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Research Article

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Comparative Studies on Biochemical Characteristics and Screening of Medium Components for Cellulase Production from *Manihot esculenta* Cranz. Ytp 1 and H740/92 Stem Using *Cellulomonas Fimi* MTCC 24

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ABSTRACT

Two varieties of cassava stems, YTP1 and H740/92, were collected Biochemical composition of were investigated by determination of total moisture, protein, lipid, cellulose, hemicellulose, crude fiber, starch and soluble sugars. Optimization of medium composition for cellulase enzyme production by Cellulomonas fimi MTCC24 from Manihot esculenta Cranz YTP 1 and H740/92 Stem as the source in submerged fermentation .Effect of different media components such as carbon, nitrogen, and minerals on cellulase production by Cellulomonas fimi MTCC24 in shake flask culture was investigated by one factor at-a-time method. The results indicated that various factors including carbon, nitrogen and minerals, influence cellulase production. The optimum fermentation medium contained a carbon source ((0.3%) pretreated cassava stem), nitrogen ((0.35%) malt extract and (0.25%) ammonium chloride), macronutrients ((0.45%) sodium chloride, Calcium chloride and magnesium sulphate) and micronutrient ((0.001%) manganese sulphate). Under these conditions, the maximum level of cellulase production was observed 8 IU/ml for YTP 1.

Keywords: Cellulose; *Manihot esculenta* Cranz*;* One factor at-a-time method; *Cellulomonas fimi*

INTRODUCTION

Renewable energy relies principally on plant and animal materials as their feedstock, of which the most dominant being the plant materials as the energy crops. An energy crop is a plant grown as a low cost and low maintenance harvest used to produce biofuels, or directly exploited for its energy content. Conventional energy crops include sunflower (*Helianthus annus*), Barbados nut (*Jatropha curcas*), maize (*Zea mays*), sugarcane (*Saccharum officinarum*), and soyabean (*Glycine max*).Cassava (*Manihot esculenta Cranz*) is a very important crop grown for food and industrial purposes in several parts of the tropics [1]. *Manihot esculenta* is the third most important source of calories in the tropics, after rice and maize. Millions of people depend on cassava in Africa, Asia and Latin America. The broad agro-ecological adaptability of cassava and its ability to produce reasonable yields makes it an important bioenergy crop. *Manihot esculenta* stems are one of the agricultural residues that could be considered for bioconversion in tropical countries [2]. Potential applications of these materials include activated carbon production and energy generation; however, the cassava stems are often left in the field, due to their low monetary value, or are burned, causing environmental problems. It can be considered to be an alternative source for the production of bioethanol, and the effects associated with leftover cassava could be mitigated [3,4].

Conversion of cellulosic biomass is a sustainable approach to develop novel products. Microbial cellulases become the focal biocatalysts due to their complex nature and wide spread industrial applications [19].Cellulases are widely synthesized by large diversity of microoraganisms of both bacteria and fungi. A number of microorganisms

(*Clostridium, Cellulomonas, Thermomonospora, Trichoderma,* and *Aspergillus*) are capable of producing extracellular cellulase enzyme and among which *Cellulomonas fimi* widely used candidates for cellulase enzyme production [20]. Cellulase is a family of at least 3 groups of enzymes endo-(1,4)-β-D-glucanase (EC 3.2.1.4) exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21).Cellulase mediates endohydrolysis of (1→4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. Cellulolytic enzymes have demonstrated their biotechnological potential in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry.

