fungal growth shows the isolated growth of bacteria on the Agar plate and fungi on potato Dextrose Agar.

Screening of cellulolytic fungi using agar plate method (zone method): Three isolated strain of fungi was Screened for cellulose Enzyme Production .The formation of Yellow zone of hydrolysis shows its ability for cellulose Production. From the IF_1 , IF_2 , IF_3 . From that Fungi IF_2 shows maximum Zone of hydrolysis which indicates CMC degradation as in figure 1.

Zone of hydrolysis



Isolated fungi IF_2

Figure 1 Isolated strain of fungi

In prepared media inoculate the isolated strain of fungi. From the three isolated fungi IF_2 was used for the enzyme production because it shows Highest Enzyme activity.

Carboxy methyl celluase assay for cellulose: In Leaf substrate at 24, 48 and 72 hours incubation the CMCase activity was found highest in D at 30˚C , 35º C and 40˚C and at 45˚C and 50˚C temperature the C Shows the highest CMCase activity. The lowest CMCase activity was found in control media at 30˚c, in the A media the CMCase activity was at 35˚C , 45˚C and 50˚C as well as the B media shows the CMCase activity at 40˚C temperature. All the media A, B, C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media. In Stem substrate at 24, 48 and 72 hours incubation the CMC activity was found highest in D at 35˚C, 40˚C, 45˚C and 50˚C and in C at 30˚C. The lowest CMC activity was found in control media at 30˚C, 35˚C, 40˚C, 45˚C and 50˚C. All the media A, B, C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media. In Root Substrate at 24, 48 and 72 hours incubation the CMC activity was found highest in D at 30˚C , 35˚C ,50˚C. At 40˚C and at 45˚C temperature the C Shows the highest CMC activity in Root substrate. The lowest CMC activity was found in control media at 50˚c, in the A media the CMC activity was at 45˚C, 50˚C as well as the C media shows the lowest CMC activity at 30˚C, 35˚C and D at 40˚c Temperature. All the media A, B,C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media.

In substrate Leaf in D media at 40˚C temperature and 72 hours (0.547 IU/ml), in substrate Stem in D media at 50˚C temperature and 48 hours (0.830 IU/ml), in substrate Root in D media at 50˚C temperature and 48 hours (2.719 IU/ml) shows highest CMCase activity.