Medium optimization by one-factor-at-a-time method involves changing one variable (nutrients, pH, temperature, etc.) while fixing the others at a certain arbitrary levels [23]. The conventional ‘‘one-factor-at-a-time’’ approach is laborious and time consuming, especially for large number of variables. Moreover, it seldom guarantees the determination of optimal conditions. In this present work biochemical composition of *Manihot esculenta Cranz YTP 1* and *H740/92* were investigated and medium components were optimized by one-factor-at-a-time method was applied to the selection of medium components that significantly influenced the production of cellulase from cassava stem by *Cellulomonas fimi MTCC24.*

MATERIALS AND METHODS

Biochemical characterization

Two varieties of *Manihot esculenta Cranz YTP 1* and *H740/92* Stem were collected from Tapioca and Castor Research Station, Yethapur, Salem District, Tamil Nadu, India. Cassava stems were dried in hot air oven at 60°C to constant weight. Then stems were ground into fine particle and sieved using sieve shaker and stored at 4°C.Moisture, ash, lipid and total solids were estimated by standard AOAC 2000 methods. Starch [9], protein [10] , cellulose [11] hollocellulose and hemicellulose [12] were determined for biochemical profiling.

Medium components

Magnesium sulphate, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate, calcium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride, yeast extract, peptone, beef extract, malt extract, sodium molybdate, ammonium nitrate, ammonium chloride, ammonium phosphate and ammonium carbonate were purchased from Hi-Media Limited, Mumbai, India.

Microorganism and culture condition

Cellulomonas fimi MTCC24 was procured from MTCC, Chandigarh, India and maintained on Nutrient agar medium at 28^0C for overnight .After the incubation the slants were kept at 4^0C and thereafter sub-cultured every 30 days.

Substrate preparation

Initially, 3mL acetic/nitric reagent was added to 1g of the sample in a test tube and mixed in a vortex mixture. The tube was placed in a water bath at 100°C for 30min. Then cooled and centrifuged the contents for 15-20min. The supernatant discarded. The residue washed with distilled water and dried in hot air oven at 60°C [11].

Cellulase Assay

Cellulase enzyme produced in the medium determined using Dension and Koehn (1997).

Optimization of production medium using one-factor-at-a- time method

Effect of carbon source: The carbon source is metabolized can often influence the formation of biomass production. Pretreated *Manihot esculenta Cranz YTP 1* Stem provided as carbon source for production media. To study the effect of carbon sources for biomass production, glucose was replaced by cassava stem was further optimized in the range of 1-5 g/L.

Effect of nitrogen sources: Various organic and inorganic nitrogen sources were added to the fermentation medium at 2 g/L. To study the effect of different nitrogen sources on biomass production, yeast extract was replaced with other organic as well as inorganic nitrogen sources such as peptone, beef extract and malt extract. Malt extract was further optimized in the range of 0.5–4.5 g/L. The inorganic nitrogen sources screened were ammonium nitrate, ammonium sulphate, ammonium chloride, ammonium phosphate and ammonium carbonate. Ammonium chloride was further optimized in the range of 0.5-4.5 g/L.

Effect of minerals: Microorganisms require certain mineral elements like macro and micro nutrients for growth and metabolism. A series of conical flasks containing 100 ml sterile medium was supplemented with macro nutrients like calcium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride and magnesium sulphate by substituting sodium chloride in the basal production medium at concentration of 3 g/L. Sodium chloride, calcium chloride and magnesium sulphate was further optimized in the range of 0.5–4.5 g/L. for improved cellulase production. Micro nutrients zinc sulphate, copper sulphate, manganese sulphate, ferrous sulphate and sodium molybdate were supplemented in the medium at concentration of 0.01g/L.

STATISTICAL ANALYSIS

One-way ANOVA technique and followed by t-test were employed for the testing of significance of the result based on p-value ($p<0.05$).

RESULTS AND DISCUSSION

The biochemical composition of dried *Manihot esculenta Cranz YTP 1* and *H740/92* Stem is given in Table1. Balamurugan and Anbuselvi (2013) reported biochemical constituents of cassava pulp, leaves and waste. Nuwamanya *et al.*,(2012) studied the amount of dry matter varied among different plant parts with dry matter content of peels (30.5%) and stems (28.77%) comparable to that of roots (38.60%). Han *et al*., (2011) revealed the chemical composition of the cassava stem varies according to the growing location, season, harvesting methods and analysis procedure, the lignin, cellulose and hemicellulose fraction was about 33.8%, 35.2% and 24.3% respectively. Sovorawet and Kongkiattikajorn (2012) proposed cellulose content of cassava stalk was relatively high compared to that of hemicellulose and cassava stalk could be a good source of cellulose. Cellulose was the major component, and it was in the range of 61-63.6% (w/w).

The second major compound was Crude fibre and in the range of 39-49% (w/w).The total moisture content varied in the range of 50.33%–51.3% (w/w). The values were somewhat consistent with the report of Magesh *et al* 2011. Total starch and soluble sugar varied in the range of 1.248-1.693 % (w/w) and 2.53-2.71 % (w/w). Hemicellulose content in the cassava stem varied in the range of 8.2-8.6% (w/w). Total protein and nitrogen content was in the range of 0.055-0.057 % (w/w) and 0.0088-0.0092% (w/w). Pectin content in the cassavas stems were varied in the range of 5.5-3.5 % (w/w). Lipid content varied as 0.811-0.836 % (w/w). YTP 1 consists 63% cellulose on its dry weight basis from the biochemical profile and selected for the cellulase production.

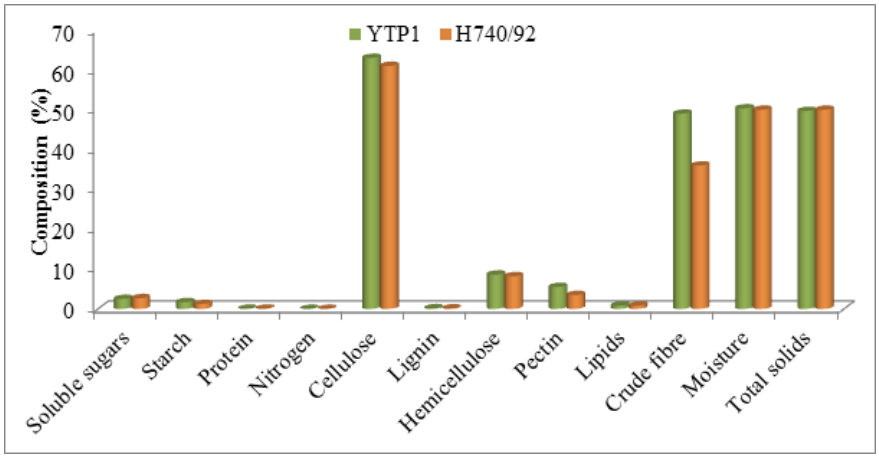

Figure 1: Biochemical profile of YTP1 and H740/92

Nitrogen sources and mineral salts have the dramatic effects on cellulase production by *Cellulomonas fimi* MTCC 24. In this study various organic and inorganic nitrogen sources and macro& micro nutrients were tested. A significant difference was observed and proved statistically by using One-way ANOVA followed by t-test.

Table 1: Biochemical composition of cassava stems

Constituents (G/100g)	Cassava stem %(w/w)	
	YTP1	H740/92
Cellulose	63±0.02	61±0.012
Hemicellulose	8.6±.06	8.2±0.026
Holocellulose	71.6±0.07	69.2±.03
Total starch	1.693±0.042	1.248±0.058
Total soluble sugar	2.53±0.03	2.71±0.075
Total protein	0.055±0.01	0.0575±0.01
Total nitrogen	0.0088±0.001	0.0092±0.001
Pectin	5.5±0.015	3.5±0.012
Lipid	0.811±0.013	0.836±0.005
Crude fibre	49±0.03	39±0.028
Moisture	50.33±0.04	51.3±0.039
Total solids	49.67±0.04	48.7±0.059

A hypothesis test in statistics, a *p*-value helps to determine the significance of results. P value is statistically significant as $P < 0.05$ and statistically highly significant as $P < 0.001$.Table 2 shows the variance analysis of organic &inorganic nitrogen sources and macro & micronutrients on cellulase activity(IU/ml).Figure 2 a-d shows the cellulase activity for various nitrogen and mineral sources.