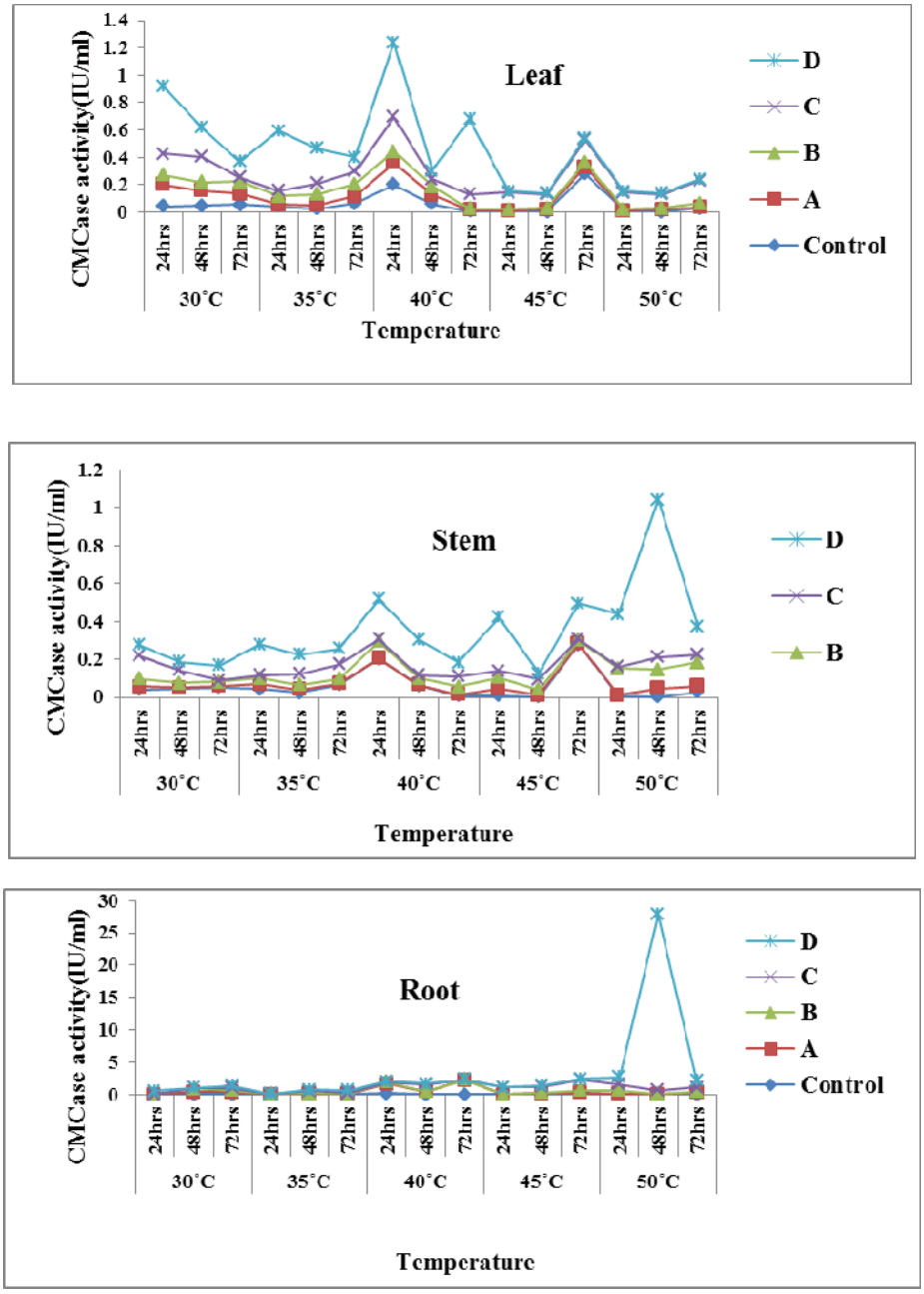


Figure 2: CMCase activity of colocasia substrate at different temperature, time and media

DNS method for Glucose Estimation: In Leaf substrate at 24, 48 and 72 hours incubation the Glucose production was found highest in D at 30˚C, 35˚C, 40˚C, the C Shows the highest Glucose production at 45˚C, 50˚C. The lowest Glucose production was found in control media at 30˚C, 35˚C, 45˚C, 50˚C. In the D media the Glucose production was at 40˚c shows the lowest Glucose production. All the media A, B ,C ,D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media. In Stem substrate at 24,48 and 72 hours incubation the Glucose production was found highest in D at 35˚C ,40˚C ,45˚C, 50˚C, the C Shows the highest Glucose production at 30˚C. The lowest Glucose production was found in control media at 35˚C, 45˚C, 50˚C. In the A media the Glucose production was at 30˚C, 40˚C shows the lowest Glucose production. All the media A, B, C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media. In Root substrate at 24, 48 and 72 hours incubation the Glucose production was found highest in D at 30˚C ,35˚C,50˚C, the C Shows the highest Glucose production at 45˚C and A media at 40˚C . The lowest Glucose production was found in control media at 30˚C,

35˚C, 40˚C, 45˚C, 50˚C. All the media A, B, C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media.

In substrate Leaf in D media at 50˚C temperature and 48 hours (1.945 mg/ml), in substrate Stem in D media at 45˚C temperature and 24 hours (3.594 mg/ml), in substrate Root in D media at 35˚C temperature and 48 hours (1.814 mg/ml) shows highest Glucose production.

Lowery method for protein Estimation: In Leaf substrate at 24, 48 and 72 hours incubation the Protein secretion was found highest in D at 30˚C, 35˚C, 40˚C, 45˚C, and B media at 50˚C temperature. The lowest Protein secretion was found in Control media at 45˚C, 50˚C. In the A media the Protein secretion was at 30˚C, 35˚c,40˚c . All the media A, B ,C ,D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media. In Stem substrate at 24, 48 and 72 hours incubation the Protein secretion was found highest in D at 30˚C, 45˚C, and B media at 35˚C, 40˚C, 50˚C temperature. The lowest Protein secretion was found in Control media at 45˚C, 50˚C. In the A media the Protein secretion was at 30˚C, 35˚C, 40˚C. All the media A, B, C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media. In Root substrate at 24, 48 and 72 hours incubation the Protein secretion was found highest in Control media at 30˚C, 35˚C, 40˚C, 45˚C and 50˚C. The lowest Protein secretion was found in the A media the Protein secretion was at 30˚C, 35˚C, 45˚C and 50˚C and in D media at 30˚C. All the media A, B, C, D at different temperature shows the highly significant ($p<0.01$) and significant difference ($p<0.05$) compared to the control media.

In substrate Leaf in D media at 35˚C temperature and 72 hours (17.505 mg/ml), in substrate Stem in A media at 30˚C temperature and 72 hours (17.057 mg/ml), in substrate Root in Control media at 30˚C temperature and 48 hours (8.047 mg/ml) shows highest Protein secretion.

Dry weight of cellulose biomass: Acidic + substrate showed highest activity at 40˚C, 50˚C at 24, 48, 72 Hours. From the result of enzyme activity the acidic + Substrate media select for the Dry Weight of biomass. At 50˚C the Leaf Substrate was incubate for 72 hours, Stem for 24 hours and Root for 48 hours in the Acidic + Substrate media (Table 2).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): Molecular weight was determined by SDS gel as described in material and method used BSA as a marker .Molecular weight of BSA is 66,000Da. Analysis of the enzyme by SDS-PAGE were shows the bands of three different sample of Acidic + Substrate media containing substrate Leaf, stem, Root.

Table 2 Dry weight of cellulase biomass (gm/100ml) of Acidic + Substrate media with different substarate

Sr. No	Sample	Temperature (50˚ C)
24 HOURS		
1	LEAF	5.1 gm
2	STEM	4.5 gm
3	ROOT	3.1gm
48 HOURS		
1	LEAF	7.4 gm
2	STEM	4.2 gm
3	ROOT	3.8 gm
72 HOURS		
1	LEAF	8.8 gm
2	STEM	3.6 gm
3	ROOT	1.4 gm

Cellulose is the most abundant renewable natural resource in the world and a potential source for the production of industrial useful materials such as fuels and chemical. Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. Cellulases are of substantial industrial interest. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting, brewing and extraction of fruit and vegetable juices and pulp and paper industries . Cellulolytic fungi can use cellulose as a primary carbone source. Pure cellulose such as cotton and avicel are good cellulase inducers, but expensive and noneconomical, consequently the use of less expensive substrates can decrease the production cost .taro is an important staple food crop grown throughout many pacific Island countries part of Africa, Asia and the Caribbean for its freshly corm and its nutritious leaves. Taro corm is excellent source of carbohydrate

and low in fat and protein whereas taro leaves contain higher source of protein and are also excellent source of carotene, Potassium, calcium, phosphorus, iron, riboflavin, thiamin, niacin, vitamin A, vitamin C and dietary. Taro is the one of the few major staple food where both the leaf and underground parts are important in human diet as well as very economical compared to the other foods. So in the present study the attempt was to use colocasia as substrate for cellulase production.

IV. CONCLUSION

Study revealed that colocasia leaf, stem and root which are very economic as well as produce large amount of cellulase enzyme when hydrolyzed by cellulytic fungi in acidic pH. The suitable conditions for cellulase production are 50°C temperature and 24 hrs, 48 hrs and 72 hrs are suitable for stem(0.830 IU/ml), root(2.719 IU/ml) and leaf(0.547 IU/ml), respectively. So instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase. For the further research aspect the cellulase production can be measured using bacterial strain as well as by changing the composition of the media preparation.

REFERENCES

[1] Saleem F, Ahmed S, Jamil A (2008). "Isolation of a xylan degrading gene from genomic DNA library of a thermophilic fungus *Chaetomium thermophile* ATCC 28076". *Pak. J. Bot*. **40**: 1225-1230.