Table 2: One-way ANOVA for effect of various sources on Cellulase activity (IU/ml)

Organic nitrogen sources							
	Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
yeast extract	Between Groups	14.07859	1	14.0785	12.9295	0.00242	4.49399
Beef extract	Between Groups	14.07859	1	14.0785	12.9295	0.00242	4.49399
Peptone	Between Groups	7.803884	1	7.80388	6.46877	0.02169	4.49399
Malt extract	Between Groups	2.88	1	2.88	1.3537	0.00169	4.49399
Inorganic nitrogen sources							
A. nitrate	Between Groups	0.905409	1	0.9054	0.55003	0.46904	4.49399
A.sulphate	Between Groups	2.136934	1	2.13693	1.37698	0.25778	4.49399
A. chloride	Between Groups	10.58	1	10.58	5.15076	0.03741	4.49399
A.phosphate	Between Groups	0.013889	1	0.01388	0.00714	0.93369	4.49399
A.carbonate	Between Groups	0.847602	1	0.8476	0.51287	0.48422	4.49399
Macronutrients							
KHPO4	Between Groups	0.013889	1	0.01388	0.00714	0.93369	4.49399
K2HPO4	Between Groups	0.956806	1	0.9568	0.58993	0.45363	4.49399
CaCl2	Between Groups	6.043606	1	6.0436	3.08783	0.09799	4.49399
MgSO4	Between Groups	6.944022	1	6.94402	3.33541	0.08652	4.49399
NaCl	Between Groups	10.42722	1	10.4272	5.13964	0.03759	4.49399
Micronutrients							
ZnSO4	Between Groups	5.700939	1	5.70093	3.02929	0.10096	4.49399
MnSO4	Between Groups	11.045	1	11.045	5.16951	0.03711	4.49399
FeSO4	Between Groups	4.940272	1	4.94027	2.7791	0.11495	4.49399
Na2MoO4	Between Groups	10.58	1	10.58	5.15076	0.03741	4.49399
CuSO4	Between Groups	0.032089	1	0.03208	0.01418	0.90668	4.49399

The pretreated cassava stem taken as acarbon source varied in the range of 1-5g/L and observed that cellulase activity was maximum at 3g/L (Figure 3). From the one-way analysis, p-values were calculated for organic nitrogen sources and varies in the range of 0.00169-0.02169.The organic nitrogen sources on cellulase production follows the sequence, malt extract > yeast extract > beef extract> peptone .At the incubation time of 16 hr and 28^{0}C, Cellulase activity was found to be 4.5,1.9, 0.89, 5.5 & 2.1(IU/ml) respectively for organic nitrogen sources Among the organic nitrogen sources tested malt extract was found to the best stimulating cellulase production by *Cellulomonas fimi* MTCC24 (Table 2). In order to determine maximum cellulase production, malt extract further optimized in the range of 0.5-4.5g/L and was found increase cellulase production 5 IU/ml with 3.5g/L (Figure 3b).

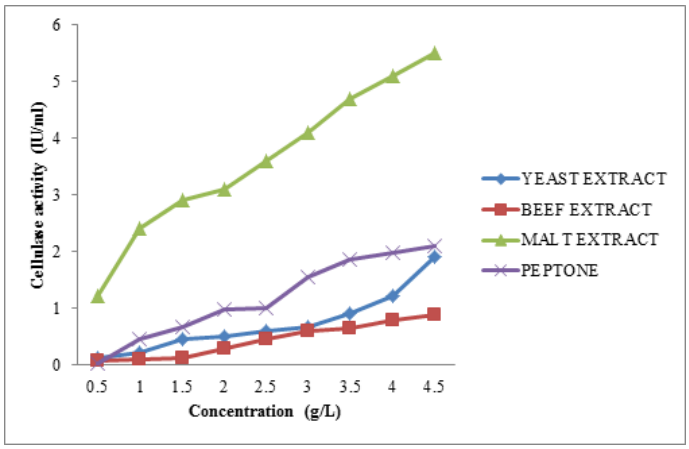

Figure 2a: Effect of organic nitrogen sources on cellulase activity (IU/ml)