[2] Ahmed S, Jabeen A, Jamil A (2007),"Xylanase From *Trichoderma Harzianum*: Enzyme Characterization And Gene Isolation". *J. Chem. Soc. Pak.* **29**: 176-182.

[3] Tuomela, M., M. Vikman, A. Hatakka, M. Itavaara (2000). "Biodegradation Of Lignin In A Compost Environment".. *Bioresource Technol*.**72**:169-183.

[4] Gardner, K.H. and Blackwell J. (1974)." The Structure Of Native Cellulose". Biopolymers., **13**:1975- 2001.

[5] Omojasola, P. Folakemi, Jilani, Omowumi Priscilla And Ibiyemi, S.A.(2008)," Cellulase Production By Some Fungi Cultured On Pineapple Waste". *Nature And Science*, **6(2)**.

[6] Watanabe H., Hiroaki Noda, Tokuda G., Lo N. (1998) ,"Role Of The Termite Gut Macrobiota In Symbiotic Digestion", *Biology Of Termites: A Modern Synthesis 2010*: Ch. 16.

[7] Sulzenbacher G, Shareck F, Morosoli R, Dupont C, And Davies Gj (1997). "The *Streptomyces Lividans* Family 12 Endoglucanase: Construction Of The Catalytic Cre, Expression, And X-Ray Structure At 1.75 Å Resolution". Biochemistry **36**: 16032–16039.

[8] Sipos B, Benko Z, Dienes D, Réczey K, Viikari L, Siika-Aho M (2010). "Characterisation Of Specific Activities And Hydrolytic Properties Of Cellwall- Degrading Enzymes Produced By *Trichoderma Reesei* Rut C30 On Different Carbon Sources". *Appl. Biochem. Biotechnol*. **161(1-8)**: 161: 347-64.

[9] Zhou J, Wang Yh, Chu J, Zhuang Yp, Zhang Sl, Yin P (2008)."Identification And Purification Of The Main Components Of Cellulases From A Mutant Strain Of *Trichoderma Viride T* 100-14". *Bioresour. Technol*. **99**: 6826-6833.

[10] Ali S, Ahmed S, Sheikh Ma, Hashmi As, Rajoka Mi, Jamil A (2009), "Lysine Production By L-Homoserine Resistant Mutant Of *Brevibacterium Flavum*". *J. Chem. Soc. Pak*. **31**: 97-102.

[11] Wagner, W. L., D. R. Herbst, And S. H. Sohmer (1999)." *Manual Of The Flowering Plants Of Hawai'I".Bishop Museum Press*.1357.

[12] Biren N. Shah.(2007)," The anti-inflammatory activity of the leaves of colocasia esculenta" .*Saudi Pharmaceutical Journal.* **15**:3-4.

[13] Jett, B. D., Hatter K. L., Huycke M. M., and Gilmore M. S. (1997)," Simplified agar plate method for quantifying viable bacteria". *BioTechniques*.**23**:648–650.

[14] Lillie RD. (1977), The Gram Stain: A quick method for staining gram positive organisms in tissue". *Arch Path*. 5:828-834.

[15] Pradeep M. Reddy And Narasimha G.(2011),"Utilization Of Pea Seed Husk As A Substrate For Cellulase Production By Mutant Aspergillus Niger". *Insight Biotechnology.* **1(2)**.

[16] Jeffries, T.W., (1996). *Production and Applications of CellulaseLaboratory Procedures*, 1-10.

[17] Mary Mandels(1974), "Production and Applications of Cellulase" Laboratory Procedures Handbook.

[18] Mandels M ,and Andreoti R .C(1976),"Measurement Of Saccharifying Cellulase ".*Biotechnol. bioen. symp*., 6:21-23.

[19] Miller ,G. L .(1959),"Use Of Dinitrosalicyclic Acid Reagent For Determination Of Reducing Sugar". *Anal. Che.* ,31:426-428.

[20] Lowery ,O .H., Rosebrough N . J., Farr A .L . and Randall R .J.(1951) " Protein Measurement With The Folin Phenol Reagent ". *Journal of Biol . chem*. 193:265-275.