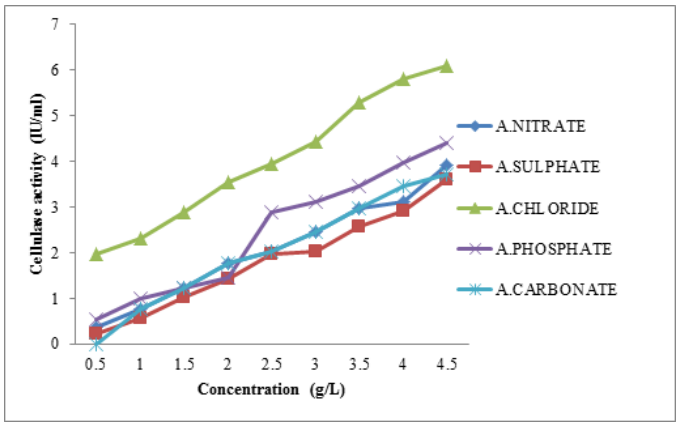

Figure 2b: Effect of inorganic nitrogen sources on cellulase activity (IU/ml)

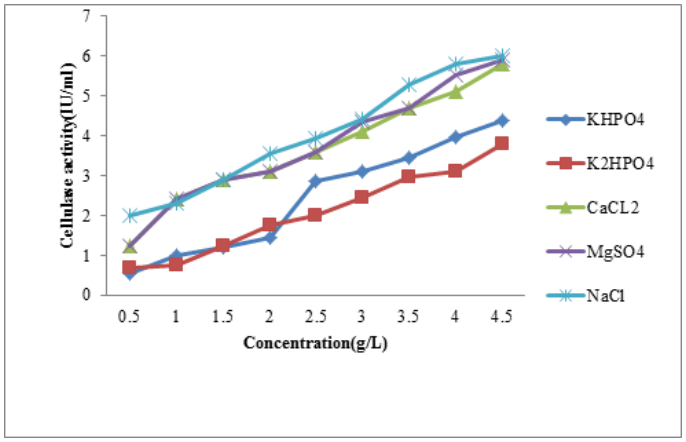

Figure 2c: Effect of macronutrient on cellulase activity (IU/ml)

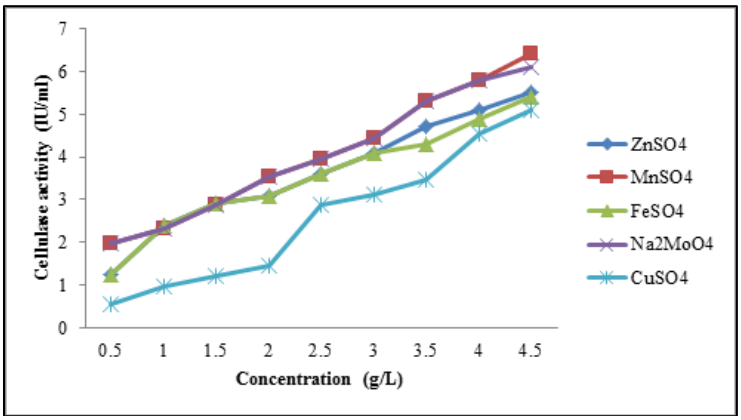

Figure 2d: Effect of micronutrient on cellulase activity (IU/ml)

Based on the statistical calculation the p-value was found to be in the range of 0.03741-0.4822 and the cellulase activity calculated as 4.5, 3.91, 3.6, 6.1, 4.4 &3.71(IU/ml) respectively for inorganic nitrogen sources. Ammonium chloride was found to be better effect on cellulase activity based on lesser p-value (shows greater effect) and the concentration varied from 0.5-4.5g/L, observed increased cellulase activity of 4.2 IU/ml with 2.5g/L (Figure 3c).

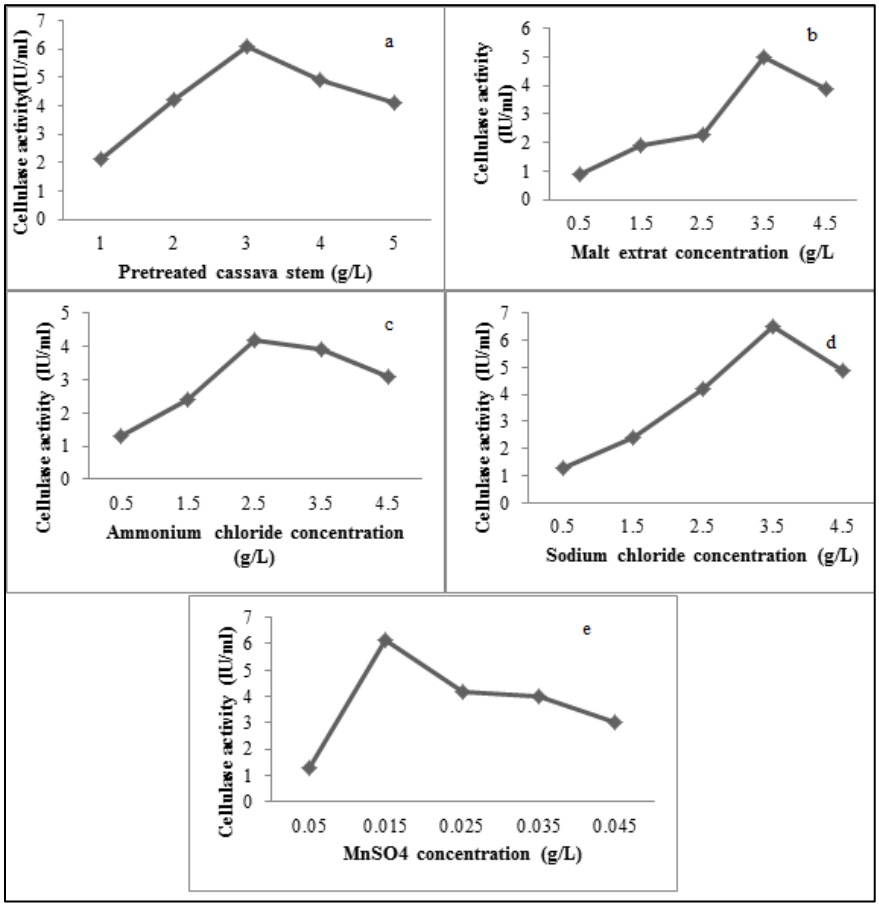

Figure 3: Effect of selected nutritions on cellulase activity (Iu/ml); a) Effect of PCS on cellulase activity (IU/ml); b) Effect of malt extract on cellulase activity (IU/ml); c) Effect of A.Chloride on cellulase activity (IU/ml); d) Effect of S.Chloride on cellulase activity (IU/ml); e) Effect of MnSO4 on cellulase activity (IU/ml)

The effect of macro and micro nutrients were studied on cellulase production calculated p-value ranged from 0.037 to 0.933. Among the five the macro nutrients was found that sodium chloride has the greater effect on cellulase

production which has the p-value of 0.037 and further varied in the range of 0.5-4.5g/L & shows the cellulase activity of 6 IU/ml (Figure 3d).

CONCLUSION

Accordingly for micronutrients p-value was found as 0.0371-0.90668 and manganese sulphate has the better effect on cellulase production with the minimized p-value of 0.037, which was selected and varied in the range of 0.5-4.5g/L& maximal activity was found to be 6.5 IU/ml (Figure 2e). Ali *et al.,* (2013) reported Calcium chloride, magnesium sulphate, ferrous sulphate and manganese sulphate were influenced on cellulase production by *Cellulomonas fimi* NCIM-5015.The selected macronutrients were further studied at the level of 0.5-4.5g/L and results, the maximum cellulase production observed at 4.5g/L. Manganese sulphate studied at the level of 0.005 to 0.045 g/L and observed increased cellulase production of 6.2 IU/ml with 0.015g/L. Under these conditions, the production of cellulase was found to be 8 IU/ml for *YTP 1*.

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